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## Below-ground fine-scale distribution and soil *versus* fine root detection of fungal and soil oomycete communities in a French beech forest

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### ABSTRACT

The use of next-generation DNA sequencing methods, which produce massive volumes of data, has transformed fungal molecular ecology. These technologies offer the opportunity to describe microbial communities in depth and to investigate fungal diversity at a large geographical scale. In particular, due to the emergence of studies conducted at a large scale (countries, continents), the definition of a compromise between the optimal and representative description of local diversity (plot scale) and the management of a greater number of sites for these studies is required. This work was performed to explore the local richness and the structure of the ectomycorrhizal (EcM) fungal and oomycete communities in a temperate beech plot using 454 pyrosequencing technology. The internal transcribed spacer (ITS-1) region was amplified and sequenced from fine roots and soil sampled from the two upper horizons. Our analyses suggested that soil could be a good substitute for fine roots in studying EcM fungi. The fungal assemblage exhibited a vertical distribution in the soil profile. Only four Pythiaceae were identified, which was insufficient to study the spatial distribution of this group. The fungal and particularly the ectomycorrhizal (EcM) richness was not correlated with any soil variable (pH, C, N or P), but the spatial structure was explained by the C:N ratio and phosphorus content. Our data reveal the importance of maintaining a minimal representative sampling at the plot scale for comparative geographical studies.

**Keywords:** spatial distribution, diversity, ectomycorrhizal fungi, oomycete, *Phytophthora*, soil, fine roots, *Fagus sylvatica*, pyrosequencing

## Introduction

Soil microorganisms are important components of forest ecosystems and can be intimately linked with forest trees through symbiotic, mutualistic or pathogenic interactions. Fungal microorganisms such as saprotrophic or mycorrhizal fungi are involved in important functions within the soil in relation to nutrient cycling (Smith & Read 2008; Courty *et al.* 2010), water dynamics and disease control that are essential for the health and growth of forest trees. In contrast, some soil microorganisms are pathogens, such as *Phytophthora* species belonging to the Pythiaceae (Stramenopiles kingdom). These species are involved in forest declines and dieback (Erwin & Ribeiro 1996), participating in tree selection and causing important economic losses. In general, soil microorganisms are highly diverse and are not randomly distributed at broad or local scales (Green *et al.* 2004). However, compared to plants and animals, little is known about the community structure and spatial distribution of microorganisms, mainly due to technological limitations. Indeed, morphological-based methods, such as fungal sporocarp monitoring (Buée *et al.* 2011), root-tip morphotype inventories (Tedersoo *et al.* 2003) and oomycete spore baiting (Hansen & Delatour 1999; Fichtner *et al.* 2007), require considerable expertise and time. Additionally, these methods may not be exhaustive, which could explain the relative underestimation of some microbial communities (Hibbett *et al.* 2011). This is particularly the case for Pythiaceae, which are important soil pathogens (Erwin & Ribeiro 1996; Hansen & Delatour 1999; Brasier 2009).

New sequencing techniques, such as high-throughput pyrosequencing (Margulies *et al.* 2005), provide a great opportunity to further investigate microbial communities at different scales. Recently, numerous studies have employed 454 sequencing to highlight the extraordinary fungal diversity in soil or on plant roots, including that of EcM fungi, which are found in the vicinity of tree roots (Öpik *et al.* 2009; Buée *et al.* 2009; Tedersoo *et al.* 2010, Tedersoo *et al.* 2011; Jumpponen *et al.* 2010; Wallander 2010; Kausrud *et al.* 2012). High-throughput sequencing represents an excellent tool for describing soil fungal communities and characterising their structure on a larger scale. However, pathogenic species belonging to the *Pythium* or *Phytophthora* genera are widespread and play important roles in natural ecosystems, but the available molecular methods for characterising their communities from environmental samples have not yet been seriously assessed (Arcate *et al.* 2006; Cooke *et al.* 2007). A recent study reported the development of a molecular approach to detect *Phytophthora* species from soil samples using novel sets of genus-specific primers designed against internal transcribed spacer (ITS) regions. However, attempts to detect *Phytophthora* species in numerous environmental samples remain unsuccessful (Scibetta *et al.* 2012). Moreover, oomycetes have not been well studied at the community level compared with fungi or bacteria (Rousk *et al.* 2010). Although baiting methods have provided some insight into the diversity of the *Phytophthora* communities in natural ecosystems, these techniques require a long time to implement, which strongly limits the ability to describe these communities (Hansen & Delatour 1999; Jung *et al.* 2000; Cooke *et al.* 2007; Balci *et al.* 2007). As a consequence, few studies have explored the distribution of the oomycete community in forest soils at the stand scale (Hansen & Delatour 1999; Gomez-Aparicio *et al.* 2012), and none of these investigations has used next-generation sequencing.

At a local scale, ectomycorrhizal (EcM) fungi may exhibit a discrete or extended patch distribution (Pickles *et al.* 2010). The relative spatial distributions of sporocarps and fungal mycelia are known to differ significantly, and has been particularly well studied among EcM fungi (Jansen & de Nie 1988; Peter *et al.* 2001). It is also reported that locally, the spatial distribution of EcM hyphae does not follow that of their mycorrhizas (Van Der Linde *et al.* 2009) and that the relative abundance of mycorrhizas and mycelia differs between fungal species (Kjøller 2006). There is also evidence that the mycelium and mycorrhiza of the same species are segregated vertically throughout the soil layers (Genney *et al.* 2006). In boreal forests, fungi are distributed in the vertical soil profile, with saprobes dominating in the litter and mycorrhizal fungi showing abundance in the humus layer (Lindahl *et al.* 2007).

Several factors may explain this heterogeneous spatial distribution of microorganisms, but such environmental factors are still poorly understood (Rousk *et al.* 2010). Ascomycetes have been suggested to respond to soil chemistry, water and resource concentrations at small scales, rather than to the type of land (Green & Bohannan 2006). Soil pathogens exhibit a spatial pattern that depends on the vegetation present and soil characteristics. For example, the soil water regime and soil texture, i.e., the proportion of sand, are major factors impacting the distributions of *Phytophthora* and *Pythium* species (Hansen & Delatour 1999; Jung *et al.* 2000; Gomez-Aparicio *et al.* 2012). However, the perception of the fungal community structure is largely dependent on the sampling effort and strategy applied, and characterisation of spatial patterns has proved to be difficult with small size samples (Withcomb & Stutz 2007). Indeed, undersampling has been stressed as biasing conclusions about the spatial scaling of microbial diversity (Green & Bohannan 2006).

A better understanding of the spatial distribution of filamentous soil microorganisms is essential both to gain knowledge about the ecology of these communities and to plan sampling for future studies (Taylor 2002). This point is particularly crucial for EcM fungi because their extramatrical structures are highly variable between different species (Agerer 2001). Moreover, while emergent large-scale studies require a multiple-stand approach, it is necessary to maintain a representative description of microbial diversity at the plot scale.

However, the sampling strategies applied at local scales differ greatly between studies comparing plots (Buée *et al.* 2009, Lilleskov *et al.* 2004), and the development of a standardised sampling method is, therefore, of great interest (Cox *et al.* 2010). Furthermore, for EcM communities, the handling of root tips (including washing and fastidious selection of living vs. dead or mycorrhizal vs. non-mycorrhizal specimens) remains a laborious component of studies that undermines the potential of high-throughput approaches. Examining soil could potentially be an alternative method for investigating the EcM community in particular. For oomycetes, baiting is classically performed directly in soil samples (Davison & Tay 2005).

