

Massively parallel 454-sequencing of fungal communities in *Quercus* spp. ectomycorrhizas indicates seasonal dynamics in urban and rural sites

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Abstract

We analysed two sites within and outside an urban development in a rural background to estimate the fungal richness, diversity and community composition in *Quercus* spp. ectomycorrhizas using massively parallel 454-sequencing in combination with DNA-tagging. Our analyses indicated that shallow sequencing (~150 sequences) of a large number of samples (192 in total) provided data that allowed identification of seasonal trends within the fungal communities: putative root-associated antagonists and saprobes that were abundant early in the growing season were replaced by common ectomycorrhizal fungi in the course of the growing season. Ordination analyses identified a number of factors that were correlated with the observed communities including host species as well as soil organic matter, nutrient and heavy metal enrichment. Overall, our application of the high throughput 454 sequencing provided an expedient means for characterization of fungal communities.

Keywords: ectomycorrhiza, fungal communities, pyrosequencing, *Quercus*, urbanization

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Introduction

The species richness of plant-associated fungal communities usually exceeds the richness of their host communities (Bruns 1995; Hawksworth 2001; Hawksworth & Rossmann 1997; Horton & Bruns 2001), but little is known about the magnitude of this richness or about its maintaining mechanisms. The number of known fungi is estimated as at least 7.0×10^4 (Hawksworth 2001). The true species richness may, however, be much greater than the number of known fungi; estimates of the global species richness vary from 0.5 to 9.9×10^6 with a proposed 'working hypothesis' of 1.5×10^6 species (Hawksworth 2001).

Community ecology explains that complex and heterogeneous communities are maintained through competition, facilitation, and niche partitioning (e.g. Bruns 1995). Various environmental parameters – including

water (e.g. Pigott 1982), temperature (e.g. Erland & Finlay 1992; Domisch *et al.* 2002), host genotypic makeup (Schweitzer *et al.* 2008), host community species composition (Molina & Trappe 1994; Kranabetter *et al.* 1999), or nutrient tolerances (e.g. Lilleskov *et al.* 2001)—constitute environmental controls of fungal community compositions. Additionally, fungal communities go through temporal transitions on long, successional time scales (Jumpponen *et al.* 2002; Nara *et al.* 2003) and do so also on shorter, intra-annual time scales (Koide *et al.* 2007; Walker *et al.* 2008). These short-term shifts in organismal abundances may lead to division of fungal communities into guilds distinguished largely by their distinct phenologies (Pringle & Bever 2002; Koide *et al.* 2007).

The underlying reasons for the temporal dynamics of the fungal communities are open to speculation. Intra-annual variability in environmental conditions (moisture, temperature, and nutrient availability) and corresponding fungal preferences or tolerances for those conditions provide a niche-based mechanism (see Rastin *et al.* 1990; Swaty *et al.* 1998; Koide *et al.* 2007).

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Additionally, vegetative growth of the host or the fungus and small scale disturbances associated with root or mycelial turnover have been considered as potential drivers of seasonal patterns in fungal communities (Bruns 1995; Izzo *et al.* 2005). However, analyses combining broad environmental data matrices with community data in a temporal context are rare and often unsuccessful in pinpointing the most likely seasonal drivers.

In the study presented here, we used a repeated measures analysis of variance (ANOVA) to explore the dynamics of fungal communities associated with ectomycorrhizas of oaks (*Quercus* spp.) growing in urban and adjacent non-urban sites. Three sampling occasions across the growing season facilitated the exploration of seasonal patterns in these communities. We chose the urban-rural study system as little is known about fungal communities in urban environments and our study presents a first large-scale molecular survey of these fungal communities. To allow a high throughput sequencing of a large number of root samples manually separated from soil cores, we applied the recently developed massively parallel 454 pyrosequencing (Margulies *et al.* 2005) and combined it with DNA-tagging (for examples see Binladen *et al.* 2007; Hoffmann *et al.* 2007).

Materials and Methods

Study sites and sampling

City of Manhattan is located in northeastern Kansas in the Flint Hills region. Manhattan has approximately 50 000 residents and 20 000 students that represent a mobile, transient population. A total of four oak trees

(*Quercus* spp.) were selected at each of two sites within (urban) and two sites beyond the city limits (rural) for a total of sixteen trees across the study (Table 1). While neither four conspecific trees could be found at all sites nor the distances to the closest ectomycorrhizal stand explicitly controlled, the tree sizes and their distribution were similar across the sites. To accomplish this, solitary and hedgerow trees were utilized outside of the city to select stand structures as similar as possible to those selected within the city limits. The sites were sampled three times (May, July and September) at six-week intervals within the growing season in 2007.

We sampled a total of 16 trees; four at each of the four sites. At each of the three sampling occasions, four evenly distributed, 15 cm-deep 5 cm (diam.) cores were sampled 3 m from the trunk of each tree resulting in a total of 64 samples for each sampling occasion. The coring locations were either flagged (rural sites) or spray-painted (urban sites) to aid in their precise relocation and the July and September samples were collected within 10 cm of those collected in May. The close proximity of the samples aimed to minimize the spatial community heterogeneity and to improve the precision in testing the temporal community dynamics. The cores were stored on ice until processed in the laboratory within 72 h of sampling. At the first sampling occasion in May, the number of ectomycorrhizas per sample was counted to test whether their density differed among the sites or land use (Table 1). As this was time-consuming and as no differences were detected among the sites (Table 1), this practice was abandoned. Ectomycorrhizal roots were manually separated from the soil cores and cleaned from the adhering soil. All living ectomycorrhizal roots were separated from the dead under 10 × magnification based on visual appearance

Table 1 Properties of the four sites sampled for *Quercus* spp. ectomycorrhizosphere communities. Different letters following the numeric values (mean ± 1 SD) in the columns indicate different means at $\alpha \leq 0.05$ (Tukey's HSD pairwise comparisons). For those parameters analysed only at one point in time (May), a one-way ANOVA with only 'Site' term as an independent variable is presented—ns $P > 0.05$; * $0.01 < P \leq 0.05$; ** $0.001 < P \leq 0.01$; *** $P \leq 0.001$

	City park	KSU campus	Kings creek	Shane creek
Land use	Urban	Urban	Rural	Rural
Location	N39°10'58 " W96°35'25 "	N39°11'46 " W96°34'53 "	N39°06'21 " W96°36'01 "	N39°06'42 " W96°33'49 "
Elevation (m asl)	312	329	344	354
EcM host(s)	<i>Q. palustris</i> , <i>Q. robur</i>	<i>Q. macrocarpa</i> , <i>Q. rubra</i> , <i>Q. stellata</i>	<i>Q. macrocarpa</i> , <i>Q. muehlenbergii</i>	<i>Q. macrocarpa</i> , <i>Q. muehlenbergii</i>
Stand structure	Single or clustered trees	Single or clustered trees	Single or clustered trees	Single or clustered trees
Ground vegetation	None or lawn	None or lawn	None or mixed prairie	None or mixed prairie
EcM Tips ^{ns, a}	398.69 ± 85.42 ^a	82.41 ± 54.24 ^a	212.50 ± 62.16 ^a	327.75 ± 276.41 ^a
DBH (cm) ^{ns, b}	77.19 ± 24.33 ^a	76.00 ± 20.69 ^a	81.97 ± 59.96 ^a	23.91 ± 7.90 ^a

^aNumber of ectomycorrhizal tips in a core sample.

