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Effects of host species, environmental filtering and forest age on community assembly of ectomycorrhizal fungi in fragmented forests

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ABSTRACT

Understanding the assembly of biological communities in space and time is a major goal in community ecology. While most studies have focused on community assembly patterns in macro-organisms, there are comparatively few studies on micro-organisms. Here, we investigated how communities of ectomycorrhizal (EcM) fungi assemble in fragmented forests. We used a space-for-time substitution as an alternative for long-term studies to investigate variation in EcM fungal communities in three host species collected from 41 forest patches of different ages. Metabarcoding of root samples revealed that community composition was affected by a combination of host plant, soil variables, and forest age. While there were no clear effects of forest age on EcM fungal communities in early-successional tree species alder and hawthorn, forest age did affect the EcM fungal communities in hazel, which is typically associated with ancient forest. EcM fungal communities in early-successional species were affected mostly by soil conditions.

1. Introduction

A major goal in ecology is to explain patterns of species diversity and community composition. The importance of both local and regional processes in structuring natural communities is well documented, and a large number of hypotheses (for a list of 120 different hypotheses see Palmer, 1994) have been put forward to explain the occurring variation in species richness and community composition (e.g. Zobel, 1992; Schluter and Ricklefs, 1993; Huston, 1994; Weiher and Keddy, 1995; Roughgarden, 2009). In an attempt to unify the various theories that have aimed at conceptualizing community ecology, Vellend (2010) recognized four key processes: selection among species, drift, speciation and dispersal (see also Vellend (2016)). These processes are analogous to the four central processes in population genetics theory, *i.e.* selection within species, drift, mutation, and gene flow. While Vellend's framework originally focused on macro-organisms such as animals and plants, it can also be applied to microbial communities (Nemergut et al., 2013). Micro-organisms have generally been considered to have very high dispersal capacities (Finlay and Clarke, 1999; Finlay, 2002; Darcy et al., 2011) and therefore to be latently present around the globe, appearing wherever environmental conditions are suitable (Baas-Becking's hypothesis (1934): 'everything is everywhere, but the environment selects'). Micro-organisms were thus assumed not to be dispersal limited and only to be affected by selection through abiotic filtering. However, recent studies have shown that this is not necessarily the case (e.g., Martiny et al., 2006, 2011), and that the relative effects of environmental factors on community composition vary across spatial and temporal scales (Nemergut et al., 2013).

Ectomycorrhizal (EcM) fungi represent an important ecological group, both ecologically and economically. They are made up of microscopic structures (hyphae), produce spores and therefore can be categorized as micro-organisms, notwithstanding the large genet size or fruit bodies of some species (Bergemann and Miller, 2002; Boddy and Jones, 2007). Although they associate with only a small proportion of plant species (ca. 2%), EcM are the dominant mycorrhizal type in temperate, boreal and some tropical forests (Smith and Read, 2008; Brundrett, 2009; Tedersoo et al., 2010; van der Heijden et al., 2015). As root symbionts, they increase nutrient uptake of their hosts, provide protection against soil pathogens (Bennett et al., 2017), and therefore are critical to the structuring of plant communities. However, how ectomycorrhizal communities disperse and assemble in current-day landscapes remains poorly understood (Horton, 2017). Particularly in landscapes that consist of habitat patches of different age, land use history and local growth

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conditions, little is known about how ectomycorrhizal communities assemble in space and time and how this is affected by environmental variables (Bahram et al., 2015). Previous research has shown that, although these root-associated symbionts show large differences in dispersal capacity, even highly dispersive species show rapidly decreasing spore loads with increasing distance from source patches (Peay et al., 2012). As a consequence, EcM fungal communities in newly established habitats may be highly affected by dispersal limitation (Peay et al., 2010) and it is therefore not unlikely that spatial isolation and overall landscape connectivity contribute to EcM fungal community assembly (Peay and Bruns, 2014; Vannette et al., 2016; Boeraeve et al., 2018).

Apart from dispersal, selection, both through biotic interactions and environmental filtering, may affect the assembly of EcM fungal communities. Obviously, the main selecting agent is the presence of a suitable host (Ishida et al., 2007; Buée et al., 2011; Urbanová et al., 2015; Vincenot and Selosse, 2017). While most EcM plant species associate with a broad range of phylogenetically diverse EcM fungi, EcM fungi vary greatly in their host range (Molina et al., 1992; Smith and Read, 2008; van der Heijden et al., 2015). For example, *Laccaria amethystina* and *Tricholoma scalpturatum* are species with a very broad host range (Roy et al., 2008; Christensen and Heilmann-Clausen, 2013), while species such as *Tricholoma cingulatum* (*Salix*), *Lactarius pyrogalus* (*Corylus*) and *Cortinarius ammophilus* (*Salix repens*) only associate with one genus or even one single species (Arnolds and Kuyper, 1995; Heilmann-Clausen et al., 2000). Furthermore, EcM fungi do not only interact with their host, but also with other soil organisms, including other EcM fungi (Kennedy, 2010), saprotrophic fungi (Cairney and Meharg, 2002), soil fauna (Anslan et al., 2018), and soil bacteria (Kluber et al., 2011; Barbieri et al., 2012), and priority effects can have a major impact on final community composition by altering competitive interactions between EcM fungi (Kennedy et al., 2009). Apart from biotic interactions, abiotic factors such as soil moisture, nutrient availability and pH also affect EcM fungal community composition (Suz et al., 2014; Erlandson et al., 2015).