The main aim of this study was to thoroughly explore the fungal (EcM) and oomycete community associated with beech trees in a relatively homogeneous plot to propose a compromise between sampling and sequencing efforts for high-throughput studies. For this purpose, we investigated the spatial (horizontal and vertical) distribution of these microorganisms in relation to a number of edaphic parameters and compared the fine root system against bulk soil as microbial reservoirs. We tested the following hypotheses: (1) the fungal and oomycete communities are not evenly distributed within the plot but instead show a spatial structure, possibly related to environmental factors; (2) sampling a limited set of trees enables the microbial community of a relatively homogeneous plot to be described; and (3) the soil fungal community is as effective as the fine root fungal community for describing ectomycorrhizal diversity using next-generation sequencing.

## Materials and methods

### Site description and sampling

The Lignéville (Vosges, France) forest is a temperate forest (48°07'32"N, 5°59'51"E) situated at 380 m elevation. The experimental plot was established in a 3500 m<sup>2</sup> area dominated by 90% beech (*Fagus sylvatica*), along with a few oak and spruce trees. All of the contiguous beech trees in this area were selected for the study (n = 20 trees). The diameter of the trees at a 1.30 m height ranged from 30 to 80 cm. The litter was removed, and four soil cores (8 cm diameter) were collected at 1 m from the trunk base of each tree. The four cores were combined, with the upper (0 to 10 cm deep) and lower (10 to 20 cm deep, sandy loam) horizons kept separate. The samples were collected on 11 December 2009 and were kept frozen until processing. Soil analyses of sub-samples were carried out by the Laboratoire d'Analyse des Sols d'Arras (<http://www5.lille.inra.fr/las>) to determine the pH (water method), total carbon (C) and total nitrogen (N) contents (NF ISO 10694 and NF ISO 13878) and phosphorus (P) content (Duchaufour & Bonneau 1959).

The root system present in each sample was separated from the soil using a 5 mm sieve. The remaining soil was gently cleaned from the roots in tap water, and the coarse roots were discarded (diameter > 2 mm). The fine roots (including root tips and residual adhering soil) were then lyophilised and ground with a mortar and pestle. Total genomic DNA extraction was carried out using the DNeasy Plant Mini Kit (Qiagen) for fine root samples (50 mg of the homogeneous powder) and the Fast DNA Spin Kit for Soil (MPBio) for soil samples (500 mg of homogeneous wet soil). Approximately 15 mg of polyvinylpyrrolidone (PVPP) was first added to each sample, and the manufacturers' instructions for the kits were then followed. The genomic DNA was then purified twice in PVPP columns.

### PCR amplification, amplicon purification and library preparation

Upper and lower horizon samples were processed independently. The fungal ITS1 region (Schoch *et al.* 2012) was amplified from both the fine root and soil DNA with the ITS1F (CTTGGTCATTTAGAGGAAGTAA) and ITS2 (GCTGCGTTCTTCATCGATGC) primers (Gardes & Bruns 1993). ITS1F was chosen over ITS1 because the latter primer binds to plant DNA more easily (Gardes & Bruns 1993 and personal unpublished results), which could decrease the fraction of fungal reads. The oomycete ITS1 region was amplified only from the soil samples using nested PCR: the first round was performed with the ITS6 (GAAGGTGAAGTCGTAACAAGG) and ITS4 (TCCTCCGCTTATTGATATGC) primers (White *et al.* 1990), and the second round with the primer pair ITS6 and ITS7 (AGCGTTCTTCATCGATGTGC) (Cooke *et al.* 2000). For 454 Titanium pyrosequencing of multiplexed samples, the forward primer (ITS1F or ITS6) was linked to a 4-base multiplexed identifier (MID) and adaptor A (CGTATCGCCTCCCTCGCGCCATCAG), whereas the reverse primer (ITS2 or ITS7) was adjoined with adaptor B (CTATGCGCCTTGCCAGCCCGCTCAG).

The amplification mixture was prepared in a 20 µl volume, obtaining final concentrations of 0.4 X enzyme buffer, 1.5 mM MgCl<sub>2</sub>, 1.12 mg ml<sup>-1</sup> BSA, 0.2 mM each dNTP, 0.2 µM each non-tagged primer (ITS6 and ITS4) or 0.8 µM each tagged primer (ITS1F and ITS2 or ITS6 and ITS7) and 0.05 U µl<sup>-1</sup> Sigma Taq polymerase. The GeneAmp 9700 system was used with the following program for fungi: 5 min at 95°C, followed by 35 cycles of 30 sec at 95°C, 40 sec at 53°C and 45 sec at 72°C, with a final step of 7 min at 72°C. For nested PCR, the first round consisted of 5 min at 95°C, followed by 20 cycles of 30 sec at 95°C, 40 sec at 53°C and 1.5 min at 72°C,

with a final step of 7 min at 72°C. The first-round amplicons were diluted 10 times, and the second-round program was as follows: 5 min at 95°C, followed by 30 cycles of 30 sec at 95°C, 40 sec at 53°C and 10 sec at 72°C, with a final step of 7 min at 72°C.

Four 20 µl reactions were run in parallel for each sample, which were then pooled and purified on an MSNU030 MultiScreen<sub>HTS</sub> PCR Plate (Millipore Corporation), with two washes using 100 µl of H<sub>2</sub>O and solubilisation of the amplicons in 40 µl of H<sub>2</sub>O. The concentration of the purified tagged amplicons was measured three times with a NanoDrop® ND-1000 spectrophotometer, and equimolar pooling was carried out. In total, we obtained three independent libraries of 40 samples, corresponding to fine root-associated fungi, soil fungi and oomycetes from soil. Thus, three independent runs were conducted with 40 MIDs each, in equal proportions. The 454 pyrosequencing was performed by Génoscope (Evry, France). The raw data were deposited on the Sequence Read Archive website (<http://www.ncbi.nlm.nih.gov/sra>) under the project identifier PRJEB583 and the study accession ERP001914. The three libraries are under the following run accessions: ERR185063 (alias run\_1.TCA.AQN\_AOTS\_GK5X8RX01 for soil microbes amplified with ITS1F-ITS2), ERR185064 (alias run\_2.TCA.AQN\_DOTS\_GRD572402 for soil microbes amplified with ITS6-ITS7) and ERR185065 (alias run\_5.TCA.AQN\_BOTS\_GJVVFH05 for fine root-associated fungi amplified with ITS1F-ITS2).

### Bioinformatics analysis

The sequences were sorted into different files according to their multiplex identifier (MID) using sfffile software, with no mismatches allowed for the MIDs, which were then removed. The sequences were cleaned (trimming and denoising) with trim.flows in Mothur version 1.20.1 with the default parameters (Schloss *et al.* 2009). The internal transcribed spacer 1 (ITS) was extracted using Fungal ITS extractor, version 2 (Nilsson *et al.* 2010). After extraction of the ITS1 region, the sequences were filtered based on a minimal length of 100 bp. Out of a total of 362 660 sequences, 212 285 (58.5%) corresponding to the three libraries, were retained for downstream analyses. Molecular operational taxonomic units (MOTUs) were generated after six successive clusterings using Uclust version 3.0 (Usearch version 5.2.32) with the parameter *iddef* = 2 (Edgar 2010) at a 97% similarity. The first clustering included all of the sequences, and subsequent clusterings were conducted with the batch of consensus sequences from the previous clustering. This allowed the redundancy of the MOTUs to be decreased (data not shown). Taxonomic assignment of a consensus sequence (available upon request) generated by Uclust was performed against a curated GenBank database (Feb 2012), excluding environmental sequences, using the Basic Local Alignment Search Tool algorithm Blastn version 2.2.23 (Altschul *et al.* 1990) with the filter turned off (i.e., segments of the query sequence with a low compositional complexity were not masked off). In addition, an R house script was used to assign MOTUs with the following selected criteria: *e*-value ≤ *e*-50, identity > 90 % for the genus and ≥ 97 % for the species level. Some synonyms were corrected using the online Species Fungorum database (<http://www.speciesfungorum.org>). The MOTUs assigned to the same species were subsequently grouped together, further reducing the total number of MOTUs. Unassigned MOTUs were considered to correspond to fungi because the applied ITS1F-ITS2 primer pair shows good specificity for the detection of Dikarya.

