New Phytologist

The composition of phyllosphere fungal assemblages of European beech (*Fagus sylvatica*) varies significantly along an elevation gradient

Tristan Cordier[1,2], Cécile Robin[1,2], Xavier Capdevielle[1,2], Olivier Fabreguettes[1,2], Marie-Laure Desprez-Loustau[1,2] and Corinne Vacher[1,2]

[1]INRA, UMR1202 BIOGECO, F-33610, Cestas, France; [2]University of Bordeaux, UMR1202 BIOGECO, F-33400, Talence, France

Author for correspondence:
Corinne Vacher
Tel: +33 05 57 12 27 24
Email: corinne.vacher@pierroton.inra.fr

Received: *1 June 2012*
Accepted: *16 July 2012*

New Phytologist (2012) **196**: 510–519
doi: 10.1111/j.1469-8137.2012.04284.x

Key words: climate change, community structure, elevation gradient, *Fagus sylvatica*, fungi, pathogens, phyllosphere, 454 pyrosequencing.

Summary

- Little is known about the potential effect of climate warming on phyllosphere fungi, despite their important impact on the dynamics and diversity of plant communities. The structure of phyllosphere fungal assemblages along elevation gradients may provide information about this potential effect, because elevation gradients correspond to temperature gradients over short geographic distances.
- We thus investigated variations in the composition of fungal assemblages inhabiting the phyllosphere of European beech (*Fagus sylvatica*) at four sites over a gradient of 1000 m of elevation in the French Pyrénées Mountains, by using tag-encoded 454 pyrosequencing.
- Our results show that the composition of fungal assemblages varied significantly between elevation sites, in terms of both the relative abundance and the presence–absence of species, and that the variations in assemblage composition were well correlated with variations in the average temperatures.
- Our results therefore suggest that climate warming might alter both the incidence and the abundance of phyllosphere fungal species, including potential pathogens. For example, *Mycosphaerella punctiformis*, a causal agent of leaf spots, showed decreasing abundance with elevation and might therefore shift to higher elevations in response to warming.

Introduction

The phyllosphere is the habitat provided by the leaves of living plants. It supports a great diversity of both epiphyllous and endophyllous microorganisms (Inácio *et al.*, 2002; Lindow & Brandl, 2003, 2003; Jumpponen & Jones, 2009; Rodriguez *et al.*, 2009; Redford *et al.*, 2010). In this study, we define phyllosphere fungal species as those species inhabiting both the surface and the interior of leaves (Jumpponen & Jones, 2009) and we define an assemblage as the addition of all fungal species inhabiting the phyllosphere of the same plants at the same time (Fauth *et al.*, 1996). Phyllosphere fungi influence the fitness of their host plants, either negatively, by acting as pathogens (Gilbert, 2002; Newton *et al.*, 2010), or positively, by increasing the stress tolerance of the plant (Redman *et al.*, 2002), reducing herbivory through the production of toxic alkaloids (especially in grasses; Wilkinson *et al.*, 2000) or reducing the infection of plant tissues by pathogens (Arnold *et al.*, 2003). They are therefore important drivers of the dynamics and diversity of plant populations and communities (Clay & Holah, 1999; Bradley *et al.*, 2008). Phyllosphere fungi also influence the dynamics of other taxonomic groups, such as phyllosphere bacteria (Suda *et al.*, 2009), phytophagous insects and their parasitoids (Omacini *et al.*, 2001). Finally, they contribute to nutrient cycling, as early colonizers of leaf litter (Osono, 2006).

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, culture-based approaches systematically exclude biotrophic species and tend to favor rapidly growing fungi, although major advances towards the resolution of this problem have been made (Unterseher & Schnittler, 2009). New culture-independent methods, such as high-throughput DNA sequencing (Shendure & Ji, 2008), have made it possible to obtain a more complete description of fungal diversity (Jumpponen & Jones, 2009, 2010), despite sequencing errors which may lead to an overestimation of species richness (Quince *et al.*, 2009). These new culture-independent methods can be used for the molecular identification of species through genetic barcoding (Nilsson *et al.*, 2009; Begerow *et al.*, 2010). They hold great promise for improving our understanding of the ecology of phyllosphere fungi and predicting their response to global warming.

Global warming is ongoing (IPCC, 2007) and has already caused species distribution shifts and extinctions, and changes to community composition and ecosystem functioning (Thomas *et al.*, 2004; Parmesan, 2006). Studies of the effects of climate

change have generally focused on higher plants (Peñuelas & Boada, 2003; Lenoir *et al.*, 2008) and animals (Root *et al.*, 2003; Devictor *et al.*, 2008). The potential response of fungal assemblages to climate change has been investigated principally in the soil system (Gange *et al.*, 2007; Meier *et al.*, 2010; Bahram *et al.*, 2011; Sheik *et al.*, 2011; Yuste *et al.*, 2011). The potential responses of phyllosphere fungal assemblages to global warming have been much less thoroughly explored (Hashizume *et al.*, 2008).

In this study, we investigated the effects of elevation on the composition of phyllosphere fungal assemblages, because elevation gradients can be used as a proxy for temperature gradients over short geographic distances (Körner, 2007). We studied the composition of fungal assemblages inhabiting the phyllosphere of European beech (*Fagus sylvatica*) over a gradient of 1000 m of elevation in the French Pyrénées Mountains. We used tag-encoded 454 pyrosequencing to test the three following hypotheses: (1) changes in the composition of phyllosphere fungal assemblages follow the elevation gradient, (2) temperature is the climatic factor accounting to the greatest extent for the elevation-related pattern and (3) many phyllosphere fungal species, including the most abundant and potential pathogens, are unevenly distributed along the gradient.