The general aim of this study was to (i) investigate patterns of ectomycorrhizal fungal community composition in forest patches of different age which occur as islands in an agricultural landscape matrix, and (ii) assess the relative importance of dispersal limitation and selection through both biotic interactions with the host plant and abiotic filtering in the assembly of EcM communities. We hypothesized that, if ectomycorrhizal fungi are dispersal limited, EcM community composition is significantly affected by forest age, with the youngest forest patches being dominated by species with high dispersal capacities, while the communities of older forest patches also comprise more slowly dispersing species. If, on the other hand, local environmental conditions or host type are the main factors determining ectomycorrhizal community assembly, differences in ectomycorrhizal communities are mainly caused by biotic and abiotic factors, and to a lesser extent by the dispersal capabilities of the fungi. To test these predictions, we used amplicon sequencing of the ITS1 rDNA region using Illumina MiSeq to identify the EcM community composition of three different host tree species that were sampled across a set of 41 fragmented forest patches of different age and covering a gradient in abiotic factors.

2. Materials & methods

2.1. Study species

Three different tree species that display different affinities to ancient forests were selected to investigate patterns of community assembly of ectomycorrhizal fungi in fragmented forest patches. Whereas alder (*Alnus glutinosa)* shows no specific affinities to forest age and can rapidly colonize newly established forest patches, hazel (*Corylus avellana*) is considered a typical ancient-forest plant species that only colonizes forest patches late in the succession stage (Honnay et al., 1998; Hermy et al., 1999). Although hawthorn (*Crataegus monogyna*) may show higher abundance in ancient forest (Dupouey et al., 2002), it is usually not considered a typical ancient-forest species in our study region (Hermy et al., 1999; Jacquemyn et al., 2003). Moreover, the three species are known to differ in the specificity of their EcM communities. While the EcM fungi associated with *Alnus* are highly specialized (Rochet et al., 2011), *Corylus* has only a few (known) specialist fungi (e.g. *L. pyrogalus* (Heilmann-Clausen et al., 2000)). For the third host species, hawthorn (*C. monogyna*), the specificity of EcM interactions is unknown, although previous research has shown that the species does form associations with EcM fungi (Newton and Haigh, 1998).

2.2. Study area

Sampling took place in 41 forest fragments located in central Belgium, 20 km east of Leuven (50°51′60″N, 4°56′50″E), in a study area of c. 50 km^2 (Fig. S1, see Jacquemyn et al. (2003) for more details). The forests in this study vary from wet forests on loamy, poorly drained soils classified as Alno-Padion forests in the valley of the river Velpe and its tributaries to forests on the hills bordering the valley with sandy loam, well drained, acidic soils that belong to the Quercion alliance. Based on nine historic topographic maps, the oldest one going back to 1775, the age of each of the selected forest fragments was determined and they were assigned to four age classes: <50, 50–100, 100–200 and > 200 years old (Jacquemyn et al., 2001).

2.3. Sampling

Forests were selected evenly over the gradient in environmental characteristics (from wet, loamy soils to dry, sandy loam soils) and over the four age classes. Tree species composition generally varied with soil conditions, but sampling was conducted in such a way that variation in tree species composition within sampling plots was minimized. In the wettest forests, the tree layer consisted mostly of *Populus* x *canadensis* (AM and EcM), *Fraxinus excelsior* (AM), *A. glutinosa* (AM and EcM) and *Quercus robur* (EcM) and in the drier forests the tree layer was made up mostly from *Q. robur* (EcM), *Betula pendula* (EcM) and *Sorbus aucuparia* (EcM). In each forest fragment, a plot of 10 by 10 m was established in which root samples from one individual of alder (*A. glutinosa)*, hazel (*C. avellana)* and hawthorn (*C. monogyna)* were taken. Plots were established 10 m away from the forest edge to exclude edge effects. As not every host was present in every forest, in some plots only one or two hosts were sampled. As alder was only found in the wettest forests, it was sampled only 20 times, while hazel and hawthorn were sampled 40 times. Root samples were taken by digging along a large root starting from the base of the tree towards the finest roots to ensure the sampled roots were from the selected host plant. The fine roots were visually inspected and 10 roots of around 5 cm were put in paper bags with silica gel. This was repeated three times on one tree, to better represent the diversity within one individual tree. All fine roots were collected within a 2 m radius of the tree and within the upper 20 cm of the soil and pooled in one sample per tree. Overall, we collected 20 pooled root samples from alder, and 40 pooled root samples from hawthorn and hazel resulting in a total of 100 root samples (Table 1).

Additionally, five soil samples for chemical analysis were randomly taken in each plot, pooled in a plastic bag and stored on ice. Samples were stored up to 4 days before processing.

Table 1
Experimental design of the study. In total, 41 forests were visited for sampling ectomycorrhizal fungal community composition associated with three tree species (*Alnus glutinosa*, *Corylus avellana* and *Crataegus monogyna*). Not all tree species were present in each forest, and alder was only present in the wettest forests. Each sample is a pooled root sample from one tree.

Age of the forest		alder *(Alnus glutinosa)*	hazel *(Corylus avellana)*	hawthorn (*Crataegus monogyna)*	
Age class 1	27	1	4	4	9
	38	2	3	3	8
	51	2	3	3	8
Age class 2	67	5	8	8	21
	97		2	2	4
Age class 3	132	1	4	5	10
	155	4	5	5	14
	202		1		1
Age class 4	>242	5	10	10	25
		$n = 20$	$n = 40$	$n = 40$	Total: 100 samples

2.4. Molecular methods

The sampled roots were brushed to remove soil particles and crushed in liquid nitrogen. DNA was extracted using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Inc, Carlsbad, CA, USA) according to the manufacturer's protocol. For each sample 0.2 g of root was used. The ITS1 region of the nuclear ribosomal RNA genes was amplified using modified versions of the primer set ITS1F and ITS2 (Smith and Peay, 2014). PCR was carried out in 20 μL reactions consisting of 1 μL genomic DNA, 0.5 μL of each 10 μM primer, 1 μL dNTPs, 4 μL 5X Green GoTaq Reaction Buffer (Promega, Madison, WI, USA), 0.2 μL GoTaq DNA Polymerase (Promega, Madison, WI, USA) and 12.8 μL of nuclease-free water. PCR cycles started with 1 min denaturation at 94 °C, followed by 30 amplification cycles of 94 °C for 30 s, 52 °C for 30 s, 72 °C for 30 s and a final extension of 72 °C for 7 min. PCR products were separated by gel electrophoresis and amplicons within the appropriate size range were cut out and purified with QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Amplicons were quantified using Qubit dsDNA HS Assay Kit (Invitrogen, Carlsbad, CA, USA) on a Qubit fluorometer. Samples were then pooled in equimolar concentrations and sent to Genomics Core UZ Leuven for 250 bp paired-end sequencing on an Illumina Miseq.

