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Spatial variability of phyllosphere fungal assemblages: genetic distance predominates over geographic distance in a European beech stand (*Fagus sylvatica*)

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ABSTRACT

Despite being major drivers of the dynamics and diversity of plant populations and communities, the spatial variability of phyllosphere fungal assemblages has been seldom explored. We used CE-SSCP and 454 pyrosequencing to quantify the spatial variability of European beech phyllosphere fungal assemblages with a hierarchical sampling design including four different spatial scales (tree, branch, group of leaves and individual leaf). Fungal assemblages were highly diverse, with high proportions of generalist and cosmopolitan fungi. The major part of the variability was at the smallest spatial scale, between individual leaves. Within a single tree canopy, dissimilarity between fungal assemblages generally increased with distance between leaves. This pattern may be driven by within-canopy gradients of leaf traits and microclimate. At the stand scale, dissimilarity between fungal assemblages was correlated with the genetic distance rather than the geographic distance between trees, consistent with the findings of community genetics studies. Our results were constant over a small simulated sequencing depth, providing opportunities for the design of large-scale studies addressing the relationship between the genetic variation of trees and the variation of associated phyllosphere fungal assemblages.

Introduction

The phyllosphere, the habitat provided by the leaves of living plants, is one of the largest microbial habitats on Earth, with an estimated global surface area of more than 4×10^8 km^2 (Morris & Kinkel 2002). It supports diverse and complex microbial communities, including many genera of bacteria and fungi (Lindow & Brandl 2003; Jumpponen & Jones 2009; Redford *et al.* 2010). Phyllosphere fungal species include both epiphytes living on the leaf surface (Inácio *et al.* 2002; Lindow & Brandl 2003) and endophytes living within the tissues of the plant (Rodriguez *et al.* 2009). However, the distinction between

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endophytic and epiphytic fungal species is ambiguous, because some epiphytic fungi also actively penetrate the epidermis or stomata and colonize internal leaf tissues (Viret *et al.* 1994). In this study, we define phyllosphere fungi as those species inhabiting both the surface and the interior of leaves (Jumpponen & Jones 2009), and fungal assemblages as the addition of all fungal species inhabiting the phyllosphere (Fauth *et al.* 1996).

Phyllosphere fungi influence the fitness of their host plants, either negatively as pathogens (Gilbert 2002; Newton *et al.* 2010) or positively, by increasing the stress tolerance of the plant (Rodriguez & Redman 2007), by reducing herbivory through the production of toxic alkaloids (Wilkinson *et al.* 2000) or by reducing the infection of plant tissues by pathogens (Arnold *et al.* 2003). Phyllosphere fungal assemblages also contribute to nutrient cycling, as early colonizers in leaf litter decomposition (Osono 2006). They are consequently major drivers of the dynamics and diversity of plant populations and communities (Clay & Holah 1999; Bradley *et al.* 2008). They also influence the dynamics of other taxonomic groups, such as phyllosphere bacteria, phytophagous insects and insect parasitoids (Omacini *et al.* 2001; Suda *et al.* 2009).

Despite the ecological importance of phyllosphere fungal assemblages, little is known about their diversity and spatial variability. Our knowledge of phyllosphere fungal diversity has long been limited by the use of culture-dependent methods, which are time-consuming and suffer from many biases. For instance, cultures systematically preclude biotrophic species and tend to be biased toward fast-growing fungi, although major advances toward overcoming this last problem have been made (Unterseher & Schnittler 2009). New culture-independent methods, such as molecular fingerprinting (Muyzer & Smalla 1998) and high-throughput DNA sequencing (Shendure & Ji 2008), have made it possible to obtain a more complete description of fungal diversity (see Buée *et al.* 2009 for soil fungal assemblages). In particular, high-throughput DNA sequencing can be used for the molecular identification of species through the use of barcode genes (Nilsson *et al.* 2010; Bengtsson *et al.* 2011). Such tools hold great promise for improving our understanding of the ecology of phyllosphere fungi.

Jumpponen & Jones (2009, 2010) used high-throughput DNA sequencing to study fungal assemblages inhabiting the bur oak (*Quercus macrocarpa*) phyllosphere. They reported the presence of highly diverse fungal assemblages. Assemblage richness and composition differed between adjacent environments (rural and urban), suggesting a non random spatial distribution of phyllosphere fungi. Very few molecular operational taxonomic units (MOTUs) were detected at high frequency and many were rare, as frequently reported in fungal metagenomic datasets (Unterseher *et al.* 2011). Their results also suggest that phyllosphere fungal assemblages are dominated by ascomycetes. However, many MOTUs could not be correctly assigned to any species in the absence of matching sequences in international nucleotide sequence databases, such as GenBank (http://www.ncbi.nlm.nih.gov/genbank/).

In the present study, we used two culture-independent techniques, capillary electrophoresis single-strand conformation polymorphism (CE-SSCP) (Zinger *et al.* 2008) and tag-encoded 454 pyrosequencing (Binladen *et al.* 2007), to assess the diversity and spatial variability of phyllosphere fungal assemblages in European beech (*Fagus sylvatica*). We used a hierarchical sampling design to estimate the spatial variability of phyllosphere fungal assemblages at tree, branch, group of leaves and individual leaf levels. Then, we investigated whether the geographic distance between leaves or trees could account for the dissimilarity of their fungal assemblages. At the stand level, we further investigated whether two alternative distances (the phenological and genetic distances between trees) were better at explaining the dissimilarity of fungal assemblages than the geographic distance.

Materials and methods

Study site and hierarchical sampling design

The forest of Laveyron (GPS +43° 45′ 51.32″, −0° 13′ 13.86″) is a mixed oak-beech stand managed by the *Office National des Forêts* (ONF: http://www.onf.fr/). Nine beech trees, separated by distances of a few meters to about 300 m, were selected on the basis of height (all about 20 m high) and circumference (from 180 cm to 250 cm). Trees were geo-referenced with a GPS system. On 27 May 2009, three branches per tree were sampled at a height of about 7 m above the ground. The three branches were chosen on the basis of their orientation (north, south-east and south-west). We collected nine leaves from each branch: three leaves about 1 m from the trunk, three leaves in the middle of the branch (about 2 m from the trunk) and three leaves from the most distal part of the branch, about 3 m from the trunk (Fig 1). Each sampled leaf was the second from the base of the twig, to minimize the effect of leaf age within trees. We thus collected a total of 243 leaves (nine trees × three branches × three groups of leaves on the branch × three leaves), which were placed in individual filter paper envelopes, brought back to the laboratory, dried and stored at room temperature (in their envelopes) until DNA extraction.