^bDiameter at breast height.

and elasticity (Aerts *et al.* 1989), and stored in MoBio Bead Tubes (UltraClean Soil DNA, MoBio Laboratories) in the bead solution and frozen at -20°C until DNA was extracted. The soil remaining after the root removal was dried at 50°C for 48 h and stored for nutrient analyses at the Kansas State University Soil Testing Laboratory, where the soils were analysed for pH (H_2O), P, K, NH_4^+ , NO_3^- , organic matter (OM), Cd, and Pb.

DNA extraction, amplicon generation and sequencing

Total DNA was extracted following manufacturer's instructions and eluted in 100 μL of the elution buffer S5 (UltraClean Soil DNA, MoBio Laboratories). All templates were quantitated with an ND1000 spectrometer (NanoDrop Technologies) and adjusted to a final 2.5 $\text{ng}/\mu\text{L}$ concentration. To allow direct 454-sequencing of the fungal Internal Transcribed Spacer1 (ITS1), the amplicons were produced using DNA-tagged primers that combined the 454-primers (Margulies *et al.* 2005) and the fungus-specific (ITS1F; Gardes & Bruns 1993) or universal (ITS2; White 1990) primers as described in Jumpponen & Jones (2009). The selected primers and their DNA tags are available in Table S1 (Supporting Information).

After optimization for template concentration, MgCl_2 concentration, and annealing temperature, fungal ITS1 was PCR-amplified in 50 μL volumes in three separate reactions to account for potentially heterogeneous amplification from the environmental template. Each reaction contained following final concentrations or absolute amounts of reagents: 200 nM of each primer, 10 ng of the template DNA, 200 μM of each deoxynucleotide triphosphate, 2.5 mM MgCl_2 , 1 unit of Platinum Taq DNA polymerase (Invitrogen), and 5 μL of PCR buffer (Platinum Blue; Invitrogen). The PCR cycle parameters consisted of an initial denaturation at 94°C for 3 min, then 25 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 1 min and extension at 72°C for 2 min, followed by a final extension step at 72°C for 10 min. All PCR reactions were performed in 96-well PCR plates on a MasterCycler (Eppendorf). Possible PCR amplification of contaminants was determined using a blank sample run through the extraction protocol simultaneously with the actual samples and a negative PCR control in which the template DNA was replaced with ddH_2O . These remained free of PCR-amplicons.

A total of 20 μL of each of the three amplicons for each sample was combined and purified (AmPure SPRI; AgenCourt Bioscience). The clean amplicons were quantitated with the ND1000 spectrometer and 75 ng combined for sequencing. The three pools representing the three seasonal samples were all adjusted to 1000ng in a

100 μL volume (10ng/ μL) and sequenced in three 1/16th sequencing reactions on a GS FLX sequencer (454 Life Sciences) at the Interdisciplinary Center for Biotechnology Research at University of Florida. We expected an average sequencing depth of 156 reads for each of the 64 samples assuming 10 000 sequence yield for each of the 1/16th regions. The GS-FLX run data are available at the Short Read Archive (<http://www.ncbi.nlm.nih.gov/Traces/sra/>) under accession SRA009804.

Bioinformatics and OTU designation

The sequence data were analysed as described in Jumpponen & Jones (2009). For quality control, sequences without valid primer sequence or DNA tag, or those that contained ambiguous bases, or were shorter than our length threshold (200 bp) were removed. To provide a comprehensive list to sequences for public access, we assigned OTUs at 99% similarity level using CAP3 (Huang & Madan 1999)—a dereplicated example of each OTU as well as an example of each singleton is available at GenBank under accession numbers FJ776018 - FJ784103.

The sequences that passed quality control were aligned with CAP3 and assigned to operational taxonomic units (OTUs) at 95% sequence identity using a minimum overlap of 100 bp (Table S2, Supporting Information) with other parameters (including those for matches, mismatches and gap penalties) left at defaults. We chose a conservative sequence identity to account for the use of only the variable ITS1 region, because the OTU richness assumes a near exponential growth above this sequence identity (Fig. S1, Supporting Information), and because 454-sequencing may overestimate richness as a result of sequencing artifacts (Quince *et al.* 2009; Reeder & Knight 2009). At this sequence identity, sequences were parsed by sample and season to calculate the frequency of all OTUs for each of the 192 samples across three seasonal samples. From this output, SAS (SAS Institute Inc.) was used to calculate richness (S) and diversity indices (including Simpson's D; Shannon's H' and Fisher's α) (see Jumpponen & Jones for details). To further explore organismal coverage within the focal trees and land use types, species accumulation (rarefaction) curves and extrapolative richness estimators were generated using EstimateS (version 8, Colwell 2006).

We used two different strategies to examine OTUs or taxa that may differ across the land use types or seasonal samples. First, a student's *t*-test at $\alpha \leq 0.05$ was used to identify OTUs, whose frequencies of occurrence were adequate to allow inferences on their distribution among the land use factors or sampling occasions. All

OTUs with an average frequency significantly greater than zero ($P \leq 0.05$), i.e. those with average frequency greater than approximately two standard deviations, were analysed using in JMP 7.0.1 (SAS Institute Inc.). Second, OTUs that occurred more than twice in the entire data set were manually assigned to families based on BLAST matches (Zhang *et al.* 2000) and their most likely nutritional mode (ectomycorrhizal, other) appended to the taxon classification. These sequences represented more than 98% of all sequences. Frequencies on family level were summed across OTUs and analysed for effects of land use and season. We chose the family level resolution for these analyses because of the poor genus level resolution for many OTUs within some of the most commonly occurring families (e.g. Thelephoraceae) and because the best BLAST matches often returned accessions annotated as ectomycorrhizal taxa assigned only to families. This is in contrast with our previous work on the phyllosphere fungal communities (Jumpponen & Jones 2009), which include a number of well-annotated anamorphs and teleomorphs that represent known foliar pathogens and plant detritus colonizing saprobes.

Test of phylogenetic signal in short MPS sequence reads

To test whether or not the short 454 sequences provided information adequate for phylogenetic placement, we selected sequences representing the most abundant OTUs from the three most abundant ectomycorrhizal families in our data set (Cortinariaceae, Thelephoraceae, and Tuberaceae) and the third most commonly occurring family Nectriaceae along with related members of Hypocreales. The sequences within an OTU were selected so that, where possible, they originated from different focal trees and represented each of the three sampling occasions. The orientation of these sequences was corrected and they were aligned with reference reads obtained from GenBank (<http://www.ncbi.nlm.nih.gov/>) in Sequencher (v.4.6; GeneCodes Inc.). The alignments were analysed in MEGA4 (Tamura *et al.* 2007) using neighbor joining (NJ) and maximum parsimony (MP). All positions containing alignment gaps and missing data were eliminated in pairwise sequence comparisons (pairwise deletion option) in the NJ analyses. All alignment gaps were treated as missing data in the MP analyses. The robustness of the acquired topologies was assessed by 1000 bootstrap replicates in both NJ and MP analyses.