Materials and Methods

Study site and sampling design

The study was conducted along an elevation gradient extending from 488 m asl (Lourdes; GPS +43°45′51″, −0°13′13″) to 1533 m asl (Lienz; GPS +42°53′32″, −00°04′24″) in the Gave valley of the French Pyrénées. We selected four stands distributed along this elevation range that contained a high proportion of beech (*Fagus sylvatica* L.) trees (> 50%) and were growing on north-facing slopes in order to avoid differences in solar exposition. Each elevation site was geo-referenced (Table 1). At each elevation, we defined three plots located about 50 m apart. Within each plot, we selected five trees that were located close together (< 10 m apart) and were at least 10 m tall. We sampled three leaves per tree, from different branches. The sampled branches were located *c.* 7 m above the ground and were oriented toward the north, south-east or south-west. The sampled leaves were located in the middle of the branch, on a current-year shoot, and were the second leaves back from bud. This sampling procedure minimized the effect of leaf age within trees. The gradient was sampled four times (July 2009, September 2009, June 2010 and July 2010), and each sampling campaign was completed within 1 wk. In total, we collected 720 leaves (4 sampling dates × 4 elevation sites × 3 plots per site × 5 trees per plot × 3 leaves per tree), which were placed in individual plastic bags, each containing 10 ml of silica gel (Sigma-Aldrich, St Louis, MO, USA) to ensure that the leaves were completely dry within a few hours. The plastic bags were brought back to the laboratory and stored at 16°C until DNA extraction.

Phyllosphere fungal assemblage data

The composition of phyllosphere fungal assemblages was characterized at the plot level for each sampling date, by tag-encoded 454 pyrosequencing. Four discs, each with an area of 28 mm^2, were cut from each side of the midrib of each leaf in a laminar flow hood. Each disc was placed in one of the wells of an autoclaved DNA extraction plate. The hole-punch used to cut the discs was sterilized after processing a leaf with 70% ethanol and flaming. A single 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 phenol/chloroform/isoamyl alcohol protocol, with the addition of β-mercaptoethanol (0.5%; Sigma-Aldrich) and proteinase K (20 mg ml^{-1}; Sigma-Aldrich). Briefly, each well was filled with 400 μl of the CTAB extraction buffer (8 mg of CTAB, 16 μl of 0.5 M EDTA, 40 μl of 1 M Tris HCl, 112 μl of 5 M NaCl, 4 mg of PVP-40 and 232 ml of ultra-pure water) and heated at 60°C for 2 h, with shaking, in an incubator (New Brunswick Scientific, Edison, NJ, USA). Samples were mixed with 320 μl of phenol/chloroform/isoamyl alcohol (25 : 24 : 1; pH 8), vortexed briefly and centrifuged at 364 ***g*** for 10 min at 4°C. The aqueous phases were transferred to a new autoclaved plate. DNA was precipitated overnight in absolute isopropanol, at −20°C. It was collected by centrifugation (364 ***g*** for 10 min at 4°C), washed twice in 70% ethanol (−20°C), dried for 1 h in a Speed Vac Plus (Savant Instruments, Farmingdale, NY, USA) and eluted in 50 μl of ultra-pure water (Sigma-Aldrich). DNA was quantified with a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA), and its concentration was adjusted to 10 ng μl^{-1} with ultra-pure water (Sigma-Aldrich).

PCR amplification targeted the internal transcribed spacer 1 (ITS1) region of the nuclear ribosomal repeat unit, which is considered to be the best available barcode for identifying fungi to species level (Nilsson *et al.*, 2009; Seifert, 2009; Schoch *et al.*, 2012). Titanium fusion primers were used for PCR amplification. The universal reverse primer ITS2 (White *et al.*, 1990) included the A adaptor, one of 24 different five-nucleotide tags and the template-specific sequence, whereas the fungus-specific forward primer ITS1F (Gardes & Bruns, 1993) contained the B adaptor (Supporting Information Table S1). Sequencing was unidirectional and started with the A adaptor. The primer design thus resulted in reverse sequences across ITS1. This sequencing strategy allowed us to minimize the sequencing of the conserved 3′ end of the nuclear small subunit RNA gene (nSSU), because the ITS2 primer binds a shorter distance into the 5.8S gene than does the ITS1F primer into the nSSU gene. Incomplete sequences (which represented a nonnegligible amount of the raw data set) had thus a higher probability of containing the informative ITS1 region.

DNA extracts were pooled by tree before PCR amplification. A single tagged primer was used for PCR amplifications of the DNA obtained from the five trees of a given plot at a given sampling date. PCR was performed in sterile 96-well plates, avoiding the wells at the edge of the plate, which were filled with 20 μl of water (to avoid Peltier effects). The reactions were performed in a volume

Table 1 Description of the elevation sites

	Lourdes	Sireix	Haugarou	Lienz
Elevation (m asl)	488	833	1190	1533
Other trees species	*Quercus petraea, Sorbus aria*	*Quercus petraea, Abies alba*	*Pinus sylvestris, Abies alba*	*Pinus sylvestris, Abies alba*
Latitude	N 43°05′46″	N 42°58′37″	N 43°00′16″	N 42°53′32″
Longitude	W 00°05′14″	W 00°08′29″	W 00°12′49″	E 00°04′24″
Average temp (°C)	12	10.2	8.9	8.1
Average precip (mm)	1504	1306	1508	1406
Mean no. of days of frost	45.1	56.3	69.1	90.1
Mean temp 2009	11.3 (6.5)	10.1 (6.8)	8.1 (7)	6.2 (7.3)
Mean temp 2010	10.4 (7)	9.2 (7.2)	7.2 (7.4)	5.2 (7.5)