DNA extraction

Under a laminar flow hood, four discs of 2 cm^2 each were cut from each leaf and placed in a well of an autoclaved DNA extraction plate. The punch was sterilized between each leaf sample using 70 % ethanol and a flame. One metallic bead was added to each well and the plant material was ground into a homogeneous powder with a Geno/Grinder 2010 (SPEX SamplePrep, Metuchen, NJ, USA). Total DNA was then extracted with a CTAB buffer, with the addition of β-mercaptoethanol (0.5 %, Sigma–Aldrich, St. Louis, MO, USA) and proteinase K (20 mg/ml, Sigma–Aldrich). Each well was filled with 400 μl of the CTAB extraction buffer, heated and shaken at 60 °C for 2 hr in an incubator (New Brunswick Scientific, Edison, NJ, USA). Samples were treated with 320 μl of phenol/chloroform/isoamyl alcohol (25:24:1, pH = 8), briefly mixed, centrifuged at 364*g* for 10 min at 4 °C, and supernatant was transferred into novel autoclaved plates. DNA was precipitated overnight using absolute isopropanol at −20 °C, pelleted

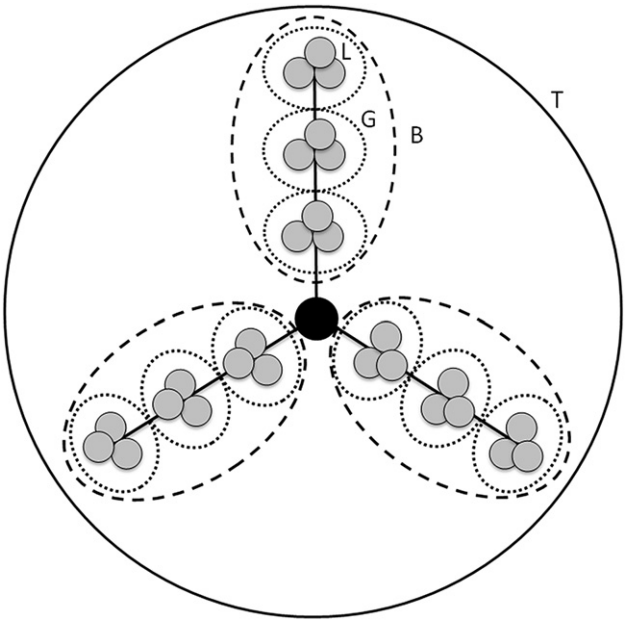

Fig 1 – Hierarchical sampling design used to estimate the spatial pattern of variability of phyllosphere fungal assemblages. The four spatial levels studied are circled with different lines: tree (T, continuous line), branch (B, dashed line), group of leaves (G, dotted line), leaf (L, circles). The diagram represents a tree canopy seen from above, with the circle in the middle representing the tree trunk and the three lines representing the three sampled branches. Branches are oriented from each other at an angle of 120°, and groups of leaves on a branch are separated from the nearest neighboring group of leaves by a distance of 1 m.

by centrifugation (5000 rpm for 10 min at 4 °C), double washed in 70 % ethanol (−20 °C), dried for 1 hr in a speed vac plus (Savant Instruments, Farmingdale, NY, USA), and eluted in 50 μl ultra-pure water (Sigma–Aldrich). DNA was quantified with a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA), and concentration was adjusted to 5 ng μl^{-1} with ultra-pure water (Sigma–Aldrich).

PCR amplification of rITS1

PCR amplification targeted the rITS1 (ribosomal internal transcribed spacer 1) of the nuclear ribosomal repeat unit, which has been identified as a candidate barcoding locus for the identification of fungi to species level (Nilsson *et al.* 2009; Seifert 2009). The primer pair used was ITS1F (Gardes & Bruns 1993), for fungus-specific amplification, and the universal primer ITS2 (White *et al.* 1990). PCR was performed in 96-well plates, with randomization and the use of a robotic pipettor (Hamilton, Reno, NV, USA). The pit boards were avoided and filled with 25 μl water (to prevent Peltier effects). Reactions were performed in a volume of 25 μl containing 1× buffer (Eurogentec, Liege, Belgium), 2 mM $MgCl_2$, 200 mM of each dNTP, 200 nM of each primer, 0.5 units of *Taq* polymerase (Bio-Rad, Hercules, CA, USA), 10× bovine serum albumin (Bio-Rad), and 2 μl of environmental DNA (i.e. 10 ng). After initial denaturation at 95 °C for 5 min, PCR was carried out with 30 cycles of 95 °C for 30 s, 54 °C for 1 min, 72 °C for 1 min 30 s, and a final extension at 72 °C for 10 min. Extraction- and PCR-negative controls were included on each plate. No PCR amplicon was detected in these negative controls.

CE-SSCP of rITS1 amplicons

Fungal diversity was characterized by CE-SSCP, with the ITS2 primer labeled at the 5′ end with a fluorochrome (6FAM). PCR was performed on the DNA sample from each leaf. Each PCR product was diluted by a factor of four in sterile water (Sigma–Aldrich) and 1 μl was added to 8.8 μl of formamide (Applied Biosystems, Foster City, CA, USA) and 0.2 μl of ROX 400HD ladder (Applied Biosystems). The samples were denatured by heating for 5 min at 95 °C and cooling on ice for 10 min, immediately before loading onto a four-capillary ABI 3130 machine (Applied Biosystems). Migration was allowed to occur for 45 min at 32 °C (Zinger *et al.* 2008) with default settings used for all other parameters.