Statistical analyses

The four samples around each of the four focal trees at each site are not truly independent. Accordingly, we

averaged the measured dependent variables for each tree ($N = 16$) and analysed the responses using repeated-measures ANOVA in a nested model with independent variables 'Land Use' and 'Site(Land Use)'. The repeated measures procedure uses ANOVA to test between subject effects using the average across the sampling times. Since seasonal dynamics was a hypothesis that could be tested using the data collected in three separate sampling occasions, we examined the seasonal or the 'within subject' terms. All these analyses were carried out in JMP 7.0.1 (SAS Institute Inc.).

To summarize and visualize fungal community and environmental data, the frequencies of the families and the environmental parameters were analysed in PC-ORD (v. 4.1, McCune & Mefford 1999). The family level frequencies were selected because the analyses of individual OTUs saturated the ordination space and because the OTU level analyses were strongly driven by low-frequency OTUs. The environmental parameters including pH, P, K, NH_4^+ , NO_3^- , OM, Cd, Pb, host species, host diameter at breast height (DBH), stand structure, ground vegetation and land use were also arranged into a secondary environmental data matrix to facilitate the exploration of the correlations among the communities and the potential environmental drivers. Pairwise community distances for averaged family frequencies and pairwise sample distances for averaged environmental parameters were estimated on a tree-level using Sørensen (Bray–Curtis) index and analysed with Non-metric Multidimensional Scaling (NMS; Mather 1976) multivariate analysis. The optimal number of dimensions (k) was selected based on Monte Carlo test of significance at each level of dimensionality comparing 40 runs with empirical data against 50 randomized runs at 100 iterations each with a step-down in dimensionality from 6 to 1 and a random seed starting value. For both the community and environmental data, $k \geq 3$ dimensional solutions yielded similar results and produced solutions with stress values smaller than those in randomized runs ($P = 0.0196$). Accordingly, the 3-dimensional solutions were selected and the data re-ordinated with $k = 3$ configuration.

Results

Environmental characterization of the study sites

The first three NMS axes represented 97.7% of the total variability in the environmental data and clearly separated the urban and rural sites on the first NMS axis (Fig. 1, Table 2) indicating distinct chemistries among them. The repeated measures ANOVA indicated that pH, P, NH_4^+ , OM, and Pb differed among the urban and rural sites (Table 2): pH, NH_4^+ , and OM were

higher in the rural sites, whereas P and Pb were higher in the urban sites. Although many of the chemical parameters that were measured in May, July and September showed seasonal variability (see pH, P, NH_4^+ , OM, Cd, Pb in Table 2 and Fig. S2, Supporting Information), the NMS analyses did not show any clear overall seasonal trends in the site properties. Additionally, the seasonal patterns often differed among the rural and urban land uses or sites within the land uses as indicated by significant interaction terms in Table 2.

General 454 sequence data characterization

Using 454 sequencing, we generated 10 782, 12 451, and 10 423 sequence reads for a total of 33 959 reads for 192 samples collected across three seasonal samples in May, July and September. After controlling for sequence quality (presence of identifiable primer, DNA tag sequences, and ambiguous bases) as well as threshold read length, we retained 9746, 11 313, and 8851 high-quality reads for a total of 29 910 sequences. Although 64 subsamples representing 16 trees at each sampling occasion were pooled into each 454-sequencing region, the numbers of reads did not differ across the land use types, sites within a land use type or among the seasons (Table 2). Most of the OTUs could be assigned to nutritional modes based on their similarities in BLAST analyses. A majority of sequences (71.6%) were ectomycorrhizal; the remaining sequences represented

potential pathogens and antagonists (12.3%), saprobes or soil fungi (8.2%), unknown fungi whose nutritional mode could not be determined (7.1%) and a mixture of arbuscular mycorrhizal (0.3%), lichenized (0.2%) and putative endophytic fungi (*Periconia macrospinosa*, *Phialocephala fortinii*—0.2%).

The observed OTUs were distributed across four fungal phyla. A total of 49.8% belonged to Ascomycota, 47.0% to Basidiomycota, 0.2% to Chytridiomycota, 0.4% to Glomeromycota. An additional 0.4% represented basal fungal lineages. The remaining OTUs (2.2% of the total) could not be assigned to a phylum and represented unknown or unannotated environmental sequences in GenBank. The detected OTUs were distributed across 34 fungal families. The most abundant families are listed in Fig. 2 and 50 most abundant OTUs in Fig. S3 (Supporting Information).

Fungal richness and diversity in *Quercus spp.* ectomycorrhizas

The acquired sequences represented a total of 1,077 unique OTUs at 95% sequence identity suggesting a diverse fungal community associated with the *Quercus* ectomycorrhizas. More than half of these (612; 57%) occurred only once indicating that our sampling effort did not adequately represent the resident richness. Similarly, rarefaction analyses at a level of a focal tree indicated that the target sequencing depth (156 reads per sample or 624 reads per focal tree) was inadequate to saturate the species richness (Fig. 3a–c). Extrapolative estimates of the richness (Chao 1 and first order Jackknife estimators) corroborated. They exceeded the observed richness two-fold (Table S3, Supporting Information) providing further indication that the fungal communities are diverse and that a far greater sequencing effort would be necessary for saturation.

Seasonal dynamics of the fungal communities

Our three seasonal samples allowed examination of seasonal dynamics among the fungi associated with the *Quercus* ectomycorrhizas. OTU richness and diversity declined in the course of the growing season (Table 2; Fig. 4). These trends did not usually differ among the land use types (repeated measures ANOVA: sampling time * land use $P > 0.05$) or among sites within the land use (repeated measures ANOVA: sampling time * site[land use] $P > 0.05$) indicating that they were mainly consistent across the study system.

We used NMS ordination to visualize seasonal dynamics within and among the fungal communities. The first two NMS ordination axes that represented 58.7% of the variation suggested strong and significant

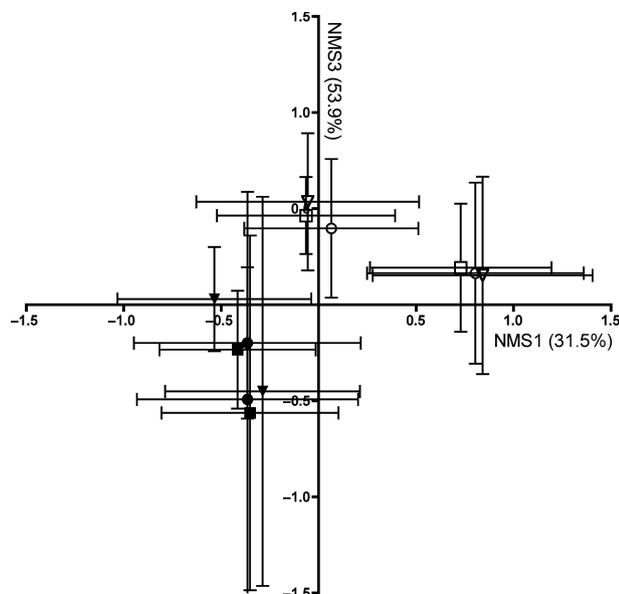


Fig. 1 Non-metric Multidimensional Scaling (NMS) of the environmental (mainly chemical) parameters in urban (closed symbols) and rural (open symbols) sites (mean \pm 1SD). Axes one and three are displayed as they explain majority of the variability. \blacksquare = May; \blacktriangledown = July; \bullet = September sampling.