### Statistical analyses

The two horizons were compared regarding their edaphic parameters with Student's *t*-test, using the *t.test* function in R. To assess the suitability of the sequencing depth and sampling effort required to estimate microbial diversity, species accumulation curves were computed using the *specaccum* function of the vegan R package (R Core Development Team 2008; Oksanen *et al.* 2011). The *rarefaction* method was applied. Prior to rarefaction, one randomly chosen sequence per MOTU was discarded to eliminate singletons (MOTUs supported by only one sequence), as suggested by Unterseher *et al.* (2011). Four sequencing efforts were tested with respect to rarefaction accumulation trees, corresponding to 100%, 75%, 50% and 25% of the 210 134 retained sequences. Additional rarefactions were performed for each habitat independently (SS, soil from the superior horizon; SI, soil from the inferior horizon; RS, fine roots from the superior horizon; and RI, fine roots from the inferior horizon) considering the ITS1F-ITS2 dataset, because only this primer pair was used in all four habitats.

The term fungal community will be used to refer to the entire set of MOTUs. The MOTUs were categorised as EcM fungi if they were assigned to a genus or a species described as such or suggested to be a putative EcM fungus in the literature (Rinaldi *et al.* 2008; Smith & Read 2008; Tedersoo *et al.* 2010). The rarefied fungal richness was estimated with rarefaction curves (*rarefy* function from the vegan R package, singletons removed) including 7 600 fungal sequences per tree, which was the minimum sequencing depth available. Then, the rarefied EcM richness was determined by multiplying the rarefied fungal richness by the proportion of EcM sequences per sample. In addition, to compare the four habitats (SS, SI, RS, RI), the rarefied fungal and EcM

richnesses per sample were estimated when applicable at a depth of 1 100 sequences (ITS1F-ITS2 dataset only, >15 samples per habitat).

The community structure was analysed at two levels: (i) the plot level, consisting of the samples obtained by merging the samples from each tree (n=20); and (ii) the tree level, considering only the sequences from the ITS1F-ITS2 dataset, with samples from the four different habitats kept separate (n=80). As one sequence does not necessarily represent one individual and because abundance is biased in other respects (Amend *et al.* 2010), the binary matrices of the presence of non-singleton MOTUs were computed from the abundance matrices. The binary matrices were used to perform correspondence analysis (CoA) at the plot and tree levels with the *dudi.coa* function of the *ade4* package (Dray & Dufour 2007). The MOTUs present in less than three unit samples, according to the level studied, were discarded to prevent distortion of the CoA analysis by rare MOTUs. The position of a tree or sample on a CoA axis will hereafter be used as a measure of the community composition.

From the binary matrix of MOTU presence at the plot level, a similarity matrix was computed using the Sørensen index, i.e.,  $2*J / (A+B)$ , with J representing the shared number of species between two trees, and A and B representing the total number of species of each of the compared trees. For this calculation, the *designdist* function of the *vegan* R package was used. The matrix of Euclidean distance was computed from the coordinates with the *dist* function of the *stats* R package. Mantel's tests and correlograms were performed using the *vegan mantel* and *mantel.correlog* functions, respectively, with the Spearman method. The distance classes were calculated following the Sturge equation, as stated by default in the *mantel.correlog* function. The *bioenv* function (*vegan* R package), with a Spearman model and Bray dissimilarity distances, was employed to determine the subset of environmental variables showing the best correlation with the fungal community dissimilarity at the plot level.

A variance analysis using the *lm* function of R was conducted to test for the effects of the horizon, (superior or inferior), the sample material, (soil or fine roots) and their interaction on the richness and community composition at the tree level. The correlations at the plot level between the estimated richness or the community composition and the edaphic parameters (mean values of the two horizons) were tested with a regression analysis, using the *lm* function in R.

## Results

### Microbial diversity

The mean number of sequences per sample after the cleaning steps was 1 768 (min: 226, max: 4 416, Table 1). Approximately 57% of the MOTUs were non-singletons. Only 36.9% of the non-singleton MOTUs could be assigned to a genus or a species. Among these MOTUs, 89% belonged to the Dikarya, and 41% were classified as EcM (Table 1). A total of 630 and 111 non-singleton MOTUs were found exclusively from the soil substratum using the ITS1F-ITS2 and ITS6-ITS7 primer pairs, respectively, while 263 MOTUs were detected by both pairs. Oomycetes were only detected with the ITS6-ITS7 primer pair and only in the soil samples. This primer pair was not as specific as suggested, as it amplified many basidiomycetes in particular (representing 129 of the 187 assigned MOTUs amplified with this primer pair) and even four plant taxa, which were removed from the dataset prior to conducting any analysis. The ITS6-ITS7 primer pair allowed the detection of three EcM MOTUs (*Russula rubra*, *Russula sp18* and *Cortinarius armillatus*) that were not detected in the soil with ITS1F-ITS2, but these three MOTUs were detected in the fine root habitat with ITS1F-ITS2. The most abundant MOTU (14 321 sequences, abundance will hereafter refer to the number of sequences per MOTU) corresponded to a Saprolegniale (Oomycota, Saprolegniaceae). Only 463 sequences from Pythiaceae (i.e., 0.8% of the sequences from the ITS6-ITS7 dataset) were detected in the plot, representing only a few species: *Phytophthora plurivora*, an unidentified *Phytophthora* species from clade 7a (cf Blair *et al.* 2008), *Pythium sylvaticum* and an unidentified species of *Pythium*.

Analysis of the sequences obtained with ITS1F-ITS2 and ITS6-ITS7 primer pairs from the soil and fine root samples together revealed that the 20 most abundant MOTUs (overall number of sequences) were EcM fungi, including seven *Russula* species (*R. brunneoviolacea*, *R. cyanoxantha*, *R. ochroleuca*, *R. vesca*, *R. nigricans*, *R. sp1* and *R. ionochlora*), two *Boletus* species (*B. pruinatus* and *B. erythropus*), *Cenococcum geophilum*, *Cortinarius sp1* and *Elaphomyces sp1* (Table 2). Other abundant MOTUs included six non-assigned MOTUs that were amplified only with ITS6-ITS7 and two *Mortierella* species (*M. sp1* and *M. humilis*). There was a significant positive correlation between the log of abundance and the frequency (Fisher's F = 2 149.5, p<0.001; the frequency refers to the number of trees or samples in which the MOTU was present). Among the MOTUs supported by more than 1 000 sequences, 74.4 % were present on more than 15 trees, while only 14 % were present on fewer than 10 trees. The abundant MOTUs present on less than 10 trees included *Cortinarius sp1*,

*Russula* sp1 and MOTU6. The community matrix, including the ecological mode for the identified MOTUs, is available as a Supplementary Table.

Table 1: Number of sequences and number of MOTUs in the datasets. The number of MOTUs in the combined dataset was not the sum of the two datasets as they were redundant.