All readable molecular fingerprint profiles were aligned with the internal ROX ladder and normalized with the R package StatFingerprints v1.3 (Michelland *et al.* 2009a; R Development Core Team 2010). This yielded a matrix in which leaf samples were indicated in rows and fluorescence values (2766 scans) in columns. A fluorescence profile may be seen as a quantitative descriptor of the fungal assemblage of a sample. Larger differences in fluorescence scans between profiles indicate a greater dissimilarity in composition between samples (Michelland *et al.* 2009b).

454 Pyrosequencing of rITS1 amplicons

Fungal diversity was also characterized by 454 pyrosequencing, with Titanium fusion primers used for PCR amplification. The ITS2 reverse primer included the A adaptor, a five-nucleotide tag and the template-specific sequence, whereas the ITS1F forward primer contained the B adaptor (Table S1). This primer design generated reverse sequences across ITS1, thus minimizing sequencing of the conserved 3′ end of the nuclear small subunit RNA gene (nSSU), because the ITS2 primer matches less far in the 5.8S gene than does the IT1F primer in the nSSU.

We did not use this method to study the variability of fungal assemblages between leaves from the same branch. We therefore pooled the nine DNA samples from each branch. We thus obtained 27 DNA samples, corresponding to the 27 branches sampled (nine trees × three branches per tree). Three PCR were performed on each sample, to account for heterogeneous amplification. The three PCR products were pooled and purified with the AMPure XP purification kit (AgenCourt Bioscience, Fullerton, CA, USA), which effectively eliminates primer dimers. The cleaned products were quantified, and 100 ng of each of the 27 samples was pooled in a single tube. This pool was sequenced in a region corresponding to one-eighth of a 454 GS-FLX Titanium sequencer (454 Life Sciences, Branford, CT, USA) sequencing plate, at the Genoscope, Evry, France. With a theoretical yield of 100 000 sequences per region, we expected a sequencing depth of

almost 3700 sequences per sample. Raw data are available upon request.

The 454 dataset was demultiplexed with the Ribosomal Database Project pyrosequencing pipeline (http://pyro.cme.msu.edu/), using the following quality filters: no mismatch was allowed in the five-nucleotide tag, the ITS2 primer sequence had to be retrieved with a maximum edit distance of two (the edit distance is the number of steps required to transform a sequence into another by insertion, deletion or substitution), a minimum sequence length of 100 bp, fewer than three ambiguous nucleotides per read and a quality score for base calling exceeding 20, on average, across the whole read. The sample files were pooled into a single Fasta file (keeping the sample information in each read entry), and the highly variable ITS1 was extracted with the perl program ITS Extractor (Nilsson *et al.* 2010). Forward and reverse pyrosequencing primers were blasted against the ITS1 dataset to check extraction efficiency, and matching sequences were removed. The ITS1 locus in fungi was shown to have a median length of 183 bp among 4185 species (Nilsson *et al.* 2008). In our dataset, the median length was of 166 bp, ranging from 52 bp to 400 bp. We thus decided to remove ITS1 sequences shorter than 100 bp.

This cleaned dataset was clustered into molecular operational taxonomic units (MOTUs) with a 97 % similarity threshold, by using the UCLUST algorithm (Edgar 2010) implemented in the *pick_otus* function of the Quantitative Insight Into Microbial Ecology toolkit (QIIME 1.1, Caporaso *et al.* 2010). Before MOTU clustering, a preliminary clustering was carried out with a 100 % similarity threshold, and the groups of identical sequences obtained were sorted in descending order of abundance. The most abundant sequences were then used as the seeds for clustering. This pre-sorting on the basis of abundance is an important step of MOTU clustering, because the most abundant sequences are more likely to be "true" biological sequences, whereas less common sequences or singletons may be PCR or sequencing artifacts. MOTU clustering was performed with the optimal flag option, which allows finding the optimal alignment between two sequences before calculating their similarity.

Each seed sequence was compared with the sequences deposited in GenBank, with the BLASTN algorithm (Altschul *et al.* 1997). Environmental sequences were first excluded for the purposes of taxonomic identification. We applied a threshold of at least 97 % similarity over at least 90 % of the query length of a fully annotated accession for the assignment of a species name to a MOTU or for the assignment of a genus name when the annotated accession contained only the genus name. Environmental sequences were then included for characterization of the remaining MOTU and the same assignment threshold was used to assign our MOTUs to environmental sequences deposited in GenBank.

The number of sequences per MOTU was considered a proxy for the abundance of the associated molecular species (Unterseher *et al.* 2011 but see Amend *et al.* 2010). The composition of the fungal assemblages was therefore represented by a quantitative matrix giving the relative abundance of each MOTU in each sample. However, as the relationship between the number of sequences and species abundance is uncertain (Amend *et al.* 2010), we also computed the corresponding presence–absence matrix. We built one dataset with "core" MOTUs only and one dataset with "satellite" MOTUs only. Core MOTUs were defined as MOTUs present in at least half the samples, whereas satellite MOTUs were MOTUs present in less than half the samples (Unterseher *et al.* 2011). Finally, four rarefied datasets, with 50, 100, 200 and 300 sequences per sample, were constructed with the *multiple_rarefaction* function in QIIME, to assess the robustness of our results to variations in sequencing depth. This function randomly samples the quantitative matrix to build the rarefied dataset.

Statistical analyses

Compositional dissimilarity of fungal assemblages between samples was estimated with the Bray–Curtis dissimilarity index (Bray & Curtis 1957), and pair-wise dissimilarities between samples were analyzed by a non-metric multidimensional scaling (NMDS) plot. NMDS was performed with the *metaMDS* function of the R vegan package (Oksanen *et al.* 2010). The Bray–Curtis dissimilarity matrix obtained from the CE-SSCP dataset was then analyzed in a hierarchical experimental design with three factors (tree, branch and group of leaves, all considered random) using permutational multivariate analysis of variance (PERMANOVA, see Anderson 2001). The Bray–Curtis dissimilarity matrices obtained from the 454 sequencing datasets were analyzed with a one-way model (tree considered random). The multivariate component of variation for each spatial scale was then calculated from the mean squares of the PERMANOVA, using the method of moments (Searle *et al.* 1992). This calculation yielded additive components of variation (Anderson *et al.* 2011). Analyses of variance were performed with the *adonis* function of the R vegan package (Oksanen *et al.* 2010), by using 999 permutations.