Table 2 Repeated measures ANOVA tables. 'Land Use' refers to urban vs. rural comparison, 'Site' to sites within urban or rural land uses (Table 1), 'Sampling' refers to comparisons among the three seasonal samples (May, July, September) and its interactions with the between subject effects. *F*-test variables are exact values for all parameters except for term 'Sampling*Site(Use)' where *F* is approximated using Wilk's Lambda. Shown are also those of the 34 detected families whose observed frequencies differed among the explanatory parameters. Responses that are significant at $\alpha < 0.05$ are boldfaced and those marginally significant at $0.05 \leq \alpha < 0.10$ italicized. Note that for the family level analysis, after Bonferroni correction for false detection, the observed *P* must be less than 0.0015 for significance at $\alpha = 0.05$

Response	Between subjects		Within subjects		
	Land use $F_{1,12}^P$	Site(Use) $F_{2,12}^P$	Sampling $F_{2,11}^P$	Sampling* use $F_{2,11}^P$	Sampling* Site(Use) $F_{4,22}^P$
Environmental NMS scores					
NMS Axis 1 (31.5%)	9.60 ^{0.0092**}	2.68 ^{0.1094}	0.53 ^{0.4147}	0.53 ^{0.6043}	1.84 ^{0.1566}
NMS Axis 2 (12.3%)	0.27 ^{0.6118}	0.42 ^{0.6672}	1.59 ^{0.2477}	0.31 ^{0.7362}	1.02 ^{0.4173}
NMS Axis 3 (53.9%)	4.68 ^{0.0514(*)}	0.64 ^{0.5424}	3.38 ^{0.0716(*)}	1.75 ^{0.2187}	0.96 ^{0.4469}
Environmental parameters					
pH	14.02 ^{0.0028**}	3.08 ^{0.0835(*)}	25.14 ^{0.0001***}	5.81 ^{0.0189*}	3.84 ^{0.0164}
P	33.23 ^{0.0001***}	1.27 ^{0.3154}	4.79 ^{0.0319*}	0.87 ^{0.4476}	0.70 ^{0.6010}
K	0.01 ^{0.9522}	0.77 ^{0.4864}	2.72 ^{0.1094}	1.64 ^{0.2379}	1.37 ^{0.2767}
NH ₄ ⁺	5.64 ^{0.0351*}	2.30 ^{0.1427}	14.89 ^{0.0007***}	10.02 ^{0.0033**}	4.08 ^{0.0127*}
NO ₃ ⁻	1.19 ^{0.2976}	6.24 ^{0.0139*}	3.41 ^{0.0703(*)}	5.48 ^{0.0223*}	3.31 ^{0.0288}
OM	40.42 ^{0.0001***}	1.19 ^{0.3383}	6.54 ^{0.0135*}	2.56 ^{0.1219}	2.80 ^{0.0510(*)}
Cd	1.07 ^{0.3220}	0.74 ^{0.4993}	22.73 ^{0.0001***}	5.14 ^{0.0266*}	1.14 ^{0.3639}
Pb	27.40 ^{0.0002***}	1.40 ^{0.2013}	5.46 ^{0.0226*}	3.29 ^{0.0759(*)}	0.42 ^{0.7951}
Sequences	1.83 ^{0.2011}	0.64 ^{0.5450}	1.49 ^{0.2678}	3.46 ^{0.0682}	1.16 ^{0.3553}
Richness	6.27 ^{0.0277*}	3.10 ^{0.0822(*)}	12.89 ^{0.0013**}	0.86 ^{0.4514}	0.97 ^{0.4441}
Singletons	2.07 ^{0.1759}	2.61 ^{0.1148}	5.83 ^{0.0188*}	0.82 ^{0.4666}	0.94 ^{0.4593}
Diversity indices					
Fisher's α	5.64 ^{0.0351*}	2.40 ^{0.1330}	6.78 ^{0.0121*}	1.39 ^{0.2887}	0.46 ^{0.7631}
Simpon's D	8.53 ^{0.0128*}	3.11 ^{0.0819(*)}	6.00 ^{0.0173*}	1.58 ^{0.2486}	3.04 ^{0.0390*}
Shannon's H'	12.50 ^{0.0041**}	3.79 ^{0.0532(*)}	5.05 ^{0.0277*}	1.26 ^{0.3224}	2.11 ^{0.1137}
Evenness	10.56 ^{0.0070**}	1.87 ^{0.1968}	2.07 ^{0.1724}	0.74 ^{0.5007}	2.04 ^{0.1233}
Community NMS scores					
NMS axis 1 (27.2%)	14.61 ^{0.0024**}	0.16 ^{0.8567}	21.92 ^{0.0001***}	0.38 ^{0.6893}	0.89 ^{0.4863}
NMS axis 2 (31.5%)	2.56 ^{0.1355}	0.14 ^{0.8741}	15.35 ^{0.0007***}	1.08 ^{0.3729}	1.68 ^{0.1904}
NMS axis 3 (29.7%)	9.71 ^{0.0089**}	0.60 ^{0.5666}	2.45 ^{0.1314}	1.29 ^{0.3130}	0.62 ^{0.6501}
Taxon responses					
Boletaceae	4.48 ^{0.0559(*)}	0.62 ^{0.5553}	0.63 ^{0.5528}	0.61 ^{0.5595}	1.27 ^{0.3134}
Lophiostomataceae	5.78 ^{0.0333*}	1.04 ^{0.3842}	0.42 ^{0.6689}	0.07 ^{0.9366}	0.12 ^{0.9740}
Mortierellaceae	0.04 ^{0.8546}	1.51 ^{0.2609}	4.22 ^{0.0438*}	0.59 ^{0.5690}	0.74 ^{0.5735}
Nectriaceae	0.84 ^{0.3778}	0.80 ^{0.4738}	17.81 ^{0.0004***}	1.05 ^{0.3823}	1.63 ^{0.2011}
Russulaceae	6.30 ^{0.0274*}	0.02 ^{0.9785}	0.83 ^{0.4635}	2	2.24 ^{0.0975(*)}
Sebacinaceae	3.57 ^{0.0935(*)}	0.32 ^{0.329}	1.65 ^{0.2368}	0.57 ^{0.5829}	0.57 ^{0.5485}
Thelephoraceae	12.89 ^{0.0037**}	0.04 ^{0.9621}	8.37 ^{0.0062**}	4.14 ^{0.0457*}	0.17 ^{0.9492}
Tuberaceae	4.10 ^{0.0656*}	0.25 ^{0.7833}	5.88 ^{0.0183*}	1.14 ^{0.3545}	2.85 ^{0.0480*}

seasonal patterns within the fungal communities (Table 2; Fig. 5). The community shifts are likely attributable to commonly observed fungal families that showed clear seasonal dynamics in the course of the sampled growing season. Indeed, Thelephoraceae were positively correlated with the first two NMS axes (Table 3) and placed among the latter seasonal samples from the rural sites (Fig. 5). In contrast, Lophiostomataceae and Nectriaceae were negatively correlated with the first axis and placed with the May and July samples

from the urban sites. Tuberaceae correlated strongly and negatively with the second axis and were placed among the September samples from the urban sites. These NMS analyses are mainly, but not completely, congruent with the repeated measures ANOVAs. The abundances of Mortierellaceae and Nectriaceae declined across the growing season, whereas the abundances of Thelephoraceae and Tuberaceae increased (Table 2; Fig. 6). The abundances of Thelephoraceae increased at different rates in urban and non-urban environments