	ITS1F-ITS2 dataset (2 libraries=80 samples)	ITS6-ITS7 dataset (1 library=40 soil samples)	Combined dataset (3 libraries=120 samples)
<b>Total number of 454 reads</b>	261 771	100 889	362 660
<b>Number of retained quality sequences</b>	156 242 (59.7%)	55 936 (55.4%)	212 178 (58.5%)
<b>Range of sequences per sample <sup>1</sup></b>	414 - 3 742	226 - 4 416	226 - 4 416
<b>Range of sequences per sampled tree <sup>2</sup></b>	4 315 - 11 570	1 435 - 5 340	7 713 - 13 860
<b>Number of MOTUs</b>	1 840	490	2 044
<b>Number of nonsingletons <sup>3</sup></b>	1 085 (59.0%)	374 (76.3%)	1 173 (57.4%)
<b>Assigned nonsingletons</b>	413	187	433
<b>Taxonomic-level assignment of nonsingletons</b>			
Not assigned	672	187	740
Genus	226	81	236
Species	187	106	197
<b>Phylum-level repartition of the assigned nonsingletons</b>			
Basidiomycota	209	129	216
Ascomycota	169	20	169
Zygomycota	6	6	6
Other or uncertain fungal lineages	29	22	32
Oomycota	0	10	10
<b>Ecological mode repartition of the assigned nonsingletons *</b>			
ECM**	177	103	177
Saprobe**	143	59	151
Plant pathogen**	15	6	18
Other or unknown mode	83	20	92

<sup>1</sup> an homogeneous sample of soil or fine-roots originating from four mixed cores, taken around a beech tree at 0-10 cm or 10-20 cm depth

<sup>2</sup> sum of the four samples from the four different habitats (see <sup>1</sup>) per tree

<sup>3</sup> MOTUs supported by two sequences at least

\* some species are classified as both saprobe and tree pathogen, thus counted twice

\*\* true and putative are summed

The rarefaction curves tended toward saturation (Fig 1A and 1B). A total of 90 % of the estimated plot richness was retrieved when only 15 out of the 20 trees were sampled with a sequencing effort of 10 500 sequences per sampled tree (Fig 1B). However, it was necessary to sample all 20 trees to achieve such a result if a sequencing effort of 7 880 sequences per sampled tree was employed (Fig 1B). An estimated richness of 1 020 MOTUs was similarly obtained when either 11 trees with 100% of the sequences or 20 trees with half of the sequences per tree were considered. When computed individually for each of the four habitats (fine roots or soil from the upper horizon and fine roots or soil from the lower horizon), the rarefaction curves also tended toward saturation (Fig 2A and 2B).

The fungal and EcM richness estimated from the rarefaction curves ranged from 196 to 308 non-singleton fungal MOTUs per tree (including unassigned MOTUs) and from 71 to 187 EcM MOTUs per tree. The rarefied fungal and EcM richness per tree were positively correlated ( $F = 4.44$ ,  $p < 0.0494$ ). The fungal richness was neither spatially structured (Mantel  $r = 0.0448$ ,  $p = 0.34$ ) across the plot nor linked to any edaphic parameter, or to the dry fine root biomass (Table 3). This was also true for the EcM richness (Table 3) (Mantel  $r = 0.0841$ ,  $p = 0.192$ ).

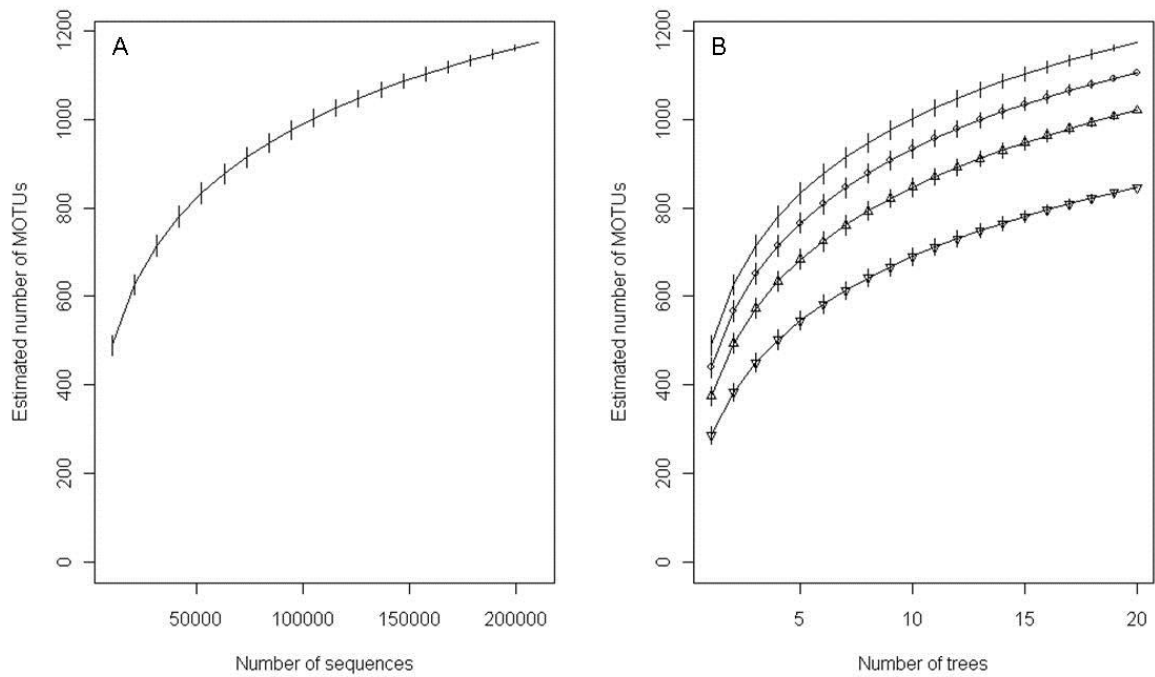


Figure 1: Rarefaction curves accumulating sequences (A) or trees (B) obtained with the function ‘specaccum’ of the *vegan* R package. Sequences, obtained with ITS1F-ITS2 and ITS6-ITS7 primer sets, were clustered at 97% and one sequence per MOTU was removed, eliminating *de facto* the singletons. Four sequencing efforts are presented: 100%, 75% (circle), 50% (triangle) and 25% (inverted triangle) of the 210134 retained sequences.

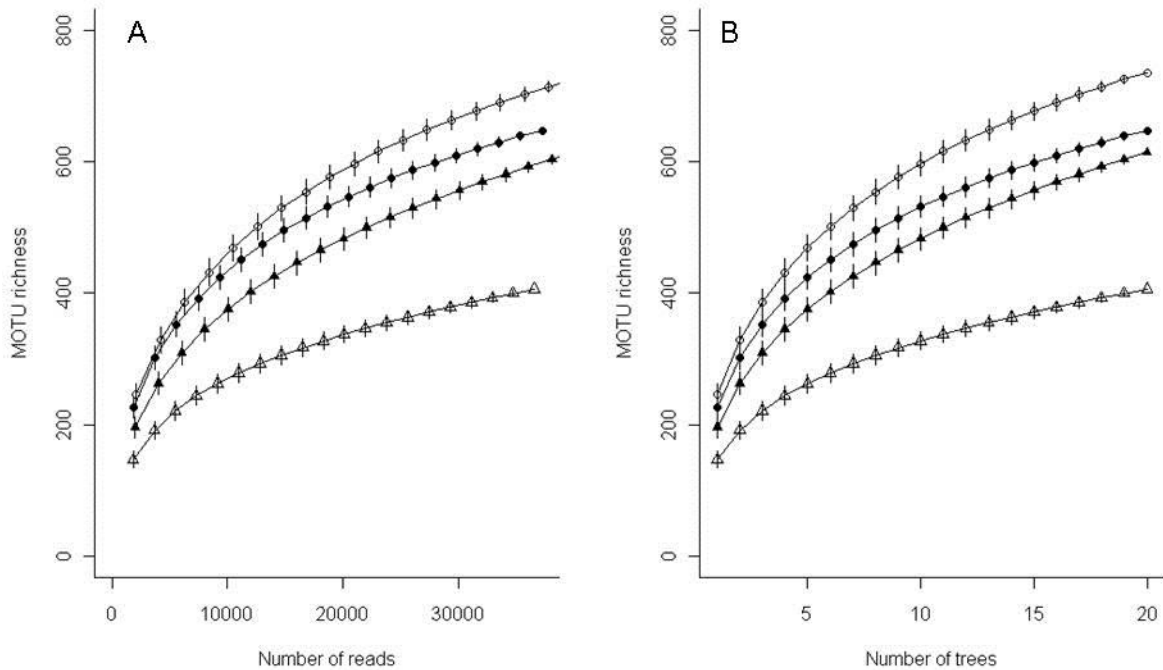


Figure 2: Saturation curves for four distinct habitats: soil (closed symbols), root (open symbols), organo-mineral horizon (circles), and mineral horizon (triangles) accumulating sequences (‘accumresult’ function of the *BiodiversityR* R package) (A) or trees (‘specaccum’ function of the *vegan* R package) (B). Sequences, obtained with ITS1F-ITS2 primer set only, were clustered at 97%. One sequence was discarded per MOTU, eliminating *de facto* the singletons.