We investigated whether the geographic distance between leaves within a tree canopy could account for the dissimilarity of their fungal assemblages, by estimating the pair-wise distances between all groups of leaves within a tree canopy. The correlation between the Bray–Curtis dissimilarity matrix (calculated from the CE-SSCP dataset averaged by group of leaves) and the geographic distance matrix was then assessed with a Mantel test (Spearman's rank correlation, 999 permutations) for each of the nine trees. Mantel tests were performed with the *mantel* function of the R ecodist package (Goslee & Urban 2007).

Finally, we investigated whether the geographic distance between trees within a forest stand could account for the dissimilarity of their fungal assemblages, by calculating the geographic distances between all trees (based on their GPS positions). The phenological distance and the genetic distance between all trees were also estimated and tested as alternative distances. Phenological distance may account for fungal assemblage structure, through differences in the early colonization of leaves (Saikkonen *et al.* 1998), whereas genetic distance may account for fungal assemblage structure through differences in plant phenotype (Whipps *et al.* 2008). The date of bud burst was defined as the day on which half the canopy buds had hatched (Vitasse *et al.* 2009) and the phenological distance between two trees was defined as the absolute

number of days between bud bursts. The trees were genotyped for eight microsatellite loci (Lefèvre et al., 2011, see Tables S2 and S3) and we calculated the pair-wise genetic distance (DAS distance, i.e. shared allele distance) between all trees, with the Population program (version 1.2.32, Langella 1999, unpublished). As the geographic, phenological and genetic distances were calculated between trees (and not between leaves or branches), we averaged the CE-SSCP profiles for each tree and calculated the relative abundance of MOTUs for each tree. We then calculated the corresponding Bray–Curtis dissimilarity matrices. We performed partial Mantel tests with the *mantel* function of the R ecodist package (Spearman's rank correlation, 999 permutations), to investigate whether the dissimilarity in the composition of phyllosphere fungal assemblages increased with the geographic, phenological and genetic distance between trees.

Results

Species richness and composition of the phyllosphere fungal assemblage

In total, 123 163 sequences were obtained from the 1/8th 454 pyrosequencing run. The cleaned dataset comprised 96 130 sequences, divided into 27 samples, each corresponding to a branch. The mean number of sequences per sample was 3560, with a range of 386–6920 sequences per sample. The clustering of these sequences, with a threshold of 97 % similarity, gave a total of 1604 MOTUs. Among them, we identified five plant MOTUs, representing 159 sequences (including 134 sequences from European beech). These MOTUs were removed from the dataset before statistical analyses. The final dataset comprised 1599 fungal MOTUs, including 560 singletons. The mean number of MOTUs per branch was 240 (SD = 76) and the mean number per tree was 468 (SD = 109). We defined 118 fungal MOTUs as constituting the "core" of the assemblage (Table S4), with 12 of these MOTUs accounting for more than 1 % of the total number of sequences of the dataset (Table 1).

We were able to assign 406 of the 1599 fungal MOTUs to species level and 128 to genus level. The remaining 1065 MOTUs were above the threshold but corresponded to insufficiently annotated accessions (111 MOTUs), were below the threshold (550 MOTUs), did not match any annotated GenBank accession but matched environmental sequences (154 MOTUs), or did not match any GenBank accession (250 MOTUs). Fifty-two of the taxonomically assigned species were represented by several different MOTUs (two to 29 MOTUs), yielding 226 unique species. The number of MOTUs per species was significantly correlated with the number of sequences assigned to the species concerned (Spearman's rho = 0.55, $p < 0.001$). Thus, more abundant species are generally divided into larger numbers of different MOTUs. The most abundant species were two ascomycetous yeasts, *Lalaria inositophila* (11 % of the total number of sequences; 29 MOTUs) and *Taphrina carpini* (10 %; 27 MOTUs). It is noteworthy that these two abundant species may be closely related because *Lalaria* is used as an anamorph genus name when the teleomorph state (*Taphrina*) is unknown (Inácio *et al.* 2004). The

Table 1 – Taxonomic assignment of the 12 most abundant MOTUs, based on BLAST analysis of MOTU seed sequences against GenBank. Coverage is the percentage of the query length covered by the alignment. Similarity is the percentage identity over the alignment. Closest matches with more than 97 % similarity over at least 90 % of the query length are shown in bold. The relative abundance of a MOTU is the number of sequences associated with that MOTU over the total number of sequences in the dataset. Source indicates the source of the sequence associated with the underlined accession

GenBank accession	MOTU relative abundance	GenBank (environmental excluded)			GenBank (environmental included)				
		Closest match	Coverage/ similarity	Putative taxon	Closest match	Coverage/ similarity	Putative taxon	Source
JF945443	19.76	AY239214	96/86	*Lalaria inositophila*	GQ999402	100/96	Uncultured fungus	Air filter sample, Germany, Fröhlich-Nowoisky *et al.* unpublished
JF946080	11.03	AY230777	94/80	*Woollsia* mycorrhizal fungus	**GQ508562**	**100/99**	**Uncultured fungus**	**Q. *macrocarpa* phyllosphere, Kansas, USA,** Jumpponen & Jones 2010
JF946013	10.48	**AY239214**	**100/100**	***Lalaria inositophila***	"	"	"	**Phylloplane, *Prunus persica*, Lisbon, Portugal,** Inácio *et al.* 2004
JF945320	9.15	**AY239215**	**100/99**	***Taphrina carpini***	"	"	"	**Phylloplane, *Quercus pyrenaica*, Caramulo, Portugal,** Inácio *et al.* 2004
JF945549	5.43	**JF440584**	**100/100**	***Aureobasidium pullulans***	"	"	"	**Xylem of *Pinus migo*, Lithuania, Lygis *et al.* unpublished**
JF945566	3.9	FN824490	66/89	*Cryptococcus stepposus*	GQ514146	65/94	Uncultured fungus	*Q. macrocarpa* phyllosphere, Kansas, USA, Jumpponen & Jones 2010
JF945021	3.41	**AB109183**	**100/100**	***Venturia hanliniana***	**FJ820756**	**100/100**	**Uncultured fungus**	**Air filter sample, Germany,** Fröhlich-Nowoisky *et al.* 2009
JF945768	2.4	**FR668008**	**100/100**	***Mycosphaerella punctiformis***	"	"	"	***Eucalyptus globulus* leaves, Spain,** Sánchez Márquez *et al.* 2011
JF945651	2.29	**EF687931**	**100/100**	***Cryptococcus* sp.**	**FJ762780**	**100/100**	**Uncultured fungus**	**Q. *macrocarpa* phyllosphere, Kansas, USA,** Jumpponen & Jones 2009
JF945444	1.4	AY239214	96/86	*Lalaria inositophila*	GQ999402	100/94	Uncultured fungus	Air filter sample, Germany, Fröhlich-Nowoisky *et al.* unpublished
JF945157	1.17	**HQ717401**	**100/100**	***Davidiella* sp.**	**HQ873349**	**100/100**	**Uncultured fungus**	**Air at 4200 m, Quilian Mountain, Tibetan plateau, Gai *et al.* unpublished**
JF945269	1.05	**AJ853459**	**100/100**	***Rhodosporidium lusitaniae***	"	"	"	**Yeast strains isolated from alpine habitat, Austria,** Bergauer *et al.* 2005