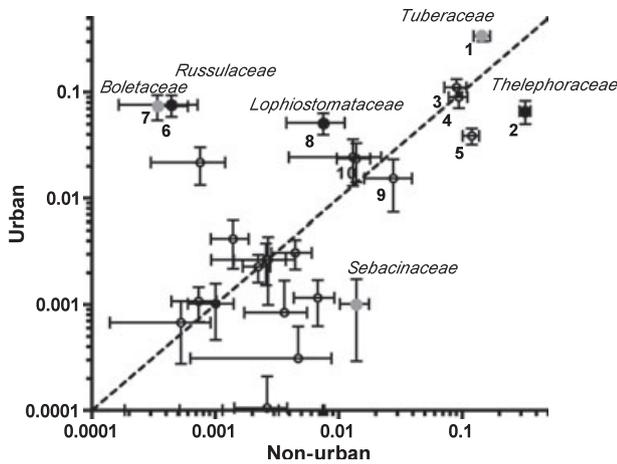


Fig. 2 Observed family-level frequencies. Log₁₀-scaled x-axis represents frequency in the non-urban sites (mean ± 1SE), log₁₀-scaled y-axis represents frequency in the urban sites (mean ± 1SE). The dashed line represents an isocline for equal frequency in both environments. Black dots emphasize $P \leq 0.05$ for the term 'land use' in Table 2; grey dots emphasize $0.05 < P \leq 0.10$. Those families whose frequencies differed among the land use types (Table 2) are spelled out in the figure. The numbers identify the most common families: 1 Tuberaceae, 2 Thelephoraceae, 3 Nectriaceae, 4 Cortinariaceae, 5 Unknown fungi, 6 Russulaceae, 7 Boletaceae, 8 Lophiostomataceae, 9 Tricholomataceae, 10 Sclerodermataceae.

(repeated measures ANOVA: sampling time * land use $F_{2,11} = 4.14$; $P = 0.0457$) and the rate of increase in the abundance of Tuberaceae varied across the sites (repeated measures ANOVA: sampling time * site [land use] $F_{4,22} = 2.85$; $P = 0.0480$).

Correlations among the communities and environmental parameters

Using NMS, we aimed to point out environmental parameters correlated with the observed community composition. In addition to the seasonal trends (above), two of the three NMS axes indicated differences among the rural and urban sites (Table 2; Fig. 5). These differences were partly confounded by the differing host communities. When the NMS data were analysed with the host species as the only main effect in a one-way ANOVA, the first NMS axis separated the host trees ($P = 0.0207$) and post hoc analyses (Tukey's HSD) indicated that fungal communities of *Q. muehlenbergii* and *stellata* differed from those of *Q. palustris*. Similarly, the second NMS axis separated the communities associated with different hosts ($P = 0.0002$), *Quercus robur* communities differed from those of hosts other than *Q. rubra* or *Q. muehlenbergii* and *Q. palustris* communities also differed from those of *Q. rubra* (Fig. S4, Supporting Information). These observations suggest that anthropo-

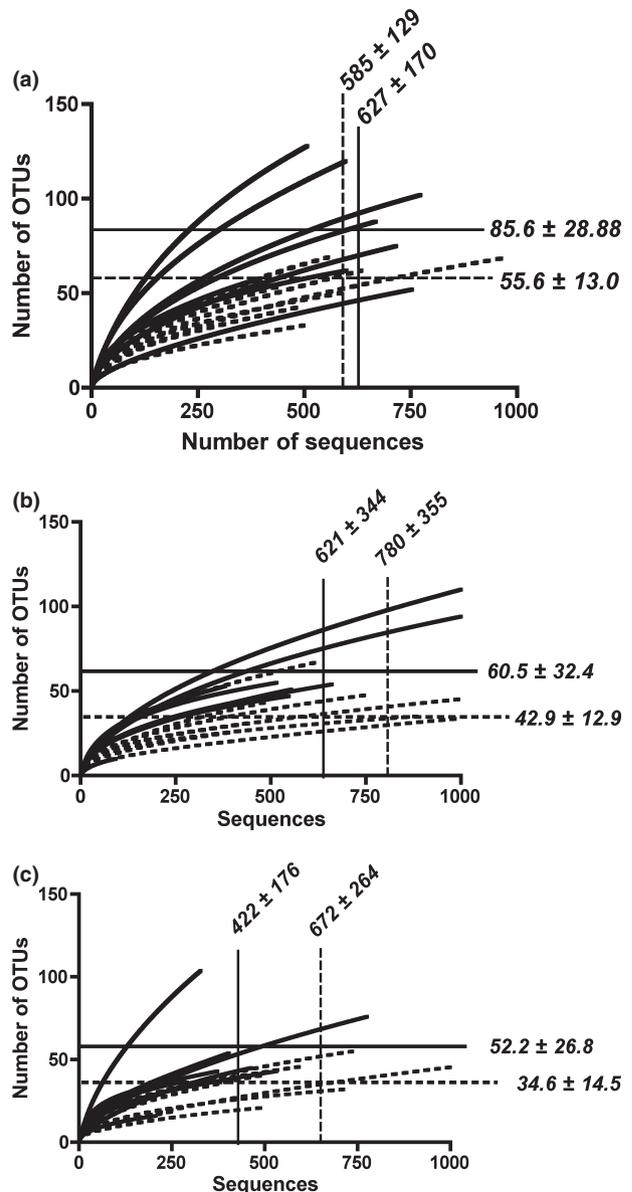


Fig. 3 Species accumulation (rarefaction) curves for OTUs in a) May, b) July, and c) September. The solid lines represent non-urban and dashed lines urban samples. The vertical and horizontal lines represent mean (± 1 SD) number of sequences and number of OTUs, respectively.

genic manipulation of macro-organism communities precipitates effects through the host-associated community and may select distinct community components (Figs 2 and S3, Supporting Information). Among the measured edaphic parameters, Pb and P were negatively correlated with the first NMS axis, whereas soil OM was positively correlated with all three NMS axes (Table 3). These correlations suggest that among the measured edaphic parameters soil OM, P and heavy metals may be the most influential drivers of the community composition.