Average climatic variables were taken from the AURELHY model (1971–2000) and mean annual temperatures in 2009 and 2010 were obtained with data loggers (SD in brackets). The tree stand at each site consisted of at least 50% European beech (*Fagus sylvatica*).

of 20 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 (20 ng). The PCR mixture was subjected to initial denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 54°C for 1 min, 72°C for 90 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 control wells. Each PCR product was purified with the AMPure XP purification kit (AgenCourt Bioscience, Fullerton, CA, USA) and quantified (NanoDrop). We combined 10 ng of each PCR product in single tubes for 2009 and for 2010.

We sequenced the PCR products in two different 454 runs. The 2009 DNA pool was sequenced using 1/8th of the area of a sequencing plate, whereas the 2010 DNA pool was sequenced using 1/4th of a plate. A 454 GS-FLX Titanium sequencer (454 Life Sciences, Branford, CT, USA) was used for sequencing, at Genoscope, Evry, France. With a theoretical yield of 100 000 sequences for 1/8th of the area of a sequencing plate and 200 000 sequences for 1/4th of a plate, we expected a sequencing depth of almost 3333 sequences per sample (a given plot at a given sampling date) for the 2009 samples and 6666 sequences per sample for the 2010 samples. One sample in each 454 run was markedly less sampled than the others (65 and 208 sequences in 2009 and 2010, respectively) and the tag-encoded primer involved was the same, suggesting an effect of the primer on pyrosequencing. We therefore included these two samples, amplified by PCR with a different tag-encoded primer, in another run as part of a different study. The 454 sff files are available from the European Nucleotide Archive (http://www.ebi.ac.uk/ena/data/view/ERP001056).

The two 454 sequencing data sets were processed identically. Each data set was demultiplexed with the *split_library* function of the Quantitative Insight Into Microbial Ecology toolkit (QIIME 1.1; Caporaso *et al.*, 2010). The following quality filters were applied: no mismatch allowed in the five-nucleotide tag, ITS2 primer sequence retrieved with no mismatch, minimum sequence length of 100 bp, no ambiguous nucleotides allowed and mean quality score for base calling > 25 across the whole read. 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 data set to check extraction efficiency, and matching sequences were removed. The ITS1 locus in fungi has been shown to have a median length of 183 bp in 4185 species from 973 genera (Nilsson *et al.*, 2008). In our data set, the median length of this locus was 162 bp, ranging from 32 to 464 bp. We decided to remove ITS1 sequences of < 100 bp, because a sufficient overlap between sequences is necessary for molecular operational taxonomic unit (MOTU) clustering.

The cleaned data set was clustered into 97% similarity MOTUs with the UCLUST algorithm (Edgar, 2010) implemented in the *pick_otus* function of QIIME. Before carrying out the actual MOTU clustering, a preliminary clustering at 100% similarity was performed and groups of identical sequences were sorted in decreasing order of abundance. The most abundant sequences thus became the seeds from which the final clustering process began. This prior sorting on the basis of abundance is important in MOTU clustering (Edgar, 2011), 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 that allows the optimal alignment to be found before calculating the similarity between two sequences.

Each seed sequence and singleton was compared with the sequences deposited in GenBank, with the BLASTN algorithm (Altschul *et al.*, 1997). We first excluded environmental sequences, for putative taxonomic identification. We applied a threshold of at least 97% similarity over at least 90% of the query length on a fully annotated accession for the assignment of a species name to a MOTU or the assignment of a genus name if the annotated accession was classified no further than the genus. Environmental sequences were then included for characterizing the environmental source of the remaining MOTUs. We used the same threshold previously used for the assignment of MOTUs to environmental sequences deposited in GenBank.

The number of sequences per nonsingleton MOTU was considered to be a proxy for the abundance of associated molecular species (Amend *et al.*, 2010; Unterseher *et al.*, 2011). The species composition of the samples was therefore represented as a quantitative sample × MOTU matrix, giving the abundance

(number of sequences) of each nonsingleton MOTU in each sample (a given plot at a given sampling date). The sample sizes in the sample × MOTU matrix were unequal (ranging from 755 to 20 495 sequences per sample). Therefore, we applied the *multiple_rarefaction* function in QIIME to the sample × MOTU matrix to build 30 rarefied quantitative data sets of 700 sequences per sample.

We also considered that the presence of a nonsingleton MOTU within a sample indicates the presence of the associated molecular species within the sample. Therefore, from the 30 rarefied quantitative data sets, we calculated 30 binary matrices. The use of binary data sets, in addition to quantitative data sets, is important because it allows one to investigate whether the variations in the composition of fungal assemblages reflect changes in the presence–absence of MOTUs, or only changes in their relative abundance.

Climatic and weather data

We characterized the local climate and the weather before sampling at each elevation site (Table 2). Climatic variables representing 30-yr averages were included as potential descriptors of the suitability of each site for sustaining populations of the different fungal species. Weather variables describing the temperature and humidity that phyllosphere fungi actually experienced during their lifetime were also included as potential factors influencing population levels at the sampling date (Bateman *et al.* 2012).