assigned MOTUs were distributed into 21 orders in Ascomycota (A) and 18 orders in Basidiomycota (B). Taphrinales (A) was the most frequently represented order, with almost 42 % of the taxonomically assigned sequences, followed by Capnodiales (A, 13 %), Dothideales (A, 12 %), Tremellales (B, 9 %), Sporidiobolales (B, 4 %), Cystofilobasidiales (B, 3 %), Erythrobasidiales (B, 2 %), Helotiales (A, 1 %), Pleosporales (A, 1 %), and Filobasidiales (B, 1 %). Each of the remaining orders accounted for less than 1 % of the taxonomically assigned sequences. Nine groups of MOTUs could not be assigned to the order level (*incertae sedis*), representing 9 % of the taxonomically assigned sequences.

A list of all the MOTUs, with their abundance and taxonomic assignment, is available in Table S4. The seeds of all MOTUs were retrieved from the demultiplexed sequence dataset (i.e. the dataset including nSSU and 5.8S in addition to ITS1) and seeds longer than 200 bp are available under GenBank accession nos. **JF944899**–**JF946081**. The entire set of fungal MOTUs ITS1 is available as a supplementary Fasta file.

Spatial pattern of variability

The NMDS plot of the Bray–Curtis dissimilarity matrix obtained from the 233 readable CE-SSCP profiles revealed that intra-host variability of phyllosphere fungal assemblages was clearly greater than inter-host variability, with the leaves of each tree spread out over the ordinate axis (Fig 2A). Trees were moderately separated on the NMDS plot. Tree #4 was clearly different from the other trees, which displayed some degree of overlap (Fig 2A). Analysis of variance showed that phyllosphere fungal assemblages differed significantly between trees and between branches of the same tree, but not within a given branch (Table 2). Identical results were obtained when tree #4 was excluded (not shown). Variance partitioning confirmed that variability was greatest at the level of leaf replicates (i.e. between leaves from the same group of leaves). This level of variability, which corresponds to the residual variance, accounted for almost 69 % of the total variance. Dissimilarity in composition between trees accounted for 24 % of the total variance, whereas dissimilarity in composition between branches accounted for 7 % of the total variance (Table 2).

The NMDS plot of the Bray–Curtis dissimilarity matrix obtained for the total quantitative 454 sequencing dataset confirmed the inter-host variability of the phyllosphere fungal assemblages. Trees were well separated on the ordinate axis, with the branches of each tree relatively close together (Fig 2B). As with the CE-SSCP dataset, analysis of variance showed that the composition of fungal assemblages differed significantly between trees (Table 2). Compositional dissimilarity between trees accounted for almost 66 % of the total variance, whereas compositional dissimilarity between branch replicates (residual variance) accounted for 34 % of the total variance. Analysis of variance on the presence–absence 454 sequencing dataset also showed that the composition of fungal assemblages differed significantly between trees. However, the proportion of the variance accounted for by tree effect was smaller than that for the quantitative dataset, at only 17 % of total variance (Table S5). Analyses of variance on the quantitative datasets including core MOTUs only and satellite MOTUs only also showed a significant effect of tree on the composition of phyllosphere fungal assemblages. Differences in the abundance of core MOTUs between trees accounted for 74 % of the total variance, whereas differences in the abundance of satellite MOTUs between trees accounted for 17 % of the total variance (Table 2). Finally, the effect of tree