2.5. Bioinformatics

Genomics Core UZ Leuven provided demultiplexed reads, which were then quality filtered, clustered into OTUs and assigned a taxonomic identity through the PIPITS pipeline (Gweon et al., 2015). In a first step, PEAR (Zhang et al., 2014) was used to join read-pairs on the overlapping regions which are then quality filtered with FASTQ_QUALITY_FILTER (Fastx toolkit: Gordon and Hannon, 2010) with minimum quality score 30 and minimum 80% of the bases that must have this quality score. In a next step, ITSx (Bengtsson-Palme et al., 2013) was used to extract the ITS1 subregion of fungal origin from the sequences after dereplication. In a third and final step of this pipeline, VSEARCH was used to remove short (<100 bp) and unique sequences, to cluster sequences with min. 97% sequence similarity into Operative Taxonomic Units (OTUs), to remove chimeras using the UNITE UCHIME reference data set (Nilsson et al., 2015) and to map the input sequences onto representative sequences. Using RDP Classifier (Wang et al., 2007) and the UNITE fungal ITS reference data set, these representative sequences were then assigned a taxonomic identity. For OTUs that where not assigned a taxonomic identity at genus level, representative sequences were used in manual BLASTn searches against the NCBI nucleotide database. Uncultured/environmental sample sequences were excluded from the search set and the search set was restricted to fungal sequences. The 20 best-matching sequences with a maximum e-value of e^{-100} and a minimum of respectively 90% and 97% sequence similarity were used for identification at genus and species level. The results were put in an OTU table (sample x OTU table with each cell containing read numbers), which was used in all further analyses. As the number of artefactual sequences is known to increase with increasing sequencing depth (Alberdi et al., 2018), OTUs represented by less than 0.01% of the reads in a sample were considered to be absent from that sample. Also, rarefaction curves were generated with the function *rarecurve* from the *vegan* R-package (Oksanen et al., 2016) and samples of which the rarefaction curve did not reach an asymptote were discarded. In order to select the ectomycorrhizal OTUs from the dataset, the OTU table was run through FUNGuild (Nguyen et al., 2016). This script compares the most highly resolved taxon of each OTU (e.g. species or genus) with a database, assigning it to an ecological guild with a confidence ranking. A subset of the OTU table (with only the ectomycorrhizal OTUs) was further analyzed as described below.

2.6. Soil chemical analysis

Soil samples were analyzed for pH, nitrate, ammonium, plant available phosphorus, gravimetric water content and organic carbon content. Soil was mixed with deionized water in a 1:10 ratio and shaken for 10 min before measuring the pH with a pH probe. Phosphorus content of the soil was determined through the Olsen P test (Olsen, 1954). Nitrate and ammonium were determined by shaking a solution of 5 g of soil in 25 mL of 1M KCl for 30 min, followed by centrifugation for 5 min at 3500*g* to clarify the sample. NH_4^+- and NO_3^--nitrogen were then measured colorimetrically from the supernatant using an Evolution 201 UV–Visible spectrophotometer (Thermo Scientific, Madison, USA). To determine the gravimetric water content, 10 g of fresh soil was dried at 105 °C for 24 h and then weighed again. The remaining dry soil was then used to determine the organic matter content by heating the samples up to 630–700 °C for 2 h and weighing them again.

2.7. Statistical analysis

For each sample, the effective number of OTUs, Hill numbers of order 1 and 2 (^1D and ^2D) (Hill, 1973), and the derived evenness of order 1 and 2 (^1E and ^2E) were calculated following Lucas et al. (2016). The general framework of Hill numbers quantifies diversity based on richness (the number of OTUs present) and evenness or abundance distribution of the OTUs present. With increasing order q, diversity measure ^qD is less influenced by rare OTUs. ^1D can be interpreted as the number of common OTUs and ^2D as the number of dominant OTUs. ^1E and ^2E are ^1D and ^2D divided by the effective number of OTUs, respectively, and will be 1 if all OTUs

are evenly present. ^1D and ^2D are mathematically transformable to the common diversity measures, Shannon diversity and Simpson diversity, respectively (Lucas et al., 2016).

The diversity measures were used as the dependent variable in generalized linear mixed models with age class (continuous variable), host species (categorical variable), $\log_{10}$-transformed fragment area (continuous variable), number of reads per sample (continuous variable) and the variation in soil characteristics as independent variables. To quantify the latter, and in order to remove correlations between the individual variables (pH, NH_4^+, NO_3^-, P, organic content, moisture), a Principal Component Analysis (PCA) was applied and the first two PC-axes were used as continuous variables in the models (Dormann et al., 2013). To detect spatial structuring, principal coordinates of neighbour matrices (PCNM) were used to transform spatial distances to a position vector which was also used as a continuous variable in the models. To calculate PCNMs, we used the *pcnm* function in the *vegan* library of R (Oksanen et al., 2016). As multiple hosts were sampled per plot, plot was added as a random factor in the models. Models were fitted separately for each diversity metric, using the *lme4* package and *lmerTest* package in R (Bates et al., 2015; Kuznetsova et al., 2017). Model selection was done using backwards selection with Wald χ^2 tests from the Type III analysis of deviance tables. To account for differences in sequencing depth, number of reads per sample was always kept in the final model, regardless of its significance. In case a factor was found to significantly affect one of the diversity metrics, multiple comparisons were carried out using the *multcomp* package (Hothorn et al., 2008).