The climate at each site was estimated from the predictions of the AURELHY model (Analysis Using the Relief for Hydrometeorology; Benichou & Le Breton, 1987) for the 1971–2000 period. The data were supplied by the French National Meteorological Office (Météo-France). This model can be used to interpolate meteorological records from 65 meteorological stations throughout France to a 1 × 1 km grid, taking local topography into account. We retrieved mean temperature, mean precipitation and the mean number of days of frost for all the sites along the gradient. Data for each variable were available for each season (winter, spring, summer and autumn).

Table 2 List of climatic and weather variables calculated for each elevation site

Abbreviation	Description
Climatic variables	
t_m_S	Mean temperature in season *S*
pp_S	Mean precipitation in season *S*
fr_S	Mean no. of days of frost in season *S*
Weather variables	
t_m_D	Mean temperature over the *D* days before sampling
t_sd_D	Temperature standard deviation over the *D* days before sampling
dew_point_D	Number of hours above dew point over the *D* days before sampling
VDP_10max_D	Mean of the ten highest vapor pressure deficit values over the *D* days before sampling

The average climatic variables were estimated from the predictions of the AURELHY model (Meteo France) for the 1971–2000 period, whereas the weather variables were measured at each site with temperature and humidity sensors. *S* indicates the season covered by the climatic variable (winter, spring, summer and autumn), whereas *D* indicates the number of days before sampling included for calculation of the weather variable (2 d before sampling, 7, 15 or 30 d).

The weather at each site over the days or weeks before sampling was assessed by taking measurements with four data loggers (HOBO Pro RH/Temp; Onset Computer Corporation, Bourne, MA, USA). Sensors were installed at a height of 1.5 m above the ground and were protected under a white plastic shelter to prevent exposure to rain or to direct sunlight. Air temperature and humidity were recorded hourly from 1 January 2009 to 31 December 2010. All sensors were intercalibrated in the laboratory before installation. We calculated the mean temperature and the standard deviation for temperature at each site over four different time periods (2, 7, 15 and 30 d) before sampling. We also calculated the mean vapor pressure deficit (which quantifies the air 'dryness') and the number of hours above the dew point at each site over the same time periods. We calculated the vapor pressure deficit (*VPD*) by subtracting the actual vapor pressure (E_a; formula 5.13 in Jones, 1992) from the saturation pressure vapor (E_s; formula 5.12 in Jones, 1992):

$$E_s = 613.75 \times \text{Exponential}\,[17.502 \times \mathit{Temperature} / (\mathit{Temperature} + 240.97)]$$

$$E_a = \mathit{Relative\ air\ humidity}/100 \times E_s$$

$$\mathit{VPD} = E_s - E_a.$$

We then calculated the mean of the 10 highest *VPD* values for the period considered. The number of hours above the dew point is the number of hours during which the *VPD* is negative over the period considered.

Statistical analyses

We calculated the compositional dissimilarity matrix between samples, based on the Canberra index calculated for each of the 30 rarefied quantitative data sets. We then calculated the average dissimilarity matrix from these 30 matrices, to describe the changes in phyllosphere fungal assemblages among sites without biases resulting from differences in sample size (sequencing depth). The Canberra dissimilarity index was selected from a list of possible indices (Bray-Curtis, Canberra, Manhattan, Kulczynski, Jaccard, Gower, Morisita and Horn) by the *rankindex* function of the R vegan package (Oksanen *et al.*, 2010; R Development Core Team, 2011), which identified this index as giving the best separation of samples along the elevation gradient. We also calculated the average dissimilarity between samples, based on the Canberra index calculated for each of the 30 rarefied binary data sets. Mean pairwise dissimilarities between samples were represented on a nonmetric multidimensional scaling (NMDS) plot. NMDS analyses were performed with the *metaMDS* function with default settings (Oksanen *et al.*, 2010).

We then assessed the effect of elevation site on fungal assemblage structure, by analyzing the average Canberra dissimilarity matrices in permutational multivariate analyses of variance

(PERMANOVAs; Anderson, 2001). These analyses were carried out with the *adonis* function of the R vegan package (Oksanen *et al.*, 2010), with 999 permutations, by using the three plots per site as replicates. Sampling date and its interaction with site were also introduced in the analyses of variance, to investigate whether the effect of site was constant through time.

We then investigated the correlations between the dissimilarities of fungal assemblages and variations of climatic and weather variables. The average Canberra dissimilarity matrices were recalculated after summing the sequence data for the three plots per site, because we had only one climate or weather measure per site. Given the high dimensionality and collinearity of the environmental data set (28 climatic and weather variables), we first selected the season (for each climatic variable) and the period before sampling (for each weather variable) best correlated with fungal assemblage dissimilarities, using the BIOENV procedure of the R vegan package. We slightly modified the *bioenv* function, in order to use the community dissimilarity matrix as input (Methods S1). From the reduced environmental data set, we selected the combination of climatic and weather variables best correlated with fungal assemblage dissimilarities, by the same procedure. The BIOENV procedure finds the best subset of environmental variables (by examining all the possible subsets of variables, from only one variable to all variables), such that the Euclidean distances of scaled environmental variables have the maximum Pearson correlation with assemblage dissimilarities (Clarke & Ainsworth, 1993). As geographic distance may also account for the dissimilarities between fungal assemblages, we added the geographic distance matrix between sites as a partial predictor in the *bioenv* function. We used the *envfit* function of the R vegan package to fit the climatic and weather variables chosen by the BIOENV procedure to the NMDS. The R script used to perform the analyses and the NMDS plots is provided in Methods S2.