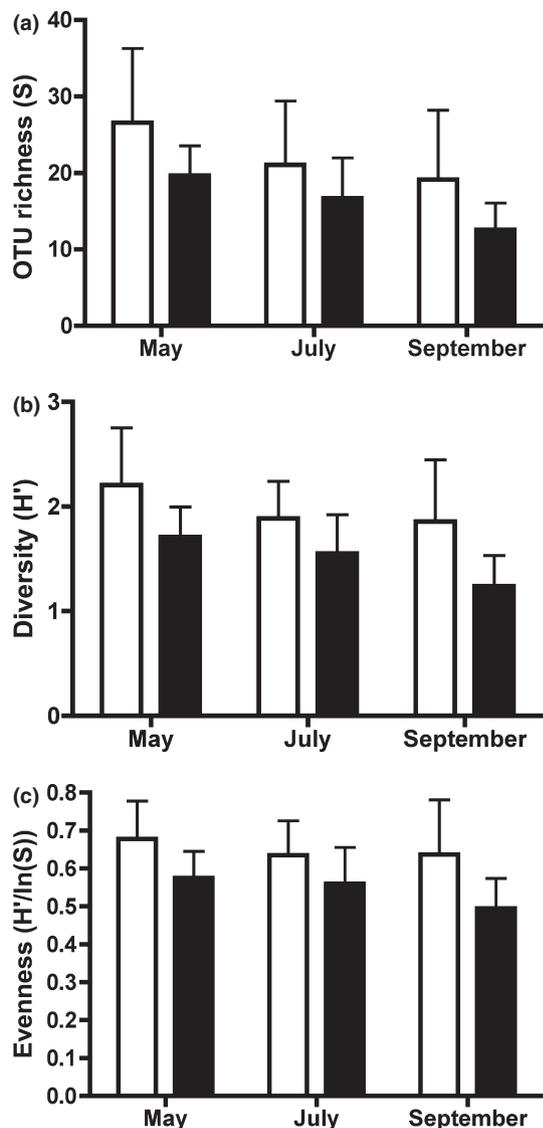


Fig. 4 a) OTU richness (S), b) Shannon's diversity (H'), and c) evenness ($H'/\ln(S)$) in the urban (closed bars) and non-urban (open bars) environments at the three different sampling occasions.

Placement of common OTUs among reference sequences

Phylogenetic analysis of the most common OTUs representing the most abundant families (Figs S5a–d, Supporting Information) suggest that the short 454-sequencing reads are adequate to provide a phylogenetic signal if sequence data for closely related taxa are available. Within Tuberaceae, the sampled sequences representing the most commonly occurring OTUs were placed (i) on a well-supported clade with *T. lyonii* or as its well-supported sister group; (ii) on a well-supported clade that included *T. whetstonense*; or, (iii) as a sister clade to *T. texense* and *T. taiyanense*. The OTUs with

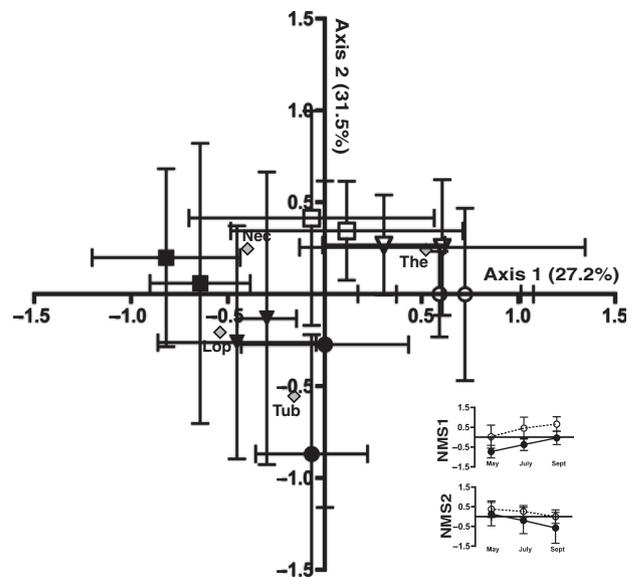


Fig. 5 Non-metric Multidimensional Scaling (NMS) of the fungal communities on a family level in urban (closed symbols) and non-urban (open symbols) sites (mean \pm 1SD). \blacksquare = May; \blacktriangledown = July; \bullet = September sampling. \blacklozenge = taxa with high correlations with the NMS axes: Nectriaceae, Lophiostomataceae, Thelephoraceae, Tuberaceae. Insets emphasize the season-related, strong increasing (NMS1) and decreasing (NMS2) linear trends.

affinities within Hypocreales were largely placed on clades that included (i) various *Nectria/Neonectria* teleomorphs or *Cylindrocarpon* anamorphs; (ii) *Gibberella* teleomorphs and *Fusarium* anamorphs; or, (iii) *Nectria* teleomorphs and *Acremonium* anamorphs. The placement of the OTUs representing the common basidiomycetous taxa was less clear. Within Thelephoraceae, most OTUs were placed on clades that received low or no support in our bootstrap analyses. However, three OTUs were placed on clades with reference sequences representing (i) *Tomentella badia*; (ii) *T. ellisii*; and, (iii) *T. lapidum* and *T. bryophila*. Within Cortinariaceae, the analysed OTUs represented sister groups to (i) the hypogeous genus *Hymenogaster*; (ii) *Inocybe obsoleta*; (iii) *I. calida*; and, (iv) a clade that included various *Inocybe* spp. including *I. dunensis*, *oblectabilis* and *decipiens*.

Discussion

Our use of 454 sequencing and DNA tagging allowed a rapid assessment of fungal communities colonizing *Quercus* roots. Similarly to the limited number of studies that have provided evidence that ectomycorrhizal communities undergo seasonal shifts (van der Heijden & Vosatka 1999; Koide *et al.* 2007; Walker *et al.* 2008), our results suggest seasonal turnover in these communities. Additionally, a number of studies have shown

Table 3 Correlations among the first three NMS axes and the measured environmental parameters and the most abundant fungal families. The parameters with r^2 greater than 0.20 are highlighted and discussed in the results. The parameters are sorted in the order of descending r^2 values on the first NMS axis

Environmental parameter	NMS axis1 (27.2%)		NMS axis2 (31.5%)		NMS axis3 (29.7%)	
	Pearson r^2		Pearson r^2		Pearson r^2	
OM	0.536	0.288	0.462	0.213	0.509	0.259
pH	0.361	0.130	-0.065	0.004	0.486	0.237
NH ₄ ⁺	0.300	0.090	0.462	0.213	0.103	0.011
K	0.095	0.009	0.157	0.025	0.259	0.067
NO ₃ ⁻	-0.060	0.004	0.130	0.017	-0.023	0.001
Cd	-0.254	0.064	-0.283	0.080	-0.134	0.018
BHD	-0.335	0.112	-0.066	0.004	-0.389	0.151
Pb	-0.525	0.275	-0.286	0.082	-0.340	0.116
P	-0.575	0.330	-0.191	0.036	-0.293	0.086
Fungal family						
Thelephoraceae	0.883	0.780	0.455	0.207	0.183	0.034
Sebacinaceae	0.315	0.099	0.230	0.053	0.109	0.012
Cortinariaceae	0.201	0.040	0.049	0.002	-0.319	0.101
Tricholomataceae	0.181	0.033	0.368	0.136	0.147	0.022
Sclerodermataceae	-0.003	0.000	-0.330	0.109	0.080	0.006
Tuberaceae	-0.244	0.060	-0.943	0.889	-0.254	0.064
Pezizaceae	-0.246	0.060	-0.142	0.020	-0.486	0.236
Russulaceae	-0.266	0.071	0.036	0.001	-0.590	0.348
Mortierellaceae	-0.295	0.087	0.223	0.050	0.098	0.010
Boletaceae	-0.317	0.101	0.068	0.005	-0.566	0.320
Lophiostomataceae	-0.485	0.235	-0.205	0.042	-0.111	0.012
Nectriaceae	-0.604	0.365	0.282	0.080	0.354	0.125