To assess the effect of host on ectomycorrhizal fungal community composition, a permutational analysis of variance (PERMANOVA) using the *adonis* function in the R package *vegan* was conducted with host species and number of reads per sample (to correct for unequal sequencing depth) as independent variables. Non-metric multidimensional scaling (NMDS) was used to visualize differences in EcM fungal community composition. Subsequently, for each tree, the species, the effect of forest age, spatial configuration and environmental variables on EcM fungal community composition were investigated using partial Redundancy Analysis (pRDA) on Hellinger-transformed OTU composition data (Legendre and Gallagher, 2001). The effect of number of reads per sample was partialled out to remove possible effects of unequal sequencing depth. The best model was selected using the *ordiR2step* function in *vegan* which performs a forward stepwise selection based on adjusted R^2 and p-values with permutation tests. A permutation test was also used to assess the significance of the axes and the terms. To test whether EcM fungal communities of forests situated close to each other were more similar than EcM fungal communities of forests lying further apart, a Mantel test was performed separately for each host species using the *mantel* function in the *vegan* library. A geographic distance matrix depicting distances between all studied forest fragments was related to a similarity matrix in Hellinger-transformed OTU composition (with Bray-Curtis dissimilarities) per host species and significance of the relationship was assessed using 1000 randomizations. The Pearson's product moment was used as correlation coefficient.

To study in more detail how host species, forest age class and environmental factors affected EcM fungal community composition, separate generalized linear mixed models (GLMM) with binomial distribution were fitted on the proportional abundances of the 15 most abundant EcM fungal genera. Host species, age class, the first PCNM spatial vector and the two soil principal components (PC1 and PC2) were added as explanatory variables, and plot was added as a random factor. Model selection was done based on AIC.

Finally, an indicator species analysis was used to determine whether certain OTUs are associated with a one of the host tree species or age classes, using the *indicspecies* library in R (Cáceres and Legendre, 2009).

3. Results

After the initial quality filtering and clustering, 9 386 408 reads were assigned to 5183 OTUs. After a second quality filtering (per sample removal of OTUs represented by less than 0.01% of the total number of reads in that sample), 2490 OTUs remained. Of these, 1648 OTUs were not identified to genus level and were subjected to a manual search against the NCBI nucleotide database. 192 of those 1648 OTUs were assigned a taxonomic identity at genus level and were, together with the rest of the dataset (842 OTUs), run through FUNGuild to determine the functional guild. 391 OTUs had an ectomycorrhizal lifestyle, 123 were arbuscular mycorrhizal fungi and the others were saprotrophs (332 OTUs), plant pathogens (64 OTUs), endophytes (60 OTUs), animal pathogens (40 OTUs), other parasites (13 OTUs) or had an unknown lifestyle (11 OTUs). Still, more than half of the OTUs were not assigned to an ecological guild mainly due to limitations in taxonomic assignment (only a limited amount of fungal species is represented in sequence databases) and due to limited knowledge of the ecology of fungi (Nguyen et al., 2016). To minimize effects of sequencing depth on statistical analyses, rarefaction curves were plotted and nine samples of which the rarefaction curve did not reach an asymptote, were removed. 68 samples remained with sequencing depth varying between 1914 and 200 774 reads. As this is still a large difference and could impact our results, number of reads per sample was included as a factor in each of the statistical models (as described below). All further results came from analyses on the 391 EcM fungal OTUs from these 68 samples.

All ectomycorrhizal fungal OTUs belonged to the phyla Basidiomycota (90.4%) and Ascomycota (9.6%). The Ascomycota came from four orders (Chaetosphaeriales, Eurotiales, Hysteriales and Pezizales) and 15 genera and the Basidiomycota came from six orders (Agaricales, Boletales, Cantharellales, Russulales, Sebacinales and Thelephorales) and 25 genera. Across all forest fragments, 1 524 476, 2 612 092 and 1 173 992 reads, divided over 160, 249 and 237 OTUs were found in the roots of alder, hazel and hawthorn, respectively. EcM fungal communities from alder roots were dominated by *Alnicola* and *Tomentella* (which make up on average respectively 34.7% and 30.9% of the reads in a sample) (Fig. 1). *Lactarius* (on average 34.0% of the reads in a sample), *Tomentella* (27.3%) and *Russula* (15.3%) were best represented in hazel root samples (Fig. 1). In hawthorn, the genera *Lactarius, Russula* and *Tomentella* had the highest abundance (on average 29.9%, 28.1% and 15.4%, respectively) (Fig. 1).

There was a strong positive correlation between pH and NO_3^- (Pearson's $r = 0.796$, $p < 0.001$), a negative correlation between pH and NH_4^+ (Pearson's $r = -0.528$, $p < 0.001$), a negative correlation between moisture and NH_4^+ (Pearson's $r = -0.243$, $p = 0.04$) and a positive correlation between moisture and organic material (Pearson's $r = 0.262$, $p = 0.031$). The PCA showed that the first PC axis explained 41.4% of the total variance and was positively associated with pH, NO_3^-, NH_4^+ and soil moisture content, while the second PC axis explained 23.0% of the total variance and was positively associated with organic matter, moisture, NH_4^+ and P. Generalized linear mixed model analyses showed a significant effect of host species, age class, soil PC2 and the interaction between soil PC2 and host species on EcM fungal richness (effective number of OTUs) (Table 2). Best-fit models on the other diversity measures found a small, non-significant effect of soil PC1 on diversity measures ^1D and ^2D (Table 2) and no significant effect of any of the variables tested on evenness ^1E and ^2E.