The rarefied fungal richness was significantly lower within the inferior horizon ( $F = 54.468$ ,  $p < 0.001$ ) and in the fine root samples ( $F = 12.299$ ,  $p < 0.001$ ). There was also a significant interaction between the two factors (horizon \* material) ( $F = 20.815$ ,  $p < 0.001$ ). However, the rarefied EcM richness was different between the two horizons ( $F = 15.9351$ ,  $p < 0.001$ ) but not between the two types of material ( $F = 0.4939$ ,  $p = 0.4846$ ). A weak interaction between the two factors was observed ( $F = 4.4014$ ,  $p = 0.0398$ ).



Notably, the edaphic parameters (pH, C:N ratio and phosphorus) were significantly different between the two horizons (data not shown), and the fine root system was more abundant in the upper horizon than in the lower horizon, which presented mean dry fine root masses of 5.8 g / tree and 1.7 g / tree, respectively ( $t = 9.2675$ ,  $p < 0.001$ ).

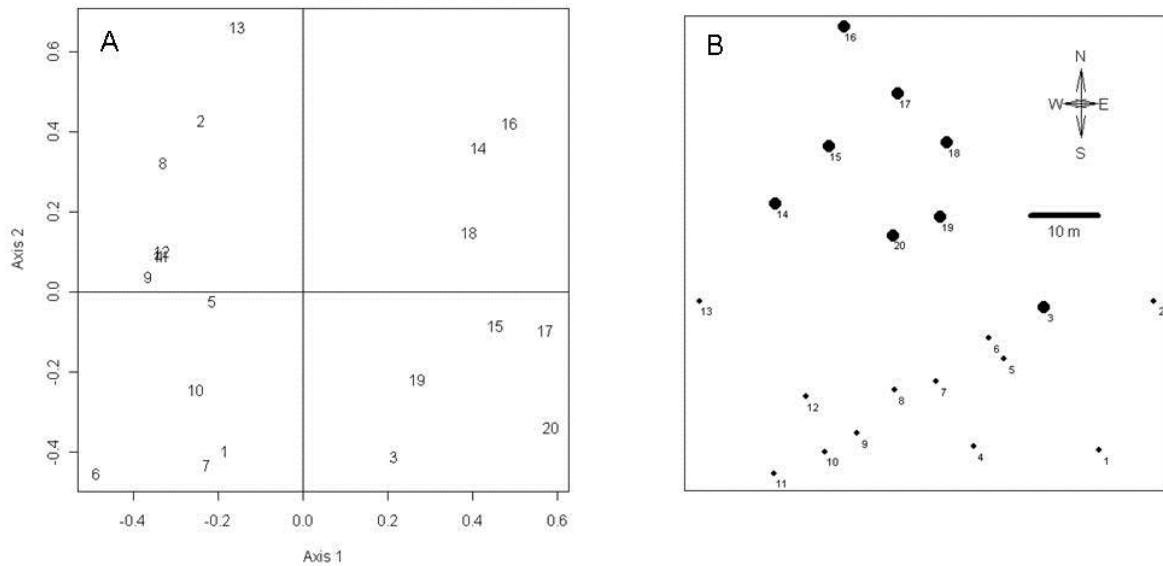


Figure 3: Distribution of the twenty beech trees according to their associated fungal and oomycete community on a correspondence analysis (CoA). The CoA was done on presence / absence of 681 MOTUs obtained with ITS1F-ITS2 and ITS6-ITS7 primers (A). The percentages of variation explained by the first and second axis are 13.2 and 9.9 % respectively; the numbers are the tree numbers. Note that trees numbered 4, 11 and 12 are superimposed. Spatial distribution of the twenty beech trees on the experimental plot (B). Dot size was related to the first axis value on the CoA beside, i.e. small dots for negative values and large ones for positive values. The axis values on the CoA were used for statistical tests presented in Table 3.

### Microbial structure at the plot level

The soil characteristics showed variability within the plot: the soil carbon content ranged from 26.4 to 59.8  $\text{g.kg}^{-1}$ , nitrogen ranged from 1.3 to 2.6  $\text{g.kg}^{-1}$ , the C:N ratio ranged from 19.9 to 24.5, phosphorus ranged from 0.08 to 0.10  $\text{g.kg}^{-1}$ , and the soil pH ranged from 4.0 to 4.3. The clay:sand ratio measured in the lower horizon was  $0.24 \pm 0.03$ .

The 681 MOTUs associated with at least three trees were retained for the CoA computed at the plot level ( $n = 20$  trees) (Fig 3A). The trees in the studied plot were clearly separated on the first axis of the CoA (Fig 3A). The distribution of the trees in the CoA was not linked to the fungal lifestyle (EcM, pathogen or saprobe), as the MOTUs from these three groups were evenly dispersed in the CoA (data not shown). The first axis was positively correlated with one of the geographical coordinates of the trees, with the trees showing a high value on this axis being mainly distributed in the northern part of the plot (Table 3, Fig 3B). The second axis of the CoA was, however, not related to any geographical parameter (Table 3). The Mantel test also clearly indicated that the fungal community showed a spatial structure, with a significant autocorrelation being detected for samples within the 0-15 m distance class (Mantel statistic  $r = -0.4972$ ,  $p < 0.05$ ) (Fig 4). The same result was obtained when considering the EcM community alone ( $r = 0.3$ ,  $p < 0.05$ ).

The first axis of the CoA was linearly related to the C:N ratio and P content, while the second axis was only related to the P content (Table 3). When only the EcM fungi were considered in the CoA, similar results were observed (indicating a significant relationship between CoA axis 1 and the C:N ratio), although there was no significant relationship with the P content (Table 3). The model showing the best correlation between the environmental variables and the fungal or EcM community data combines two parameters: the C:N ratio and P content. These two variables were positively correlated with the latitude ( $F = 6.865$ ,  $p = 0.0174$  and  $F = 5.782$ ,  $p = 0.0272$  for C:N ratio and P soil content respectively).

The EcM fungi that presented a high value on the first axis of the CoA (over 1) were *Amanita fulva*, *A. porphyria*, *Cortinarius angelesianus*, two unidentified *Cortinarius* species and *Russula mairei*. These fungi were almost exclusively found associated with trees located in the northern part of the plot, where the soil exhibited both a higher phosphorus content and higher C:N ratio. Previously mentioned abundant EcM species, such as *R. brunneoviolacea*, *R. cyanoxantha* and *Boletus pruinatus*, were not discriminated in the CoA, which was also the case for MOTU1. No clear pattern was seen for the few observed Pythiaceae. *Phytophthora plurivora*,

*Phytophthora* clade 7a, *Pythium sylvaticum* and *Pythium* sp1 were associated with 4, 3, 1 and 2 non-adjacent trees out of the 20, respectively.

Table 2: The twenty most abundant MOTUs in the plot. The abundance was the number of sequences and the frequency was the number of trees (max=20) around which the MOTU was found.