Finally, we investigated the elevation distribution of the three most abundant taxonomically assigned MOTUs and the species known to be potentially pathogenic to beech. These latter species included *Phyllactinia guttata* (powdery mildew), *Neonectria coccinea* (bark canker), *Mycosphaerella punctiformis* (leaf spot) and *Apiognomonia errabunda* (anthracnose). The two latter species were shown to be foliar endophytes which can turn into pathogens under certain conditions (Verkley *et al.*, 2004; Bahnweg *et al.*, 2005; Unterseher & Schnittler, 2010). We tested the effects of site, sampling date and their interaction by fitting generalized linear models with a quasi Poisson error distribution and a log link function to the abundance data (number of sequences assigned to the considered species or order), by using the *GENMOD* procedure of the SAS/STAT® software (SAS Institute Inc, 1997). The log of the total number of sequences per sample was included as an offset, to account for differences in sample size.

Results

The data set comprised 206 073 quality sequences distributed into 48 samples (4 sampling dates × 4 elevation sites × 3 plots per site). The mean number of sequences per sample was 2400 for 2009 (ranging from 755 to 4033) and 6195 for 2010 (ranging from 946 to 20 493). The clustering of these sequences, with a threshold of 97% similarity, gave a total of 3729 MOTUs (Table S2). We identified 12 plant MOTUs, corresponding to 439 sequences (including 411 sequences from European beech) and four MOTUs that best matched protists (five sequences). These MOTUs were removed from the data set before statistical analyses. The final data set comprised 3713 fungal MOTUs, including 1662 singletons, which were also removed before statistical analyses. The mean number of MOTUs per plot was 268 (SD = 74) for 2009 and 360 (SD = 165) for 2010. The mean number per elevation site was 546 (SD = 107) for 2009 and 708 (SD = 274) for 2010.

We were able to assign 367 of the 2051 nonsingleton MOTUs to species and 156 to genera. We were also able to assign 210 of the 1652 singleton MOTUs to species and 90 to genera (Table S2). The taxonomically assigned MOTUs accounted for 31% of the total number of sequences. The 577 MOTUs assigned to species corresponded to a total of 335 species, because several MOTUs were assigned to the same species. As previously found and discussed by Cordier *et al.* (2012), the number of MOTUs assigned to the same species was significantly correlated with the number of sequences assigned to the species concerned ($R^2 = 0.85$, $P < 0.001$). Thus, larger numbers of different MOTUs were generally identified for the more abundant species.

Among the 3713 fungal MOTUs, 12 MOTUs each accounted for > 1% of the total number of sequences (Table 3). We were able to assign five of these abundant MOTUs to species, and one to a genus. The three most abundant assigned MOTUs were the ascomycetous yeast *Taphrina carpini* (6% of the sequences), the ascomycetous black yeast *Venturia hanliniana* (5% of the sequences) and the ascomycetous saprobe *Mycosphaerella flageoletiana* (3% of the sequences). The six MOTUs which could not be taxonomically assigned matched unidentified environmental sequences obtained either from beech leaf litter in early decomposition in Austria (S. R. Moll *et al.*, unpublished), from beech phyllosphere sampled in May 2009 in southern France (Cordier *et al.*, 2012) or from *Quercus macrocarpa* phyllosphere in the USA (Jumpponen & Jones, 2010).

A list of all the MOTUs (singletons and nonsingletons), with their abundance and taxonomic assignment, is available in Table S2. The corresponding sequences are available under GenBank accession numbers JN904149–JN906968. These sequences include nSSU and 5.8S in addition to ITS1, whereas the sequences which were used for taxonomic assignment include ITS1 only. Only the sequences longer than 200 bp are available in GenBank.

The NMDS plot shows that the composition of fungal assemblages differed considerably between sites, with dissimilarity increasing with increasing difference in elevation between sites. It also indicates that the composition of fungal assemblages differed between the two years of sampling (Fig. 1a). Based on the 30 rarefied presence–absence data sets, we found that 65% of the nonsingleton MOTUs were specific to a particular site whereas 16% of the MOTUs were common to two adjacent sites, both in 2009 and in 2010. In 2009, only 6% of the MOTUs were common to all sites

Table 3 Taxonomic assignment of the 12 most abundant molecular operational taxonomic units (MOTUs), based on BLAST analysis of MOTU seed sequences against GenBank