PERMANOVA showed that the mycorrhizal communities

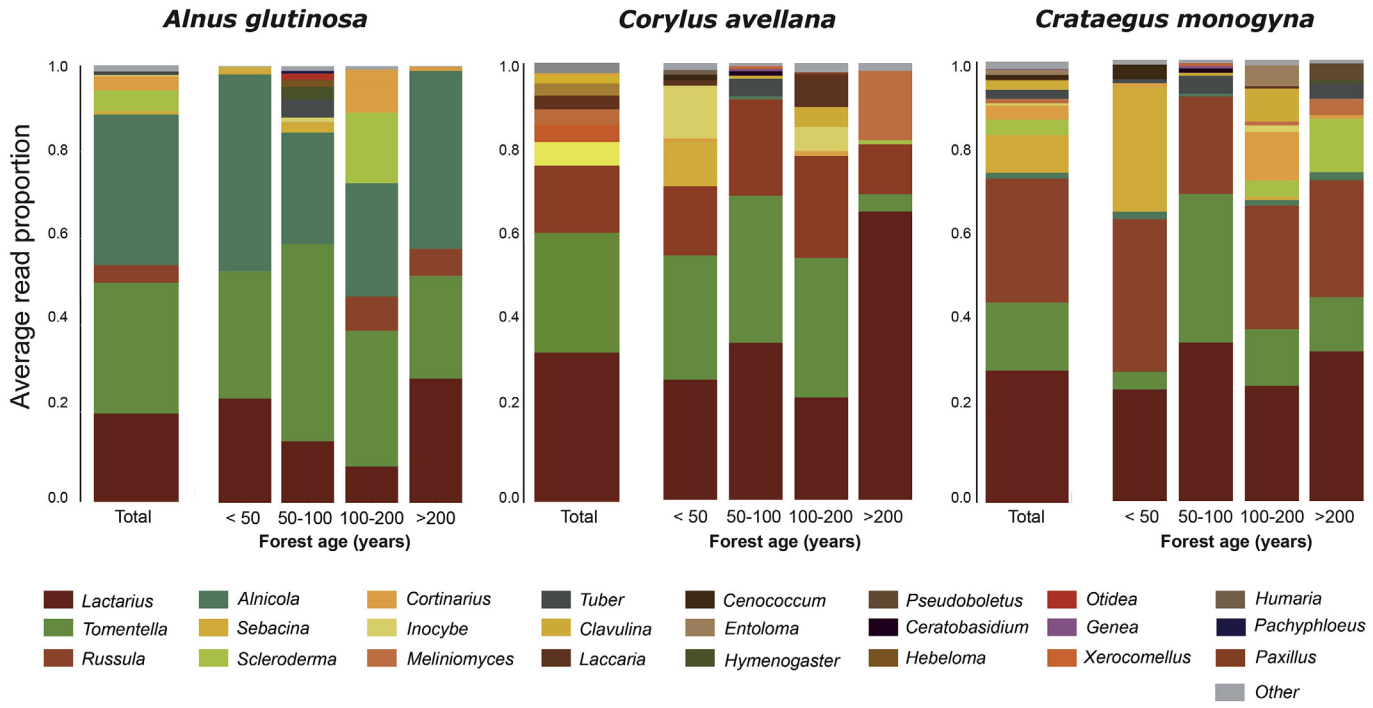

Fig. 1. Average ITS1 read proportions of each ectomycorrhizal fungal genus per host. The first of every 5 bars shows read composition over all root samples per host (respectively 17, 27 and 24 samples), while the next 4 bars show read composition split up into four age classes per host (*Alnus glutinosa:* respectively 4, 3, 5 and 5 samples, *Corylus avellana:* respectively 7, 4, 10 and 6 samples, *Crataegus monogyna:* respectively 7, 6, 6 and 5 samples). When analyzed separately, only the communities of *Corylus avellana* were significantly affected by forest age (RDA: $F_{3,22} = 1.569$, $p = 0.023$, $R^2_{adj} = 0.064$).

differed (pseudo-$F = 5.41$, $p < 0.001$) significantly between the three host species. Number of reads per sample did not affect community composition (pseudo-$F = 1.14$, $p = 0.27$). Differences in mycorrhizal communities were visualized in an NMDS (Fig. 2) and were largest between alder and hazel, whereas the mycorrhizal communities associating with hawthorn were somewhat intermediate between these two species. Separate partial RDAs per host species followed by permutation tests revealed a difference in effect of soil PC1 and age class on EcM fungal community composition. Soil PC1 had a significant effect on the EcM fungal community composition in the roots of alder and hawthorn (respectively $F_{1,14} = 1.825$, $p = 0.037$, $R^2_{adj} = 0.051$ and $F_{1,21} = 1.771$, $p = 0.014$, $R^2_{adj} = 0.033$), while age class significantly affected EcM fungal community composition in hazel roots ($F_{3,22} = 1.569$, $p = 0.023$, $R^2_{adj} = 0.064$). The Mantel tests per host, relating similarities in ectomycorrhizal fungal community composition to the distances separating forest patches, revealed no structuring along a spatial gradient in the communities associated with alder and hawthorn (respectively $r = -0.0892$, $p = 0.717$ and $r = -0.003$, $p = 0.524$) and some indication of spatial structuring in the communities associated with hazel ($r = 0.104$, $p = 0.085$).

Separate generalized linear mixed models for the 15 most abundant EcM fungal genera showed a significant effect of age class on the proportional abundance within the genera *Russula, Scleroderma* and *Sebacina* and a marginally significant effect of age class on the proportional read abundance within *Lactarius* (Table 3). Host species had a significant effect on the read numbers within most of the genera tested (Table 3). The average read proportions varied among host species over the four age classes (Fig. 1). The first soil PC had a significant effect on the proportional read abundance within seven genera while the second soil PC significantly affected proportional read abundance within two genera. Some genera showed some spatial structuring with proportional read abundances of *Cenococcum, Clavulina* and *Lactarius* significantly differing along spatial vector PCNM1 and proportional read abundances of *Russula* affected by $\log_{10}$-transformed area. Proportional read abundances of *Laccaria* and *Pseudoboletus* were not significantly affected by any of the studied variables.