MOTU	Abundance	Frequency	GI number	Best BLAST hit	Identity	E-value
MOTU1	14321	19	323302256	<i>Saprolegnia megasperma</i>	90.3	1,00E-45
<i>Russula brunneoviolacea</i>	12069	17	91199853	<i>Russula brunneoviolacea</i>	98.4	2,00E-90
<i>Russula cyanoxantha</i>	11088	19	22024448	<i>Russula cyanoxantha</i>	99.3	2,00E-66
MOTU2	9257	16		No hit found		
<i>Boletus pruinatus</i>	7833	20	61657552	<i>Boletus pruinatus</i>	98.9	3,00E-92
MOTU3	6964	19	323302256	<i>Saprolegnia megasperma</i>	87.8	4,00E-36
<i>Cenococcum geophilum</i>	6917	20	55832969	<i>Cenococcum geophilum</i>	97.8	3,00E-64
MOTU4	5220	20	150439140	<i>Homobasidiomycete sp</i>	90.5	3,00E-27
<i>Mortierella sp1</i>	5213	20	183013810	<i>Zygomycete sp</i>	93.3	6,00E-53
<i>Russula ochroleuca</i>	5193	19	308055381	<i>Russula ochroleuca</i>	100	2,00E-75
MOTU5	4761	18	323302256	<i>Saprolegnia megasperma</i>	89.2	3,00E-36
<i>Mortierella humilis</i>	4512	20	119444359	<i>Mortierellales sp</i>	98.1	3,00E-70
<i>Russula vesca</i>	3868	13	308055461	<i>Russula vesca</i>	100	9,00E-90
<i>Cortinarius sp1</i>	3733	7	57869258	<i>Cortinarius cagei</i>	99.4	6,00E-84
<i>Russula nigricans</i>	3541	15	91199858	<i>Russula nigricans</i>	99.2	8,00E-59
<i>Boletus erythropus</i>	3409	19	300387792	<i>Boletus erythropus</i>	97.9	7,00E-91
<i>Elaphomyces sp1</i>	3396	18	189306999	<i>Elaphomyces cf. granulatus</i>	91.8	2,00E-53
<i>Russula sp1</i>	3137	8	23393838	<i>Russula rosea</i>	96.9	1,00E-82
MOTU6	3013	9	48766839	<i>Saprolegnia semihypogyna</i>	88.5	6,00E-38
<i>Russula ionochlora</i>	2871	12	307159052	<i>Russula ionochlora</i>	97.5	2,00E-72

Table 3: Relationships between richness or community composition of MOTUs or of EcM species and environmental variables at the plot level (n=20).

	Spatial variables		Edaphic variables			Biotic variable
	X	Y	soil pH	Carbon:Nitrogen ratio	Phosphorus content	dry root mass
Fungal richness <sup>1</sup>	0.0487 (0.83)	0.2848 (0.60)	0.1553 (0.70)	2.4047 (0.14)	2.1968 (0.16)	1e-04 (0.99)
EcM richness <sup>1</sup>	0.2304 (0.64)	0.3443 (0.56)	0.0245 (0.88)	0.701 (0.41)	0.5146 (0.48)	1.828 (0.19)
MOTUs community composition : CoA Axis1 <sup>2</sup>	0.2385 (0.63)	57.208 (<0.001)	2.9838 (0.10)	7.7554 (<0.05)	5.834 (<0.05)	0.0357 (0.85)
MOTUs community composition : CoA Axis2 <sup>2</sup>	3.2323 (0.09)	1.1868 (0.29)	1.7193 (0.20)	4e-04 (0.98)	9.538 (<0.01)	0.4049 (0.53)
EcM community composition : CoA Axis1 <sup>3</sup>	0.1029 (0.75)	17.026 (<0.001)	2.1135 (0.16)	8.9674 (<0.01)	1.3713 (0.26)	0.01 (0.92)
EcM community composition : CoA Axis2 <sup>3</sup>	0.0985 (0.76)	0.003 (0.96)	0.3806 (0.55)	0.2979 (0.59)	0.0128 (0.91)	0.0368 (0.85)

<sup>1</sup> rarefied richness estimated for 7200 sequences per sampled tree

<sup>2</sup> axis values of the CoA based on 681 MOTUs and <sup>3</sup> axis values of the CoA based on 107 EcM species

Community composition was defined as the position of the tree on the CoA axis at the plot level (see Figure 3A for CoA with the MOTUs, CoA with EcM species not shown).

Fisher's F statistics with the probability value into brackets. Significant values (p<0.05) in bold. Each variable was tested independently.

### Structure of the fungal assemblage at the tree level

Among the 1 085 non-singleton MOTUs obtained, 681 (58.0%) were shared by the two horizons, whereas 340 (29.0%) were exclusively found in the upper layer, and 152 (13.0%) were exclusive to the lower layer (based only on data obtained with ITS1F-ITS2, as this was the only primer pair used for fine root samples). More than 66 % (118 / 177) of the EcM MOTUs were found in both horizons.

A total of 700 non-singleton MOTUs (64.5%) were shared by the fine root and soil samples, while 193 (17.8%) were exclusively found in the soil samples and 192 (17.7%) in the fine root samples. Among the MOTUs exclusively found in the fine root samples, only 28 (14.6%) were supported by at least ten sequences, and the

most abundant corresponded to an ericoid mycorrhizal fungus, *Oidiodendron* sp3 (116 sequences). Notably, 86 % of the EcM MOTUs found in the fine root samples were also retrieved in the soil samples (134 / 155). The EcM MOTUs that were not detected in the soil samples corresponded to six *Cortinarius* species including *C. delibutus*, *C. sp18*, three *Boletus* (*B. sp2*, *B. sp3*), three *Russula*, two *Cladophialophora*, *Cenococcum* sp3, *Exophiala* sp2, *Tuber puberulum*, *Hebeloma* sp1, *Hydnum* sp1, *Meliniomyces* sp2 and *Tomentella* sp8. In contrast, some EcM MOTUs were detected in the soil samples, but not in the fine root samples (22 / 156, or 14 %, corresponding to nine *Cortinarius*, four *Russula*, four *Lactarius*, *Amanita* sp5, *Hebeloma radicosum*, *Hydnum ellipsosporum*, *Inocybe lacera* and *Tomentella* sp7).

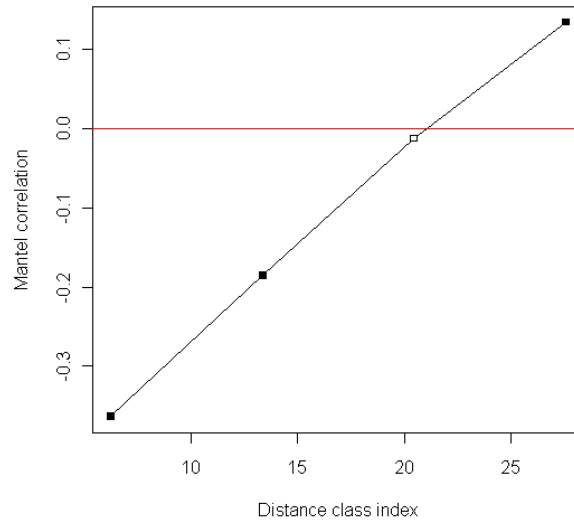


Figure 4: Mantel correlogram for the MOTUs community at the plot level, based on the binary Sørensen similarity coefficient. Distance class index were the values delimiting distance classes. Negative Mantel statistics correspond to positive autocorrelation. Dark symbols indicate significant Mantel statistics ( $\alpha=0.05$ ).