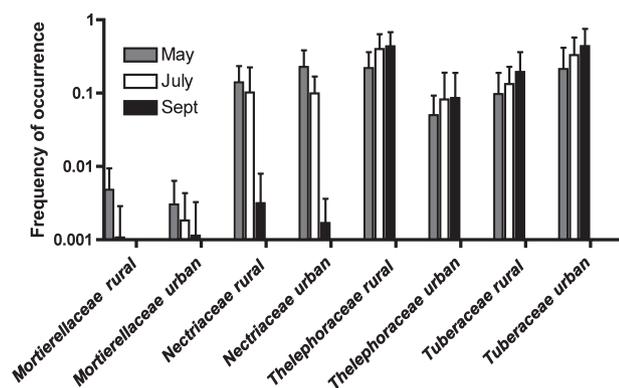


Fig. 6 Occurrence of the families whose frequencies showed seasonal responses (Table 2). Note that the Y-axis is \log_{10} -scaled.

intra-annual correlation among ectomycorrhizal colonization and climatic variability (Harvey *et al.* 1978; Rastin *et al.* 1990; Swaty *et al.* 1998). Surprisingly, our data showed that the fungal richness and diversity

tended to decline in the course of a growing season. While factors such as root turnover, stochastic vegetative growth patterns, and small scale disturbances associated with root growth and mycelial dieback in response to environmental extremes have been put forth as potential mechanisms behind seasonal dynamics (Bruns 1995; Izzo *et al.* 2005), we are unable to pinpoint drivers with any certainty.

A closer look at the families whose abundances shifted provides some insight though. The fungi with declining trends across the growing season were Mortierellaceae and Nectriaceae with related members in the order Hypocreales. The ascomycetous Hypocreales that were detected in our analyses included anamorphic genera *Cylindrocarpon* and *Fusarium* as well as the closely related teleomorphs *Nectria*, *Neonectria*, and *Gibberella*. These fungi are often antagonists and pathogens (Vujanovic *et al.* 2007) but may also reside benignly or sub-symptomatically in plant tissues or behave as saprobes (Samuels 1996; Rossman *et al.* 1999). The Mortierellaceae are commonly encountered in soil (O'Donnell *et al.* 2001; Benny & Blackwell 2004), soil-borne organic substrates (O'Donnell *et al.* 2001; Thormann *et al.* 2001) or roots (Domsch *et al.* 1980) and therefore are likely encountered in the roots. Many of the Mortierellaceae possess enzymatic capabilities that allow their use of a variety of carbohydrates including cellulose, pectin and starch (for examples see Thormann *et al.* 2001) making them likely decomposers in the roots and the surrounding soil.

Although substantial variability exists in fine root development and decomposition, new roots develop mainly during the growing season (Burton *et al.* 2000; Joslin *et al.* 2001; Baddeley & Watson 2004; Satomura *et al.* 2006; Guo *et al.* 2008). The root decomposition is likely to be more variable (Burton *et al.* 2000). However, Satomura *et al.* (2006) showed that, in a temperate deciduous forest ecosystem, the fine root decomposition peaked in the fall followed by a lesser peak in spring indicating that much of the root decomposition occurs outside the growing season. Similarly, Ponti *et al.* (2004) showed that oak (*Quercus robur*) roots developed rather steadily from early spring regrowth to mid-autumn and that the root mortality peaked during winter. While it is impossible to completely decouple the disturbance from repeated soil sampling and the seasonal dynamics of the fungal communities, we find it most unlikely that the sampling-related disturbance would lead to increase in the frequencies of ectomycorrhizal families at the cost of the non-ectomycorrhizal families and not *vice versa*. Based on the reported seasonal dynamics of the host roots, on our observations on the non-mycorrhizal families that decline, and on our observations on the ectomycorrhizal families that increase, we propose that the abundance of the weak pathogens and saprobic fungi in

the early season is associated with the spring fine root turnover. These fungi are replaced during the growing season by the resident ectomycorrhizal fungi, such as the Thelephoraceae and Tuberales that colonize the emerging new fine roots and thus increase in abundance. Note that these two families represent the two most frequent ectomycorrhizal taxa in our sampling suggesting that they simply occupy the root space that becomes available once new roots develop.

Koide *et al.* (2007) propose alternative and additional mechanisms for seasonal patterning in ectomycorrhizal communities and speculate that it is the environmental tolerances that mainly dictate the seasonal partitioning of the communities. Our broader sampling that includes both mycorrhizal and non-mycorrhizal fungi strongly suggests that host root turnover may be among the primary drivers of shifts among non-mutualist and mutualist communities. However, within this broader host-controlled seasonal dynamic, fungal environmental tolerances likely dictate which community components most effectively compete for the emerging, new root tissues. Although our NMS analyses of the environmental, secondary data matrix provided no support for overall seasonal shifts in the soil chemistries, many repeatedly measured variables tended to show minor shifts within the growing season. For example, P, Cd, and Pb tended to decline in the course of the growing season possibly indicating their biological immobilization. These dynamics may be correlated with the fungal community dynamics, as the fungal communities are sensitive to macronutrient (Wallenda & Kottke 1998; Treseder 2004; Parrent & Vilgalys 2007) and heavy metal enrichment (Jones & Hutchinson 1986; Bell *et al.* 1988; Blaudez *et al.* 2000; Kieliszewska-Rokicka *et al.* 2000; Fomina *et al.* 2005). It is also of note that we did not measure the most likely and fundamental controls of fungal physiology and growth rates: moisture and temperature fluctuate more wildly in the course of a growing season than soil chemistry and may therefore be more influential in structuring the fungal communities (Swaty *et al.* 1998).

Similarly to studies on other organisms that have suggested changes in aquatic and terrestrial species richness and evenness within cities (e.g. Paul & Meyer 2001; McKinney 2002), our results suggest that anthropogenic activities related to urbanization may lead to community level shifts in root-associated fungal communities. These results corroborate the results of an ectomycorrhizal morphotyping study in oak stands distributed along a human population density gradient in New York (Baxter *et al.* 1999) as well as a comparison among urban, suburban and rural deciduous forest stands in Japan (Ochimaru & Fukuda 2007). The mechanisms underlying the observed differences among the urban and rural fungal communities are difficult to elucidate and likely include

a variety of mechanisms ranging from stand size and isolation (Peay *et al.* 2007), variation in susceptibility to fungal colonization among heterogeneous host genotypes and species (Kranabetter *et al.* 1999; Schweitzer *et al.* 2008), or edaphic differences among the sampled stands (see Baxter *et al.* 1999; Craul 1992; Poyat & McDonnell 1991). The NMS analysis of the environmental data indicated that the soil chemistries among the land uses (rural vs. urban) were biochemically distinct (see also Craul 1992; Poyat & McDonnell 1991; Kaye *et al.* 2006), therefore likely selecting distinct fungal communities. However, as our community analyses were confounded by the host selection, the true drivers of the community responses remain open to speculation and will require robust empirical testing.

Phylogenetic signal of the short 454-sequences

To address the question whether or not the 454 sequencing reads averaging 265 bp in our analysis would be adequate to place the observed common OTUs into a phylogenetic context, we selected a limited number of common sequences and aligned them with known, well-annotated references. Our findings echo those of Liu and collaborators (2007), who found that these reads are adequate for community analyses and concluded that sequencing effort is better placed in a larger number of shorter reads than fewer but longer reads.