Species indicator analysis found 30 OTUs to be specifically associated with alder, 17 OTUs associated with hazel and 6 OTUs associated with hawthorn (Table S1). Most of the indicator OTUs for alder belonged to the genera *Alnicola* and *Tomentella.* Indicator OTUs for hazel belonged to the genera *Tomentella, Lactarius,* and *Cenococcum* and indicator OTUs for hawthorn belonged to the genera *Clavulina, Lactarius, Russula, Sebacina* and *Tomentella.* A separate species indicator analysis found 8 OTUs to be associated with forests younger than 50 years (age class 1), 6 OTUs associated with forests between 50 and 100 years old (age class 2), 5 OTUs associated with forests between 100 and 200 years old, 1 OTU associated with forests of the second and third age class together and 2 OTUs associated with forests of the two oldest age classes together (older than 100 years) (Table S2). No indicator OTUs were found for the oldest age class (forests older than 200 years).

4. Discussion

By using a space-for-time substitution as an alternative for long-term studies we characterized ectomycorrhizal (EcM) fungal community assembly over a time period of >200 years. Although this approach has its limitations (Pickett, 1989), the results showed that within a given landscape EcM fungal communities differed significantly between host species, and that community composition of EcM fungi of individual tree species was affected by variation in environmental conditions (alder and hawthorn) or the age of the forest (hazel).

Ectomycorrhizal fungal community composition differed significantly between the three investigated host species,

Table 2
Results of the generalized linear mixed models on the diversity metrics. None of the variables tested had a significant effect on evenness of the first and second order, so these were not included in the table. Reads per sample was kept as a variable in each model, regardless of significance in order to correct for unequal sequencing depth. Variables that were not included in any of the reduced models were not included in the table. Est. = estimate, "-" indicates the factor was not retained in the reduced, most parsimonious model, significant effects ($p < 0.05$) are in bold. The intercept of the first GLMM corresponds to host: alder in age class 1.

Diversity metric	Model type	Intercept	Host: hazel	Host: hawthorn	Age class: Age class 2	Age class 3	Age class 4	Soil PC1	Soil PC2	Host: hazel* Soil PC2	Host: hawthorn* Soil PC2	Reads per sample
Richness	GLMM (Poisson)	est. = 3.353 ± 0.179	**est. = -0.246 ± 0.074** **$p = 0.0009$**	est. = 0.148 ± 0.081 $p = 0.067$	**est. = -0.457 ± 0.213** **$p = 0.032$**	est. = 0.171 ± 0.187 $p = 0.359$	est. = −0.114 ± 0.198 $p = 0.954$	–	**est. = -0.218 ± 0.090** **$p = 0.015$**	**est. = 0.464 ± 0.080** **$p < 0.001$**	est. = −0.032 ± 0.070 $p = 0.645$	est. = −4.65E-7 ± 1.05E-6 $p = 0.658$
Diversity $q = 1$	GLMM (negative binomial)	est. = 1.882 ± 0.153	–	–	–	–	–	est. = 0.230 ± 0.128 $p = 0.073$	–	–	-	**est. = -0.365 ± 0.115** **$p = 0.002$**
Diversity $q = 2$	GLMM (negative binomial)	est. = 1.505 ± 0.132	-	-	–	–	–	est. = 0.193 ± 0.112 $p = 0.085$	–	–	-	**est. = -0.381 ± 0.105** **$p < 0.001$**

Fig. 2. Non-metric multidimensional scaling (NMDS) visualizing EcM fungal community composition. Black diamonds depict samples from *Alnus glutinosa*, red squares samples from *Corylus avellana* and green triangles samples from *Crataegus monogyna*. Ellipses depict standard deviation from the centroid for each host species.

confirming previous results that host range (Molina et al., 1992; Molina and Horton, 2015) and the distribution of host plants largely determine the distribution of EcM fungi, and thus form an important filter in the assembly of EcM fungal communities (Buée et al., 2011; Scheibe et al., 2015; Urbanová et al., 2015; Saitta et al., 2018). Besides differences in host, abiotic conditions formed another important filter for EcM fungal communities. In this study, soil PC2, which correlated with soil organic matter, moisture, NH_4^+ and P, had a significant effect on EcM fungal richness, which interacted with host species, and soil PC1 (correlated with pH, NO_3^-, NH_4^+ and soil moisture content) had a significant effect on community composition in two host species (alder and hawthorn). Acidification and increased N availability have been shown before to negatively affect diversity and evenness of EcM fungal communities (Wallenda and Kottke, 1998; Toljander et al., 2006; Suz et al., 2014).

Previous research has already shown that variation in pH is an important driver of EcM fungal community composition (Kutszegi et al., 2015; Matsuoka et al., 2016). Soil pH can influence EcM community composition directly since EcM fungi differ in their pH optima, but also indirectly by affecting nutrient availability (Erland and Taylor, 2002). As a result, both soil pH and nutrient availability can have a strong impact on richness of soil biota (Tedersoo et al., 2016). Soil moisture is also a known factor to cause shifts in EcM fungal community composition (Cavender-Bares et al., 2009; Erlandson et al., 2015) and in the levels of colonization by EcM fungi versus arbuscular mycorrhizal fungi (Gehring et al., 2006). The latter could also play a role in the communities of hawthorn and alder as they are capable of dual mutualisms with both arbuscular and ectomycorrhizal fungi (Molina et al., 1992).