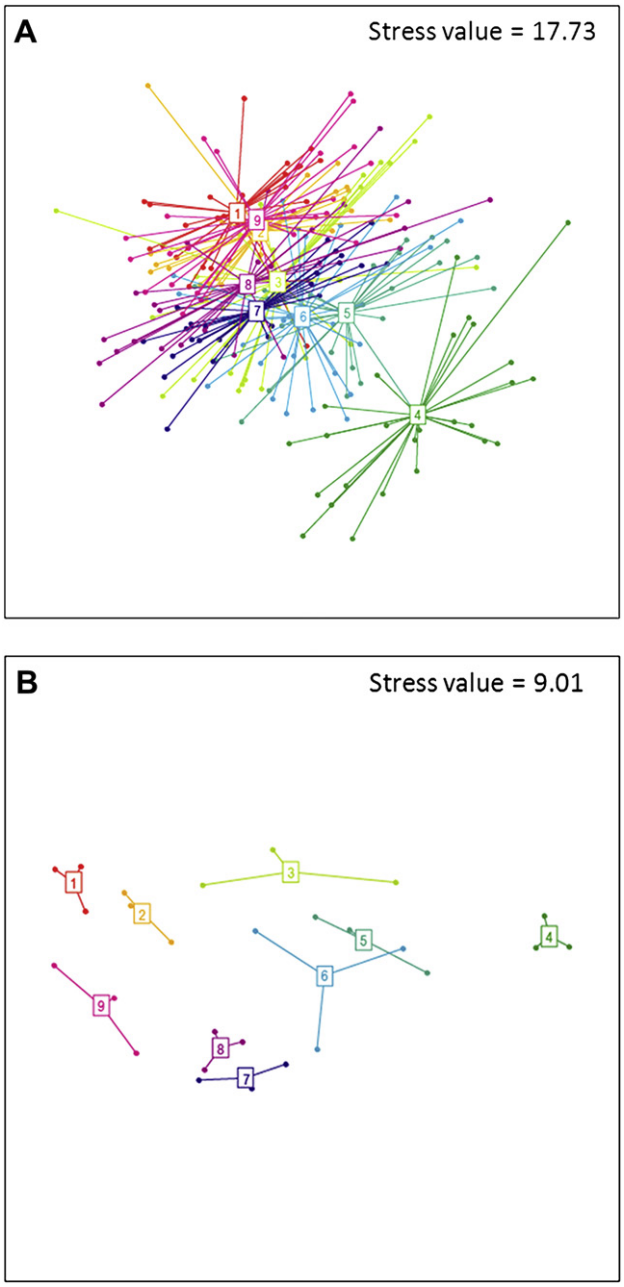

Fig 2 – NMDS plot of phyllosphere fungal assemblages associated with nine beech trees. Each colour represents a tree and dots represent samples. In A, each sample is a leaf ($n = 233$). Fungal composition of each sample was assessed by CE-SSCP on rITS1 amplicons. In B, each sample is a branch ($n = 27$). Fungal composition of each sample was assessed by the 454 pyrosequencing of rITS1 amplicons. The compositional dissimilarity between samples was assessed with the Bray–Curtis dissimilarity index.

Table 2 – Permutational multivariate analysis of variance of the compositional dissimilarity between phyllosphere fungal assemblages associated with beech leaves ($n = 233$, CE-SSCP) and beech branches ($n = 27$, 454 pyrosequencing). The analyses are based on the sample × fluorescence (CE-SSCP) and on the 454 quantitative sample × MOTU matrix including all MOTUs (All), core MOTUs only (Core) or satellite MOTUs only (Sat). The compositional dissimilarity between assemblages was assessed with the Bray–Curtis dissimilarity index.

Data	Source	*df*	Mean Sqs	*F*	*p*	Explained variance (%)
CE-SSCP	Tree	8	0.45776	11.3216	**0.001*****	23.68
CE-SSCP	Branch	18	0.08032	1.9864	**0.001*****	7.34
CE-SSCP	Group of leaves	54	0.0413	1.0215	0.369	0.49
CE-SSCP	Residuals	152	0.04043			68.49
CE-SSCP	Total	232				
All	Tree	8	0.261333	6.7465	**0.001*****	65.7
All	Residuals	18	0.038736			34.3
All	Total	26				
Core	Tree	8	0.232056	9.4359	**0.001*****	73.8
Core	Residuals	18	0.024593			26.2
Core	Total	26				
Sat	Tree	8	0.52917	1.5974	**0.001*****	16.6
Sat	Residuals	18	0.33126			83.4
Sat	Total	26				

Significant tests are shown in bold, and * $0.01 \leq P < 0.05$; ** $0.001 \leq P < 0.01$; *** $P < 0.001$.

was also significant in the four rarefied 454 sequencing datasets. The proportion of the variance accounted for by the tree effect decreased slightly with the simulated sequencing depth, from 55 % with 300 sequences per sample to 40 % with 50 sequences per sample (Table S6).

Geographic distance and dissimilarity between fungal assemblages

Mantel tests on the sample × MOTU matrix obtained by CE-SSCP showed that the compositional dissimilarity between phyllosphere fungal assemblages increased significantly with the distance between leaves within the tree canopy, for five of the nine trees (Table 3). By contrast, geographic distance between trees could not account for the compositional dissimilarity between trees. Phenological and genetic distances between trees were also insufficient to account for dissimilarity in composition between trees (Table 4).

Partial Mantel tests on the quantitative sample × MOTU matrix obtained by 454 pyrosequencing confirmed that the geographic distance between trees could not account for phyllosphere fungal assemblage structure at the stand scale. However, a significant correlation was observed between the genetic distance between trees and dissimilarity in the composition of their associated assemblages (Table 4, Fig 3). Significant correlations were also obtained with compositional dissimilarity matrices derived from the core MOTU dataset (Table S7) and the four rarefied 454 sequencing datasets (Table S8).

Table 3 – Mantel correlation between the geographic distance between group of leaves within a tree canopy and the compositional dissimilarity of fungal assemblages associated these groups of leaves. Species composition of each leaf was assessed by CE-SSCP of rITS1 amplicons and profiles were averaged by group of leaves before Mantel test. The compositional dissimilarity between assemblages was assessed with the Bray–Curtis dissimilarity index.

	Spearman rho	*p*-value
Tree #1	0.197	0.183
Tree #2	0.3202	0.067
Tree #3	0.3483	**0.046***
Tree #4	0.3393	**0.047***
Tree #5	−0.009	0.51
Tree #6	0.479	**0.007*****
Tree #7	0.5086	**0.003*****
Tree #8	0.4142	**0.018***
Tree #9	0.3109	0.065

Significant tests are shown in bold, and * $0.01 \leq P < 0.05$; ** $0.001 \leq P < 0.01$; *** $P < 0.001$.

Discussion

The beech phyllosphere houses highly diverse fungal assemblages

We showed, with high-throughput sequencing techniques, that a couple of dozen leaves from a single beech tree housed approximately 400 fungal molecular operational taxonomic units (or MOTUs). The fungal assemblages associated with beech leaves are therefore highly diverse, but it is difficult to determine the exact number of species within the tree canopy from the observed number of MOTUs. The true species richness is likely lower than the richness in MOTU, due to the combined effects of methodological and biological biases. First, PCR and sequencing artifacts are known to overestimate the perceived species richness (Quince *et al.* 2009; Reeder & Knight 2009; Dickie 2010), and such artifacts are even more likely to occur with abundant amplicon copies. Second, intragenomic variations of ribosomal genes and rITS, i.e. within a single isolate genome, are more common than was

Table 4 – Mantel correlations between the geographic, phenological and genetic distance between trees within a forest stand and the compositional dissimilarity of phyllosphere fungal assemblages associated with trees. Species composition of each tree was assessed by CE-SSCP of rITS1 amplicons or 454 pyrosequencing of rITS1 amplicons. The analyses are based on the sample × fluorescence (CE-SSCP) and on the quantitative sample × MOTU matrix including all MOTUs (454). The compositional dissimilarity between assemblages was estimated with the Bray–Curtis dissimilarity index.