GenBank accession no.	MOTU relative abundance	GenBank (environmental sequences excluded)			GenBank (environmental sequences included)			
		Closest match	Similarity/ coverage	Putative taxon	Closest match	Similarity/ coverage	Putative taxon	Source
JN906440	14.38	AY239214	86/96	*Lalaria inositophila*	**JF495183**	100/100	**Uncultured *Taphrina***	Austria, *Fagus sylvatica* leaf litter, S. R. Moll *et al.*, unpublished
JN904440	14.34	GQ411291	95/100	*Articulospora tetracladia*	**JF945438**	100/100	**Uncultured fungus**	France, *Fagus sylvatica* phyllosphere, Cordier *et al.* (2012)
JN906683	10.46	AY971723	76/94	Fungal sp.	**JF495199**	100/100	**Uncult. Pezizomycotina**	Austria, *Fagus sylvatica* leaf litter, S. R. Moll *et al.*, unpublished
JN905902	6.11	**AY239215**	100/100	***Taphrina carpini***	**AY239215**	100/100	***Taphrina carpini***	Portugal, *Quercus pyrenaica* phylloplane, Inácio *et al.* (2004)
JN905258	5.2	**AB109183**	100/100	***Venturia hanliniana***	**JF945021**	100/100	**Uncultured fungus**	France, *Fagus sylvatica* phyllosphere, Cordier *et al.* (2012)
JN904579	3.6	**EU167597**	100/100	***Mycosphaerella flageoletiana***	**JF945447**	100/100	**Uncultured fungus**	France, *Fagus sylvatica* phyllosphere, Cordier *et al.* (2012)
JN905085	2.79	AY239214	86/96	*Lalaria inositophila*	**JF945443**	99/100	**Uncultured fungus**	France, *Fagus sylvatica* phyllosphere, Cordier *et al.* (2012)
JN904832	2.56	AY230777	80/94	*Woollsia* mycorrhizal fungus	**JF946080**	99/100	**Uncultured fungus**	France, *Fagus sylvatica* phyllosphere, Cordier *et al.* (2012)
JN905448	1.93	**HQ909089**	100/100	***Aureobasidium pullulans***	**HQ909089**	100/100	***Aureobasidium pullulans***	NA, Y. Li & M. Yang, unpublished
JN904996	1.46	EU252549	100/38	*Cryptococcus skinneri*	**GQ508475**	97/100	**Uncultured fungus**	*Kansas, USA, Q. macrocarpa* phyllosphere, Jumpponen & Jones (2010)
JN904818	1.14	**HQ717406**	100/100	***Cryptococcus sp.***	**HQ267064**	100/100	**Uncultured fungus**	Quebec, Maple tree, Filteau *et al.* (2011)
JN904663	1.03	**AY808308**	100/100	***Dothistroma rhabdoclinis***	**JF945040**	100/100	**Uncultured fungus**	France, *Fagus sylvatica* phyllosphere, Cordier *et al.* (2012)

Coverage is the percentage of the query length covered by the alignment. Similarity is the percentage identity over the alignment. Closest matches with > 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 data set. Source indicates the source of the sequence associated with the underlined accession. NA, not available.

(7% in 2010). These latter MOTUs were very abundant, accounting for 77% of the total number of sequences in the rarefied data sets. Similar results were obtained with the nonrarefied data set.

The fit of the environmental variables selected by the BIOENV procedure to the NMDS plot shows that climatic variables, especially temperatures, were the variables best correlated with fungal assemblage dissimilarities along the gradient of elevation, after the effect of geographic distance was accounted for. In particular, the average temperature in spring and the average number of days of frost during spring were the variables best correlated with variations in the composition of phyllosphere fungal assemblages along the gradient of elevation ($R^2 = 0.98$, $P = 0.001$ and $R^2 = 0.89$, $P = 0.001$, respectively). Weather variables were not correlated with fungal assemblage dissimilarities along the gradient of elevation but with fungal assemblage dissimilarities among sampling dates (Fig. 1b).

Permutational multivariate analyses of variance confirmed that site and sampling date were significant factors of variation of the composition of phyllosphere fungal assemblages. The effect of site was statistically significant with both the abundance and presence–absence data sets. A statistically significant interaction between site and sampling date was observed (Table 4).

Generalized linear models revealed significant site effects for the three most abundant MOTUs assigned to species (*Taphrina carpini*, *Venturia hanliniana* and *M. flageoletiana*). The interaction between site and sampling date was statistically significant for *T. carpini* and *V. hanliniana* but not for *M. flageoletiana* (Table 5). This latter species had a significantly lower abundance at the two highest elevations (Fig. 2a). Generalized linear models also revealed significant site effects for two species described as pathogenic to beech, *Apiognomonia errabunda* and *M. punctiformis*. For both species, the interaction between site and sampling date was not statistically significant (Table 5). *Mycosphaerella punctiformis* was found predominantly at the lowest elevation (Fig. 2b), whereas *A. errabunda* did not display monotonous variation in abundance along the gradient in elevation (Fig. 2c). The *GENMOD*