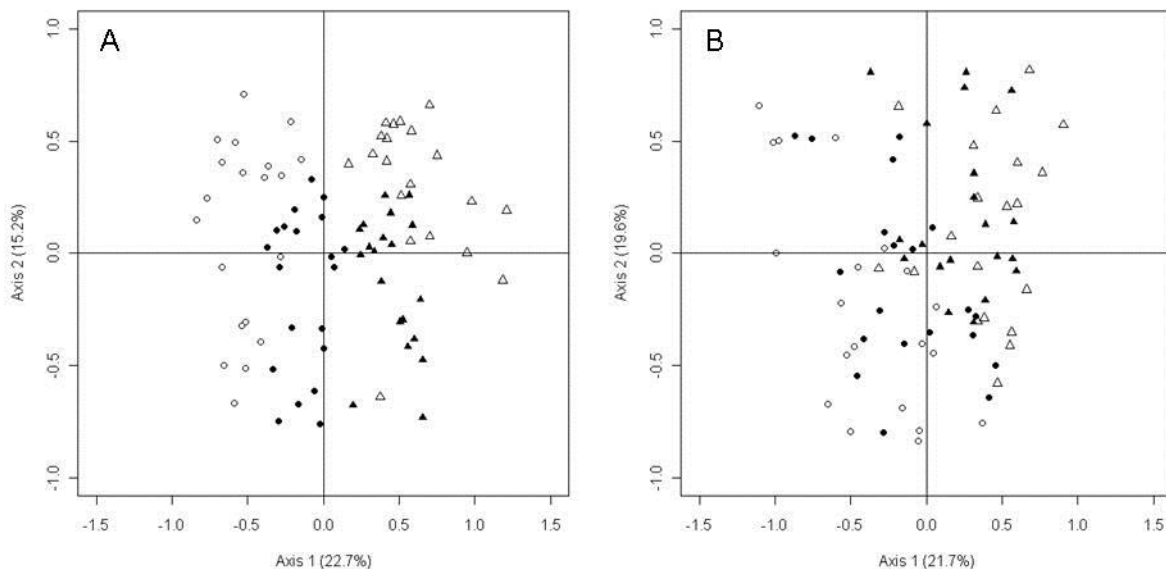


Figure 5: Correspondence analysis for fungal (690 MOTUs) (A) or ectomycorrhizal (127 EcM MOTUs) (B) communities in four different habitats of a beech plot ( $n=80$ ): soil (closed symbols), root (open symbols), superior horizon (circles), and inferior horizon (triangles). A presence/absence matrix of the MOTUs present in at least three samples was used for the correspondence analysis. All the MOTUs considered were obtained with ITS1F-ITS2 primers. The percentages indicate the variation explained by each axis.

The 690 MOTUs present in at least three samples were retained for the CoA. The four investigated habitats, i.e., fine roots or soil from the upper or lower horizon ( $n = 80$ ), were distinct regarding the microbial community (Fig 5A). The first axis of the CoA explained 22.7% of the variance. It separated the four habitats,

particularly the samples of the upper horizon (negative values of axis 1), from the samples of the lower horizon (positive values of axis 1) ( $F = 242.15$ ,  $p < 0.001$ ). There was a significant interaction between the horizon (superior or inferior) and material (fine roots or soil), with the difference between the horizons being far less pronounced for the soil material ( $F = 36.94$ ,  $p < 0.001$ ). The second axis explained 15.2% of the variation and was related to the material ( $F = 19.65$ ,  $p < 0.001$ ), but not to the horizon ( $F = 2.22$ ,  $p = 0.14$ ). The CoA computed only taking into account the 127 EcM fungi present in at least three samples showed a notably different pattern (Fig 5B). The distribution of the samples from the four habitats was different for the EcM MOTUs compared to what was observed for all of the fungal MOTUs. While the distribution on axis 1 was similar, with a significantly higher value observed for the lower horizon, ( $F = 53.86$ ,  $p < 0.001$ ) as well as a significant horizon \* material interaction ( $F = 7.53$ ,  $p = 0.0075$ ), the distribution on axis 2 was different. Indeed, the fine roots and soil samples were not significantly different regarding their position on axis 2 ( $F = 0.78$ ,  $p = 0.38$ ). Only the horizon factor significantly influenced the position on axis 2 ( $F = 12.93$ ,  $p < 0.001$ ).

## Discussion

A description of the fungal community at the plot level was achieved using 454 sequencing, which therefore allowed a sampling strategy to be outlined, including the corresponding sequencing effort required for future large-scale community studies where the plot is the basic unit. Remarkably, our work suggested that the soil habitat can be a good proxy for studying mycorrhizal assemblages. Additionally, this study was among the first to investigate oomycetes in forest soils using high-throughput sequencing, although these organisms are relatively difficult to detect compared to fungi. The fungal community observed in the present study showed spatial autocorrelation at a distance of  $< 15$  m and exhibited a differentiated vertical distribution along the soil profile.

To study the microbial community at a plot level, a compromise was required between the number of samples and the sequencing depth per sample (Taylor 2002, Cox *et al.* 2010). Indeed, as the number of sequences or samples increased, the number of MOTUs increased, with a similar proportion of singletons being recorded. In our case, the number of sequences was sufficient to describe the fungal community at the plot level and at the tree level, for which the community was described in the four habitats separately (RS, SS, RI, SI). Hence, under our experimental conditions, we suggest that four soil cores (20 cm depth) and approximately 15 trees with a sequencing depth of 10 500 sequences per tree unit allowed a good description of the fungal community within our relatively homogeneous 3 500 m<sup>2</sup> plot to be obtained. The large number of basidiomycetes observed may represent a bias due to the use of the ITS1F primer (Bellemain *et al.* 2010).

Our work suggested that the soil habitat was a good proxy for studying mycorrhizal assemblages. Our description of the EcM assemblage was based only on the taxonomically assigned MOTUs and the still incomplete literature addressing the ecological mode of fungi. Despite this limitation, our findings are in accordance with previous studies (Rosling *et al.* 2003; Landeweert *et al.* 2005). In particular, the EcM richness was not dependent on the type of material (fine roots or soil), and the CoA of EcM MOTUs was not able to separate the samples. Moreover, most of the MOTUs that were absent from the soil samples were supported by a low number of sequences (less than 120 / MOTU), and only 11% (21 / 192) were EcM MOTUs. Thus, the MOTUs that were not detected in the soil were not found at a high abundance in the fine roots. High-throughput sequencing appears to smooth out the niche differentiation between fine roots and soil, as we could detect almost all of the MOTUs found in the fine root samples in the soil samples as well. Nevertheless, this method allowed us to investigate the fungal community present on the fine roots more thoroughly and to detect rare MOTUs associated exclusively with fine roots. However, Dickie *et al.* (2002) thought it unlikely that the hyphae of a fungal species present on root tips in a sample could be totally from the surrounding soil. Additionally, Landeweert *et al.* (2005) did not detect any fungal species that were present on root tips but absent from soil. Some EcM species appear to be more frequent as mycelia in the soil than as mycorrhizal root tips (Kjøller *et al.* 2006), which was the case for many *Tomentella* species. In the present study, we indeed found that some EcM MOTUs were detected exclusively in the soil. However, this was not the case for *Tomentella*: *T. sublilacina* and *Pseudotomentella tristis* were more frequent and more abundant in the fine root samples compared to the soil samples. Other *Tomentella* MOTUs were similarly frequent in the fine root and soil samples, with a higher number of sequences in the fine root samples. *Boletus* spp. were also found as frequently in the soil as in the fine root samples, although this genus has been reported to be more frequent as mycelia in soil than as mycorrhizas (Kjøller *et al.* 2006). The findings reported in the literature are more conflicting for the *Cortinarius*. While Kjøller *et al.* (2006) retrieved some species from root tips, but not from the soil, Genney *et al.* (2006) detected extramatrical mycelia of *Cortinarius* more frequently compared to their presence at root tips. Our data confirmed these conflicting reports, as among the 39 *Cortinarius* MOTUs observed, nine were recorded exclusively from soil, while six were observed exclusively in the fine root samples. *Cortinarius* is probably the

largest agaricoid genus (Smith, 1973), and it is therefore not surprising that species with large differences in ecology can be found in this group.