Compared to biotic filtering through host plant species and abiotic filtering, ectomycorrhizal fungal communities were less affected by the age of the forest, suggesting that most species disperse easily through the landscape and establish well when a suitable host plant is present. *Sebacina*, *Scleroderma* and *Russula*

Table 3
Results of the GLMMs with binomial distribution on proportional abundances per genus, for the 15 most abundant genera. For *Laccaria* and *Pseudoboletus*, no significant effect of any of the variables tested was found, so they were not included in the table. Est. = estimate, "-" indicates the factor was not retained in the final model, significant effects ($p < 0.05$) are in bold. The intercept corresponds to host: alder for the first seven GLMMs and host: alder in age class 1 for the last four GLMMs.

Genus	Intercept	Host: hazel	Host: hawthorn	Age class: Age class 2	Age class 3	Age class 4	Soil PC1	Soil PC2	log10 Area	PCNM1	ΔAIC
Alnicola	est. = −2.927 ± 0.558	**est. = -6.218 ± 0.022 *p* < 0.001**	**est. = -4.387 ± 0.012 *p* < 0.001**	–	-	-	**est. = 2.798 ± 0.853 *p* = 0.001**	–	–	–	11.58
Cenococcum	est. = −15.214 ± 1.657	**est. = 1.685 ± 0.049 *p* < 0.001**	**est. = 2.217 ± 0.049 *p* < 0.001**	–	–	–	–	**est. = 1.949 ± 0.986 *p* = 0.048**	-	**est. = -4.311 ± 1.969 *p* = 0.029**	7.45
Clavulina	est. = −22.237 ± 1.804	**est. = 6.405 ± 0.093 *p* < 0.001**	**est. = 6.846 ± 0.094 *p* < 0.001**	–	–	–	–	–	-	**est. = 5.922 ± 2.755 *p* = 0.032**	11.04
Cortinarius	est. = −10.165 ± 1.078	**est. = 0.120 ± 0.014 *p* < 0.001**	**est. = -1.847 ± 0.014 *p* < 0.001**	–	-	-	**est. = 4.659 ± 1.559 *p* = 0.003**	–	–	–	8.58
Entoloma	est. = −25.878 ± 1.944	**est. = 2.721 ± 0.132 *p* < 0.001**	**est. = 7.969 ± 0.146 *p* < 0.001**	–	–	–	–	–	–	–	13.86
Genea	est. = −17.403 ± 1.223	**est. = 7.094 ± 0.275 *p* < 0.001**	**est. = 1.666 ± 0.272 *p* < 0.001**	–	–	–	–	–	–	–	7.58
Inocybe	est. = −15.440 ± 1.125	**est. = 5.878 ± 0.042 *p* < 0.001**	**est. = 3.517 ± 0.043 *p* < 0.001**	–	-	-	**est. = 4.002 ± 1.623 *p* = 0.014**	–	–	est. = 3.661 ± 1.983 $p = 0.065$	5.91
Lactarius	est. = −2.874 ± 0.833	**est. = 1.906 ± 0.004 *p* < 0.001**	**est. = 1.153 ± 0.004 *p* < 0.001**	est. = −2.182 ± 1.241 $p = 0.079$	est. = −1960 ± 1.130 $p = 0.083$	est. = 1.747 ± 1.209 $p = 0.148$	–	**est. = -0.932 ± 0.435 *p* = 0.032**	-	**est. = 1.612 ± 0.810 *p* = 0.047**	3.21
Russula	est. = −5.344 ± 0.790	**est. = 1.467 ± 0.008 *p* < 0.001**	**est. = 1.852 ± 0.007 *p* < 0.001**	**est. = -2.024 ± 1.001 *p* = 0.043**	est. = 1.762 ± 1.225 $p = 0.150$	**est. = 1.762 ± 1.225 *p* = 0.008**	**est. = -1.685 ± 0.615 *p* = 0.006**	–	**est. = -1.377 ± 0.481 *p* = 0.004**	–	2.59
Scleroderma	est. = −3.209 ± 0.565	est. = 0.154 ± 0.399 $p = 0.700$	**est. = 1.342 ± 0.385 *p* = 0.0005**	**est. = -2.314 ± 0.898 *p* = 0.010**	est. = 1.061 ± 0.565 $p = 0.061$	est. = 0.151 ± 0.629 $p = 0.810$	**est. = -1.023 ± 0.372 *p* = 0.006**	–	–	–	4.47
Sebacina	est. = −3.068 ± 0.716	**est. = 0.930 ± 0.345 *p* = 0.007**	**est. = 1.764 ± 0.345 *p* < 0.001**	est. = −1.398 ± 0.966 $p = 0.148$	**est. = -2.297 ± 1.055 *p* = 0.030**	est. = −1.770 ± 1.015 $p = 0.081$	**est. = 1.646 ± 0.618 *p* = 0.008**	–	–	–	2.38
Tomentella	est. = −1.572 ± 0.517	**est. = -0.499 ± 0.004 *p* < 0.001**	**est. = -1.323 ± 0.005 *p* < 0.001**	–	–	–	–	–	–	–	6.17
Tuber	est. = −2.951 ± 0.412	est. = −0.112 ± 0.291 $p = 0.701$	**est. = 0.700 ± 0.278 *p* = 0.012**	–	–	–	**est. = 1.571 ± 0.536 *p* = 0.003**	est. = 0.539 ± 0.309 $p = 0.081$	–	–	4.36