Dataset	Variable of interest	Partial predictor	Spearman rho	*p*-value
CE-SSCP	Geographic	Genetic, phenological	0.06683017	0.37
CE-SSCP	Phenological	Geographic, genetic	0.38810239	0.054
CE-SSCP	Genetic	Geographical, phenological	0.22474231	0.125
454	Geographic	Genetic, phenological	0.23632214	0.096
454	Phenological	Geographic, genetic	0.3193117	0.075
454	Genetic	Geographic, phenological	0.3496958	**0.03***

Significant tests are shown in bold, and * $0.01 \leq P < 0.05$.

initially thought, with more than 5 % variation in some cases (Lindner & Banik 2011). These two biases increase the probability of observing rare rITS variants with increasing sequencing depth, and thus overestimate the MOTU richness. Third, intraspecific rITS1 variability may exceed 3 %, as this threshold is an operational compromise rather than a biological reality (Nilsson *et al.* 2008). These three factors may account for the representation of 52 species by several different MOTUs in our dataset. Conversely, the true species richness in a tree canopy may be much higher than the MOTU richness observed, for two reasons. First, we sampled only 0.02 m^2 of leaf surface per tree (four disks of 2 cm^2 each taken from 27 leaves). This constitutes probably only slightly more than 0.1 ‰ of the leaf area of a single beech tree (Bréda 1999). Second, even with the sampling of such a small area, the recovery of the phyllosphere fungal assemblage was not exhaustive, as shown by the high proportion of singletons. For

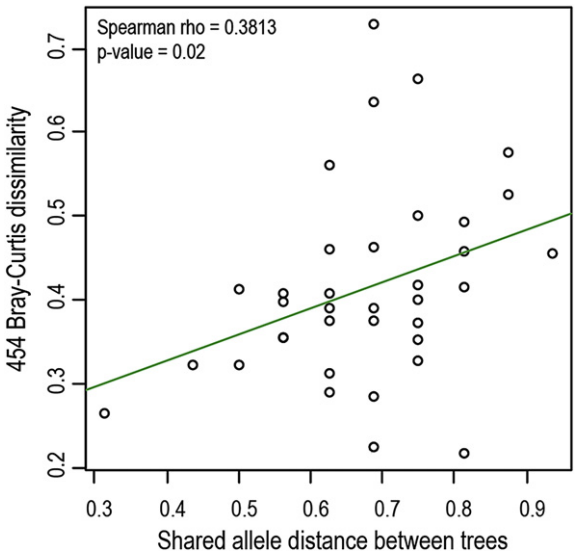

Fig 3 – Compositional dissimilarity of phyllosphere fungal assemblages (454 dataset) as a function of shared allele distance between trees. Spearman's correlation coefficient and the *p*-value of the Mantel test are indicated on the figure. The correlation remains significant when geographic distance and phenological distance between trees are included as partial predictors (Table 4).

these reasons, and despite methodological biases potentially leading to the overestimation of fungal species richness, we believe that the fungal species richness within the canopy of a single beech tree is greater than the number of MOTUs found in our samples.

Many fungal species of the beech phyllosphere are generalists and have a cosmopolitan distribution

Our study was conducted in a mixed oak-beech forest stand in the South of France. Seven of the 12 dominant MOTUs (Table 1) had previously been detected as phyllosphere fungi on other plant species and/or in other regions of the world. This suggests that the most abundant fungal species of the beech phyllosphere are generalist fungal species with a cosmopolitan distribution. *L. inositophila*, previously reported as an abundant phylloplane yeast on various plant species (Inácio *et al.* 2010), was the most abundant species in our dataset. This species, which is a *Taphrina* anamorph (Inácio *et al.* 2004), has also been found in the *Q. macrocarpa* phyllosphere in Kansas, USA (Jumpponen & Jones 2010). The latter study revealed significant seasonal trends of *Taphrina* spp. with a peak of abundance in early or mid-season. The high proportion of Taphrinales in our dataset could then be due to the early date of sampling (May). The ubiquitous yeast *Aureobasidium pullulans* and the plant pathogen *Mycosphaerella punctiformis* have already been identified as endophytes of beech in Germany (Sieber & Hugentobler 1987; Unterseher & Schnittler 2010). Two of the 12 most abundant MOTUs had previously been obtained from the *Q. macrocarpa* phyllosphere in Kansas (Jumpponen & Jones 2009; 2010) and two MOTUs displayed more than 95 % similarity with sequences obtained in a study on aerial fungal assemblage in Germany (Fröhlich-Nowoisky *et al.* unpublished). Among these four latter MOTUs, two may be taxonomically assigned (to *Cryptococcus* sp. and *Venturia hanliniana*).

We found that 162 MOTUs could be assigned to those obtained by Jumpponen & Jones (2009, 2010) from the *Q. macrocarpa* phyllosphere. These 162 MOTUs accounted for 18 % of the sequences in our dataset and included 112 MOTUs for which taxonomic assignment was possible. The assigned MOTUs common to both environments mostly belonged to *Cryptococcus* spp. (Tremellales, Basidiomycota), representing 55 % of the sequences of the 112 assigned MOTUs. The other

shared assigned MOTUs belonged to the Sporidiobolales (14 %), Erythrobasidiales (10 %) and Taphrinales (8 %). In addition, 46 MOTUs, accounting for 27 % of all the sequences in our dataset, could be assigned to those obtained in two studies carried out by Fröhlich-Nowoisky *et al.* (2009 and unpublished) on aerial fungal assemblages in Germany. We found that 66 % of the MOTUs common to these two studies belonged to the taxonomically ambiguous group of Dothideomycetes *incertae sedis*, 11 % to the Cystofilobasidiales, 9 % to the Helotiales and 6 % to the Tremellales. A comparison of our results with those of these previous studies further confirmed the presence in the beech phyllosphere of a large number of ubiquitous fungal species (Levetin & Dorsey 2006).