As is commonly observed, rare MOTUs dominated the community, apart from a few abundant MOTUs. Among the most abundant EcM MOTUs, a number are generalists (*C. geophilum*, *B. pruinatus*), are species associated with acidoclines (e.g., *R. ochroleuca*, *B. erythropus*) or are preferentially present in deciduous forests (*R. ionochlora* under *Fagus* especially, *R. nigricans*, *R. vesca*). Other, less abundant, species were also recorded that are preferentially associated with beech trees (e.g., *L. subdulcis*, *R. fellea*, *A. citrina*, *R. mairei*) (Courtecuisse & Duhem, 1994). Additionally, the presence of young individuals of several other tree species on the plot could explain the detection of *L. quietus*, an oak-specific fungus, and *Russula puellaris*, which preferentially exists in interactions with coniferous species. Additionally, *Oidiodendron* sp., which was the most abundant MOTU found only in the fine root samples, forms ericoid mycorrhizas with *Vaccinium* species (Dalpé 1986), which were identified in our forest site.

To our knowledge, this study is the first to investigate oomycetes in forest soils using high-throughput sequencing. We detected only a few MOTUs of Pythiaceae species compared to those that are potentially present in beech forests, which include *Phytophthora cactorum*, *P. cambivora*, *P. gonapodyides*, *P. inflata*, *Pythium sylvaticum*, *P. acrogynum*, *P. inflatum* and *P. anandrum* (Nechwatal & Osswald 2001; Jung *et al.* 2005; Brasier & Jung, 2006; Vettraiño *et al.* 2008; Brasier 2009). However, Brasier (2009) estimated the number of potential *Phytophthora* species to be between 200 and 600, and 301 species of *Pythium* have been listed in the Index Fungorum to date. Compared to the 100 000 fungal species that have been described thus far (Hibbett *et al.* 2011), the proportion of four Pythiaceae MOTUs for 2000 putative fungal MOTUs was therefore somewhat expected. However, the Pythiaceae community is relatively difficult to detect (Scibetta *et al.* 2012) compared to fungi in general. Technical considerations could explain the small number of Pythiaceae detected. The specific primer sets used for nested PCR amplification (Cooke *et al.* 2000) were not as appropriate for environmental samples as for pure cultures. Indeed, a majority of the Dikarya were identified along with oomycetes within large (*Achlya*, *Phytophthora*, *Saprolegnia*, *Pythium*) and other unassigned MOTUs. New primers were recently reported for the detection of *Phytophthora* from soil and water samples that could improve the detection of these species in environmental samples (Scibetta *et al.* 2012). Furthermore, as for other fungi, we were not able to assign all of the MOTUs to the species level. Additionally, the sequences from *Phytophthora* clade 7a (approximately 230 bp) corresponded to *P. alni* subsp. *uniformis*, *P. fragariae* or *P. cambivora* reference sequences at a  $\geq 97\%$  identity and 100% coverage, and the sequences from *Phytophthora plurivora* (approximately 186 bp) also partially matched those of *P. inflata*. As few Pythiaceae species have been characterised thus far (Kroon *et al.* 2012) compared to EcM fungi, taxonomic affiliation remains a serious constraint in studying the Pythiaceae community. Improvements in technical and taxonomic methods for studying the Pythiaceae community are therefore clearly needed. From an ecological point of view, *Phytophthora* and *Pythium* have not been found to be widespread at a stand scale (Davison & Tay 2005). The trees in our study site were very healthy, showing no symptoms of decline, such as crown transparency or small or yellowish foliage, suggesting that they were not infested by pathogenic *Phytophthora* species. The plot conditions were moderately favourable for the presence of *Phytophthora* and *Pythium*. Fine and coarse sand represented more than 60% of the soil in the lower layer, and the soil pH range was between 4.0 and 4.3. *Phytophthora* have been found to be less frequent in acidic or sandy soils (Jung *et al.* 2000; Gomez-Aparicio *et al.* 2012). Furthermore, the date of sampling (beginning of December) was probably not the time when the highest load of Pythiaceae occurs in the soil habitat. However, the use of molecular techniques might have partly overcome this problem, as hard life stages and DNA may persist in the soil after the active season, as for fungi.

This study is among the few investigations to explore the spatial distributions of EcM species (Courty *et al.* 2008) and Pythiaceae species (Davidson & Tay 2005; Gomez-Aparicio *et al.* 2012) in temperate forests. No spatial structure could be found for Pythiaceae. The fungal community in our study was spatially autocorrelated at a distance of < 15 m., and autocorrelation has also been reported elsewhere (Lilleskov *et al.* 2004; Toljander *et al.* 2006). Toljander *et al.* (2006) found that the EcM community structure varied along a nutrient gradient characterised based on NH<sub>4</sub>, base saturation, C:N ratio and pH data, although they could not identify the relative importance of these factors. In the present study, the fungal and EcM communities were structured by the C:N ratio and phosphorus, but not by the pH, which was more homogeneous within the plot.

The vertical distribution of mycorrhizas has been reported in several studies in podzolic mixed and coniferous boreal forests (Tederloo *et al.* 2003; Rosling *et al.* 2003; Lindahl *et al.* 2007), but it has also been explored rarely in temperate deciduous forests (Courty *et al.* 2008). At the plot level, the vertical abundance distribution was conserved, regardless of the examined material (fine roots or soil). In the present study, the dry fine root biomass, pH, C:N ratio and P were found to be confounding factors that varied with depth and that could be linked to the composition of the fungal community along the vertical profile. The higher fine root biomass observed in the upper organic horizon is in accordance with previous studies (Rosling *et al.* 2003; Bakker *et al.* 2008, Courty *et al.* 2008). These data were in agreement with those Taylor (2002), Rosling *et al.* (2003) and

Tedersoo *et al.* (2003), who advocated sampling the mineral horizon in addition to the organic horizon. Similar to most previous reports, *Cenococcum geophilum* was abundant and ubiquitous in the two horizons investigated. Some groups were, however, more abundant in one of the two layers. Indeed, the Cantharellales, such as *Clavulina cristata*, *Hydnum rufescens*, *Craterellus tubaeformis* and *Botryobasidium subcoronatum*, were more abundant in the first 10 cm, where they could play a role in organic matter processing. Likewise, the Atheliaceae *Piloderma fallax*, *Byssocorticium atrovirens*, *B. sp1* and *Tylospora asterophora*, which were more abundant in the upper layer, have previously been recorded in an organic substratum (Tedersoo *et al.* 2003). *Tomentella sublilacina* was also mostly found in the organic layer (Courty *et al.* 2008), along with the several MOTUs assigned to the *Cladophialophora*. Two Helotiales, *Botrytis sp1* and *Cudoniella sp1*, were also recorded in this upper layer, which is not consistent with the previous observation of the presence of *Cudoniella* in deeper soil (Jumpponen *et al.* 2010), but both fungi have been reported to show a preference for wet conditions. In contrast to the above species, *Armillaria sp1*, *Leotia lubrica*, *L. atrovirens* and *Microglossum sp1* were confined to the lower horizon.

## CONCLUSION

Our results showed that in an apparently homogeneous plot comprising 20 beech trees, the fungal and EcM communities exhibited a spatial variability linked to the examined edaphic parameters. Consequently, a sufficient sampling effort at a local scale should be applied to fully capture the fungal diversity present. In our temperate beech forest plot, we estimated that approximately 15 trees should be sufficient for this purpose. However, the laboratory work could be facilitated by employing bulk soil as a good, easy-to-handle substitute for the fine roots to study EcM assemblages using the pyrosequencing technique, although the relative abundance was not similar. The fungal and EcM communities also exhibited a vertical soil distribution, which could be explained by differences in soil parameters between the lower and upper horizons. Sampling several soil horizons is, therefore, encouraged for analyses of diversity, although it could be difficult for studies conducted at a large geographical scale, which encompass samples from several soil types with structurally different profiles. Care should be taken in extrapolating these findings to other situations (especially in different forest types or under different host trees), as only one plot was studied in the present work, and additional studies are required to confirm our findings.

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