were most strongly affected by the age of the forest, the first decreasing and the others increasing with forest age. Also a small, non-significant effect of age class was found on the proportional abundance of *Lactarius*. *Sebacina* are known to be efficient dispersers and have a very low host specificity (Weiβ et al., 2011; Bokati and Craven, 2016) and therefore can rapidly colonize vacant habitat patches. *Scleroderma*, *Russula* and *Lactarius*, on the other hand, germinate poorly (Nara, 2009), implying they do not easily establish new populations. Fine-scale analyses of the correlation between genotypic similarity and geographic distance in several *Russula* species found limited gene flow via spore dispersal (Wang et al., 2015). In a study on EcM fungal communities in recent oak stands that were either isolated from or connected to ancient forest, *Russula* was more abundant in recent stands connected to ancient forest than in isolated stands (Boeraeve et al., 2018), suggesting that *Russula* species are unable to travel large distances across a hostile landscape matrix. Indicator species analysis on the four age classes revealed a similar pattern with indicator OTUs of the youngest age class belonging to *Cenococcum*, *Tomentella* and *Sebacina,* all known to be efficient dispersers. The observed effect of forest age might thus be attributable to differences in dispersal capacities among ectomycorrhizal fungi. It is, however, possible that other factors associated with forest age, but not measured in the environmental variables, contributed to this effect. Increased microhabitat availability (Iwański and Rudawska, 2007; Tedersoo et al., 2008), stand development (Twieg et al., 2007) or priority effects (Kennedy et al., 2009) have been shown to affect fungal communities and therefore may have contributed to the observed differences in community composition between forests of different ages.

The effect of forest age on EcM fungal community composition interacted with host species. Separate analysis of the three host species revealed a difference in how they were affected by forest age and environmental conditions. While the communities of alder and hawthorn were mainly affected by soil PC1, those of hazel were significantly affected by age class. Especially the youngest (<50 y old) and oldest age class (>200 y old) were clearly different from each other, whereas the two other age classes (50–100 and 100–200 y old) were more similar. *Sebacina, Cenococcum* and *Humaria* were only present in the youngest age class, while *Scleroderma* and *Meliniomyces* were only present in the oldest age class. On average, a third of the reads belonged to *Tomentella* in the first three age classes, compared to less than 5% in the oldest age class. The genus *Inocybe* was represented by 12% of the reads in the youngest age class and was absent from the oldest. Around one third of the reads in the three youngest age classes belonged to *Lactarius*, in the oldest forests this increased to two-thirds. These shifts in EcM fungal community composition are comparable with known patterns across chronosequences (Smith and Read, 2008; Dickie et al., 2013). EcM fungal communities of hazel also showed some indication of spatial structuring, which is most likely caused by dispersal effects. As alder species are early-successional and associate with a limited number of host-specific EcM fungi (Rochet et al., 2011), we can expect those EcM fungi not to be dispersal-limited and to be adapted to environmental conditions of young forest soils. Also, the preference of alders for alluvial habitats along rivers could improve connectivity via the river, but more research is needed to test this. The absence of an effect of forest age on EcM fungal communities in alder is thus not surprising.

We found very limited spatial effects on EcM communities. Distance did not affect diversity or community composition in the communities of hawthorn and alder and was only marginally significant in the communities of hazel. Proportional abundances of three genera (*Cenococcum, Clavulina* and *Lactarius*) were significantly affected by the spatial vector and *Russula* reached significantly higher proportions in smaller fragments. Spatial structuring in the abundance of *Lactarius* could be due to low germination capacities and vegetative expansion as the main dispersal strategy, which would also explain why it was much more abundant in the hazel communities of the oldest age class. Increased dominance of *Russula* in smaller fragments could be due to decreased competition or due to rare species disappearing in smaller fragments because of ecological drift. In a relatively homogeneous system of tree islands, Peay et al. (2007) found a strong species-area relationship for ectomycorrhizal communities with increasing diversity with habitat size and with communities from small islands nested within those of larger islands. This nested pattern, where species-poor communities of small habitat patches consisting of common species are a subsample of the species-rich communities of larger habitat patches, suggests rare species are the first to disappear with decreasing habitat size.

Other landscape scale studies have revealed similar patterns, with host and soil variables as main selecting agents (Gao et al., 2015; Matsuoka et al., 2016). Matsuoka et al. (2016) found that EcM community composition along an elevation gradient was affected by host community, spatial variability, environmental factors and a combination of those three. Studying the EcM communities in a subtropical secondary forest succession, Gao et al. (2015) found effects of a combination of forest successional stage, herb layer functional group composition, upper tree layer cover, elevation and total P. These results nicely fit within the framework proposed by Vellend (2010) with ectomycorrhizal fungal community assembly affected by a combination of selection and dispersal. Selective forces include biotic interactions (mainly host species) and abiotic conditions and dispersal limitation results in spatial structuring and differential occurrences among forests of varying age. The two other key processes described by Vellend, speciation and ecological drift, are very difficult to study. While speciation is unlikely to play a role at the studied spatial and temporal scales, ecological drift can be expected to have contributed to the unexplained variation in community composition. Especially rare taxa are vulnerable for ecological drift (Zhou and Ning, 2017) and most EcM fungal communities are made up of a small number of abundant taxa and a large number of rare taxa (Taylor, 2002), making it very likely that ecological drift affects EcM fungal communities.

5. Conclusion

The assembly of ectomycorrhizal fungal communities is the result of a complex interplay between selective processes (biotic interactions and abiotic filtering) and dispersal processes. We showed that the effect of abiotic filtering and dispersal processes can differ between communities of different host species. While no age effect was found in the EcM fungal communities of the early-successional host alder, there were clear differences in EcM fungal community composition on ancient forest species hazel between young and old forests. While it is clear that environmental filtering plays a role in the assembly of EcM fungal communities, more (experimental) research is needed to determine the exact effects of each of the different environmental factors, their interactions and how they differ between host species.

Data accessibility

Sequences used in the final dataset are available under GenBank accession numbers: KY654755 - KY654953, raw sequence data is available in the Sequence Read Archive under Bioproject PRJNA378411.

Author contributions

M.B., O.H. and H.J. conceived and designed the project. M.B. performed the field sampling and the sequence and statistical analyses. All authors contributed to the writing and editing of the manuscript.

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Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.funeco.2018.08.003.

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