Cultivable endophytes may represent only a small proportion of the fungal biomass in the beech phyllosphere

The order Diaporthales, Helotiales and Dothideales, which are thought to be the dominant orders of cultivable endophytic fungi associated with beech (Sieber 2007), represented only 0.26 %, 1.5 % and 11.5 % of the assigned sequences, respectively. *Apiognomonia errabunda* (Diaporthales), a well known fungal species considered as the most abundant beech cultivable endophyte (Unterseher & Schnittler 2010), was represented by only 57 sequences, even if it was one of the core MOTUs. While cultivable endophytes are mainly composed of ascomycetes (Sieber 2007), cultivable epiphytic fungi are mainly composed of basidiomycetous yeasts (Inácio *et al.* 2010). Basidiomycetes represented 22 % of the taxonomically assigned sequences, suggesting that many of the ascomycete MOTUs in our dataset may represent uncultivable endophytes or epiphytic fungi. Taken together, this suggests that cultivable endophytes represent only a small proportion of the fungal biomass in the phyllosphere.

The fungal assemblages of the beech phyllosphere are spatially structured within the canopy

The hierarchical sampling was designed to estimate the spatial variability of phyllosphere fungal assemblages at the scale of entire trees, single branches, groups of leaves and individual leaves. Our results suggest that the leaf level accounted for the greatest variability. By characterizing fungal assemblages with CE-SSCP, we found that the dissimilarity of fungal assemblages between leaves from the same group of leaves accounted for most of the total variance. This very high variability at such a fine spatial scale may be due to a sampling effect related to the small amount of leaf-surface sampled (four leaf discs, corresponding to 8 cm^2). Indeed, epiphytic microbial communities tend to aggregate on individual leaves, due to the heterogeneity of the leaf surface, which provides multiple microhabitats (Kinkel 1997; Lindow & Brandl 2003). A small sampling surface may lead to an incomplete image of the fungal assemblage. Another explanation for such a high variability could be the stochastic early colonization just after bud burst, and subsequent priority effect possibly leading to particular assemblages on each leaf (Almany 2003). Joshee *et al.* (2009) isolated endophytic fungi from 50 leaves per tree on five different plant species. They, too, found that the greatest variance was at the smallest spatial scale (i.e. between leaf replicates).

Distance between leaves within the canopy was shown to be associated with a greater dissimilarity of fungal composition for five of the nine trees. This effect could be due to a microclimatic gradient within the canopy (leaf-surface temperature and humidity), to a gradient in leaf traits within the canopy or to constraints on fungal dispersal within the canopy. Leaves in the outer canopy are directly exposed to sun light, ultraviolet and wind whereas relative humidity may be higher in the inner part of the canopy. This drastically different environment between inner and outer canopy is known to generate a high phenotypic plasticity in leaf traits (Garcia-Verdugo *et al.* 2009). For instance, leaf water content and leaf toughness are more variable within trees than between trees in birch (Suomela & Ayres 1994). Leaf macronutrient content (Jumpponen & Jones 2010) and leaf-surface sugars (Mercier & Lindow 2000) may also vary within the canopy. These differences in microclimate and leaf traits between the inner and the outer may impact the composition of fungal assemblages (Unterseher *et al.* 2007). For instance, the successful colonization of beech leaves by *A. errabunda* is strongly dependent on light exposure, probably through an effect of light on phenolic compounds (Bahnweg *et al.* 2005). In addition, small-scale dispersal of yeast cells or fungal spores, due to rain splash or insect vectoring (Coluccio *et al.* 2008), may account for within-canopy variations in fungal assemblages. However, our results show that many fungal species are ubiquitous and generalist, suggesting that fungal dispersal is not a constraint in the studied forest stand. Therefore, we believe that within-canopy variation in microclimate and in leaf traits are more likely explanations than constraints on dispersal for the observed pattern.

Phyllosphere fungal assemblage structure at the stand scale may be shaped by the genetic variation of beech trees

Despite the use of two entirely different molecular approaches to characterize fungal assemblages (454 pyrosequencing and CE-SSCP), both methods revealed significantly differing fungal assemblages between trees. Variability of MOTU abundance was greatest between trees, accounting for 66 % of the total variance, whereas the proportion of the between-tree variance was much lower when only presence-absence was considered (only 17 % of the total variance). Furthermore, the core MOTUs accounted for a higher proportion of the variance between trees than did the whole MOTU dataset. Thus, variability mostly reflected differences in the relative abundance of core MOTUs. Core MOTUs may therefore correspond to fungi actively interacting with beech trees, whereas satellite MOTUs may correspond mostly to passive and stochastically occurring fungi (Unterseher *et al.* 2011).

Neither geographic nor phenological distance could explain the structure of beech phyllosphere fungi. Instead, genetic distance of host trees could play a major role. The significant correlation between genetic distance of host trees and dissimilarity of fungal assemblages (for 454 data) suggest an effect of tree genotype on the associated phyllosphere fungal assemblages. It has been suggested that the microbial communities developing on leaves must relate to a large

extent to the phenotypic characteristics of the plant, which are controlled at least in part by genetic background (Whipps *et al.* 2008). Our study gives a first hint that the genetic variation of trees affects phyllosphere fungal assemblages. However, further studies addressing this topic will need to include the sampling of a larger number of trees and the analysis of a larger number of molecular markers, for the calculation of a more robust genetic distance matrix. It is noteworthy that we detected significant correlations between genetic variation of the host trees and the fungal assemblage structure, even at low simulated sequencing depth (50 sequences per sample). This suggests that greater multiplexing could be used, rather than increasing sequencing depth, to explore such a link.

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Supplementary material

Supplementary material associated with this article can be found in the online version, at doi:10.1016/j.funeco.2011.12.004.

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