Our NJ and MP analyses indicate that, in many cases when appropriate and close reference sequences are available, the short reads spanning across the ITS1 are adequate for a well-supported placement. This was largely exemplified in the case of Tuberales, Hypocreales and, to a lesser extent of our selection of Cortinariaceae. However, the most saturated data set, Thelephoraceae, often failed to produce a reliable assignment but still yielded well-supported clades in three cases. The inability to assign Thelephoraceae is not uncommon: Smith *et al.* (2007), similarly, were unable to resolve Thelephoraceae using restriction fragment length polymorphisms of PCR-amplified ITS regions. It is worth noting here that the alignments using exclusively the variable ITS1 regions are problematic as the only conserved region in most reads included only little more than the 5.8S-residing ITS2 priming site.

Conclusion

To our knowledge this is the first study reporting the use of 454-sequencing technology to directly sequence fungal communities associated with ectomycorrhizas. Our approach produced nearly 30 000 sequences from 192 samples and provided a cross-section of the fungal communities inhabiting the *Quercus* spp. ectomycorrhizas.

While a large proportion of the detected species belonged to non-mycorrhizal taxa (e.g. Nectriaceae, Lophiostomataceae among the most common), our study provided a view of the ectomycorrhizal community (dominated by Tuberales, Thelephorales, Cortinariaceae, Russulaceae and Boletaceae). More importantly, our combination of the 454-sequencing and DNA-tagging allowed analyses to test specific hypotheses about the root-inhabiting communities. Those analyses indicate temporal shifts within a growing season but also suggest anthropogenically driven community shifts. Overall, the direct 454-sequencing proved an efficient and expedient tool to estimate fungal diversity and richness parameters. Our goal to maximize the number of samples at the cost of sequencing depth lead to incomplete saturation of the richness on the level of a sample. However, such saturation may be attainable but will require substantial additional sequencing effort.

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Conflicts of interest

The authors have no conflicts of interest to declare and note that the sponsors of the issue had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Jumpponen is interested on the controlling mechanisms of microbial communities. He is currently investigating on the primary mechanisms that dictate the compositional differences between urban and rural fungal communities inhabiting tree roots and foliage. Jones is a research scientist at the University of Georgia's Georgia Genomics Facility, where he leads the next generation sequencing group. His current interests are in the application of next generation sequencing to population and community genetics. Mattox is the City Forester for the City of Manhattan. He is interested in the maintenance of a diverse arboreal portfolio in urban environments. Yaeger is interested in genetics of fungi used in industrial and food fermentation. She has investigated genetics and transformation of red rice mold *Monascus purpureus* for pigment production and its potential as a fungal model for production of fungal metabolites.

Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1. List of the Internal Transcribed Spacer (ITS) primers that combine fungus specific (ITS1F; Gardes & Bruns 1993) and universal (ITS2; White 1990) primers and the 454sequencing endemic (Margulies *et al.* 2005) A- and B-primers plus a 5 bp DNA-tag for post-sequencing sample identification.

Table S2. OTU assignments and summaries at 95% sequence identity. Included are sample read length as well as Blast-derived coverage and sequence identity. For each best match GenBank accession and taxon information (Genus and Specific epithet, family, order and phylum as well as nutritional mode) are listed. OTUs with significant differences in the repeated measures ANOVAs are highlighted with listing of the significant terms.

Table S3. OTU richness estimators, number of singletons, proportion of singletons, number of doubletons, and two extrapolative estimators (Chao 1 and first order jackknife). Standard deviations and 95% confidence intervals displayed where appropriate. Singletons comprise on average ~50% of the observed richness indicating that the sampling on a level of a tree did not reach saturation. Extrapolative estimators corroborate

rate and exceed the observed estimators two-fold. ACE and ICE estimators provided richness estimates far exceeding those presented here and were omitted.

Fig. S1. Total richness (S) of Operational Taxonomic Units (OTUs) for the three sampling occasions (May, July, and September) across sixteen CAP3 (Huang & Madan 1999) sequence similarity (identity) thresholds. Note that 95% sequence identity was selected for the OTU and taxon level analyses as the OTU richness assumes an exponential growth above this level.

Fig. S2. Soil chemical parameters in urban (closed symbols) and rural (open symbols) sites (mean \pm 1SD) sampled repeatedly in May, July and September. (a) soil pH, (b) soil organic matter, (c) soil ammonium, (d) soil nitrate, (e) soil phosphorus, (f) soil potassium, (g) soil lead, and (h) soil cadmium. The asterisks indicate results of one-way ANOVAs comparing land use (urban vs. rural) at each sampling. * $0.01 < P \leq 0.05$; ** $0.001 < P \leq 0.01$; *** $P \leq 0.001$

Fig. S3. Rank abundances (mean \pm 1 SD) of the 50 most abundant OTUs among those that were determined to occur at frequencies > 0 based on Student's *t*-test at $\alpha < 0.05$. Asterisks indicate OTUs that differ significantly among the land uses. The best Blast matches for the shown OTUs are 237 *Nectria mauritica*; 431 *Tuber whetstonense*; 454 *Tuber whetstonense*; 506* *Tuber lyonii*; 236 *Boletus cf. rubelus*; 401 Uncultured Lophiostomataceae; 341* *Tuber lyonii*; 4 *Tuber talyanense*; 23 *Tomentella stiposa*; 520 *Tomentella cf. coerulea*; 69* *Russula pulverulenta*; 389 *Tomentella ellisii*; 74 *Hebeloma velutipes*; 8 *Hymenogaster subalpinus*; 36 *Scleroderma cepa*; 82 *Inocybe aff. oblectabilis*; 30* *Inocybe fibrosa*; 189* *Tomentella cf. coerulea*; 13 *Leohumicola minima*; 491 *Tuber talyanense*; 12 *Russula puellula*; 2* Uncultured Fungus; 57* *Thelephoraceae*; 56 *Thelephoraceae*; 141 *Tomentella sp.*; 101 *Russula pectinatoides*; 180 *Tomentella fuscocinerea*; 73 *Tomentella stiposa*; 196* *Tomentella cf. coerulea*; 109 *Thelephoraceae*; 182 *Peziza depressa*; 15 *Tomentella sp.*; 232 *Tomentella sp.*; 89 Nectriaceae; 34 Uncultured Fungus; 334 *Hebeloma velutipes*; 44 Uncultured Fungus; 65 *Xylaria sp.*; 159* *Thelephoraceae*; 7 Uncultured Fungus; 111 *Sebacina aff. incrustans*; 265 *Phialocephala fortinii*; 107* Uncultured Fungus; 100 *Fusarium oxysporum*; 217 *Saccharomyces cerevisiae*; 103 *Tuber talyanense*; 202* *Thelephoraceae*; 228 *Heterochaete shearii*; 329 *Tuber lyonii*; 78 Uncultured Fungus.

Fig. S4. Non-metric Multidimensional Scaling (NMS) of the fungal communities of the sampled hosts (mean \pm 1 SD) suggesting partial host control of the fungal community. The superscripted letters following taxon names indicate results of Tukey's HSD at $\alpha = 0.05$ for the two displayed NMS axes. Hosts followed by different letters differ on either or both of the NMS axes.

Fig. S5. Phylogenetically inferred taxon affinities of the most commonly occurring families: (a) Tuberales; (b) Thelephorales; (c) Nectriales and related Hypocreales; and (d) Cortinariales. Neighbor-joining (NJ) topologies with bootstrap support ($>50\%$) for NJ and maximum parsimony (MP) analyses are shown. The nodes that provide well-supported placement of the short environmental sequences are highlighted for emphasis.

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