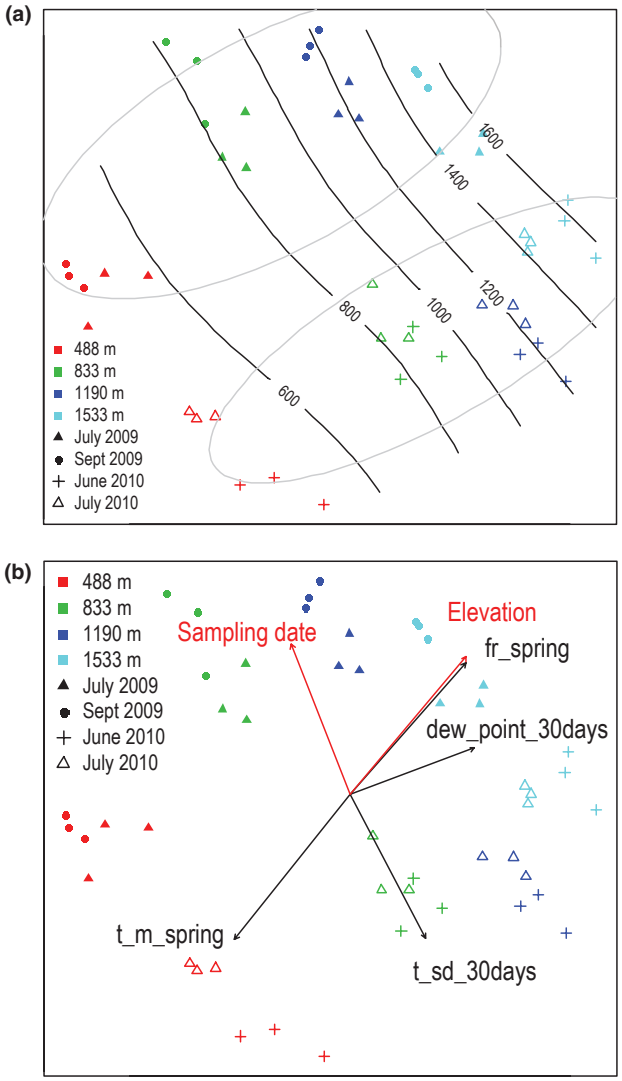

Fig. 1 Phyllosphere fungal assemblage dissimilarity among beech tree plots (n = 12) located along an elevational gradient, represented by nonmetric multidimensional scaling (NMDS). The NMDS plots represent the average Canberra dissimilarity matrix computed from 30 rarefied quantitative sample × molecular operational taxonomic unit (MOTU) matrices. In (a), the black lines represent elevation and the gray ellipses highlight the year of sampling. In (b), the black arrows indicate the environmental factors best correlated with fungal assemblage dissimilarities whereas the red arrows indicate the direction of the gradient of elevation and sampling date. The arrow indicating sampling date points toward the end of the vegetative season (September).

procedure did not converge for the two other species potentially pathogenic to beech (*Phyllactinia guttata* and *Neonectria coccinea*), which had a very low relative abundance (0.015% and 0.18%, respectively) and were absent from most samples (67% and 73%, respectively).

Discussion

Our results show that the composition of fungal assemblages inhabiting the phyllosphere of European beech varied considerably over a gradient of 1000 m of elevation in the French Pyrénées Mountains. More than 60% of the MOTUs were specific to a particular elevation site whereas < 10% of the MOTUs were common to all sites. The few MOTUs common to all sites were very abundant MOTUs, representing together > 75% of the total number of sequences. The ascomycetous yeast *Taphrina carpini*, the ascomycetous black yeast *Venturia hanliniana* and the ascomycetous saprobe *M. flageoletiana* were the three most abundant and taxonomically assigned MOTUs. Their abundance varied significantly between elevation sites. Hence, variations in the

Table 4 Permutational multivariate analyses of variance of the compositional dissimilarity between phyllosphere fungal assemblages along an elevation gradient

		Abundance data		Binary data	
Source	df	Sums of sqs	Pseudo *F*	Sums of sqs	Pseudo *F*
Elevation site	3	2.7848	**2.9667*****	2.8991	**3.7652*****
Sampling date	3	2.0411	**2.1744*****	1.7463	**2.2679*****
Site × date	9	3.4795	**1.2356*****	2.7552	**1.1928****
Residuals	32	10.0126		8.2130	

Analyses were based on a mean distance matrix computed from 30 rarefied sample × MOTU matrices. Distances were calculated from abundance matrices or binary matrices with the Canberra index.
*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Table 5 Results for generalized linear models testing the effect of elevation site, sampling date and their interaction on the abundance of the three most abundant molecular operational taxonomic units (MOTUs) assigned to species (*Taphrina carpini*, *Venturia hanliniana* and *Mycosphaerella flageoletiana*) and two species potentially pathogenic to beech (*Mycosphaerella punctiformis* and *Apiognomonia errabunda*)

	Deviance	Num df	Den df	*F* value	*P* > *F*
Taphrina carpini					
Elevation site	3151.32	3	32	30.88	< **0.0001*****
Sampling date	1435.99	3	32	27.97	< **0.0001*****
Date × site	654.12	9	32	4.25	**0.0011****
Venturia hanliniana					
Elevation site	6542.9	3	32	38.35	< **0.0001*****
Sampling date	1141.86	3	32	149.13	< **0.0001*****
Date × site	386.31	9	32	6.95	< **0.0001*****
Mycosphaerella flageoletiana					
Elevation site	12842.6	3	32	79.53	< **0.0001*****
Sampling date	995.81	3	32	164.43	< **0.0001*****
Date × site	768.51	9	32	1.05	0.423
Mycosphaerella punctiformis					
Elevation site	107	3	32	20.96	< **0.0001*****
Sampling date	105.2	3	32	0.28	0.8375
Date × site	68.09	9	32	1.94	0.0818
Apiognomonia errabunda					
Elevation site	203.18	3	32	7.77	**0.0005*****
Sampling date	123.42	3	32	10.01	< **0.0001*****
Date × site	85.03	9	32	1.6	0.156

*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

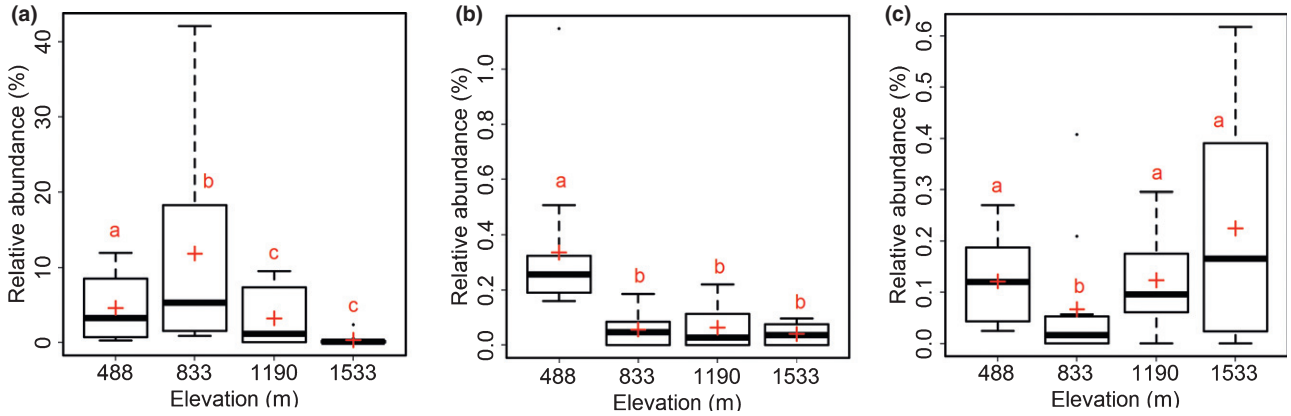

Fig. 2 Relative abundance (number of assigned sequences/total number of sequences of the sample) of (a) *Mycosphaerella flageoletiana*, (b) *Mycosphaerella punctiformis* and (c) *Apiognomonia errabunda* as a function of elevation site. The abundance data of the four sampling dates were pooled as the interactions between sampling date and elevation site were not significant (Table 5). The red crosses indicate the mean relative abundances and red letters indicate the results for the tests of differences of least square means.

composition of phyllosphere fungal assemblages over the gradient of elevation were caused not only by variations in the presence–absence of the numerous rare MOTUs but also by variations in the abundance of the few abundant MOTUs.

Our analyses show that climatic variables, representing 30-yr averages (1971–2000), better accounted for these variations than weather variables, describing temperature and humidity 2–30 d before sampling. Of the climatic variables examined, the mean temperature in spring and the mean number of days of frost in spring were the variables best correlated with fungal assemblage dissimilarities along the gradient of elevation. Our analyses took into account the geographic distance between sites (26 km between the lowest and highest sites) as a partial predictor of the spatial structure of fungal assemblages along the gradient. In addition, dispersal constraints over this distance are unlikely to be a strong structuring factor because many phyllosphere fungi have a high capacity for dispersal and a cosmopolitan distribution (Levetin & Dorsey, 2006; Helander *et al.*, 2007; Cordier *et al.*, 2012). Our results therefore confirm that phyllosphere fungal assemblages have a spatial structure despite the high capacity for dispersal of the species (Jumpponen & Jones, 2009; Cordier *et al.*, 2012). They suggest that the spatial structure at the regional scale might be shaped by variations in abiotic factors, especially temperatures.

However, several environmental parameters covarying with temperature were not taken into account in our analyses and may also influence the structure of phyllosphere fungal assemblages along the studied gradient. First, variations in atmospheric pressure and UV-B radiation along the gradient of elevation (Körner, 2007) may affect the structure of airborne and epiphytic fungal assemblages (Marchisio *et al.*, 1997; Newsham *et al.*, 1997; Moody *et al.*, 1999). Secondly, variation in the composition of the plant community may affect the structure of the phyllosphere fungal assemblages of European beech along the studied gradient. The percentage of beech trees varied among elevation sites, as well as the species composition of neighboring trees (*Quercus robur* at the lower sites and *Abies alba* at the upper sites). We nevertheless tried to minimize this potential neighborhood effect by sampling only forest stands containing > 50% beech trees. Thirdly, variation in the functional traits of leaves, controlled by the environment and the genetic background of the trees, may also structure phyllosphere fungal assemblages along the gradient of elevation. For example, photosynthetic capacity, leaf mass per unit area, leaf nitrogen content and leaf wettability vary with elevation (Bresson *et al.*, 2009, 2011; Aryal & Neuner, 2010). Genetic variation along a gradient of elevation has been demonstrated for various tree species (review by Ohsawa & Ide, 2008), including European beech (Löchelt & Franke, 1995; Sander *et al.*, 2000; Jump *et al.*, 2007). Such variation may influence the structure of associated communities (Bailey *et al.*, 2009), including phyllosphere fungal assemblages (Cordier *et al.*, 2012).

To conclude, our results show that the composition of phyllosphere fungal assemblages of European beech varied significantly along a steep gradient of elevation, in terms of both the relative abundance and the presence–absence of species. Variations in temperature might account for this pattern, although we cannot exclude the influence of other environmental parameters varying along the gradient and not included in our analyses. We found that the dominant species, as well as two species described as pathogenic to beech, were unevenly distributed along the gradient of elevation. If the constraints controlling species distributions along the elevation gradient are mostly abiotic (e.g. temperature and frost), species currently present predominantly at lower elevations, such as the endophytic latent pathogen *M. punctiformis* (a causal agent of leaf spots) and the saprobe *M. flageoletiana* (a highly dominant species), might move upward as the climate warms up. If the constraints are also biotic (e.g. presence of antagonists), species distribution change is more uncertain, because the outcome of biotic interactions in conditions of global warming is difficult to predict (Tylianakis *et al.*, 2008).

Acknowledgements

We thank Sylvain Delzon for providing the weather data along the gradient. We also thank Frederick Gavory and Corinne Cruaud from Genoscope, Evry, for the 454 sequencing of the samples. We

thank three anonymous reviewers for helpful comments on the first version of the manuscript. This study was supported by a grant from the Forest Health Department of French Ministry of Agriculture (Convention E17/08, no. 22000285), by a European project, Biodiversity And Climate Change, A Risk Analysis (BACCARA, no. 22000325) and by Genoscope project (no. 42 AP09/10).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 454 pyrosequencing primer sequences (forward) used for the amplification of fungal rITS1 and concatemer scheme

Table S2 List of all molecular operational taxonomic units (MOTUs), their relative abundance, and taxonomic assignments by BLAST in GenBank (environmental sequences excluded or included)

Methods S1 Modified version of the *bioenv* function of the R vegan package.

Methods S2 Full R script and data files.

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