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Seasonally dynamic fungal communities in the *Quercus macrocarpa* phyllosphere differ between urban and nonurban environments

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Summary

• The fungal richness, diversity and community composition in the *Quercus macrocarpa* phyllosphere were compared across a growing season in trees located in six stands within and outside a small urban center using 454-sequencing and DNA tagging. The approaches did not differentiate between endophytic and epiphytic fungal communities.

• Fungi accumulated in the phyllosphere rapidly and communities were temporally dynamic, with more than a third of the analyzed operational taxonomic units (OTUs) and half of the BLAST-inferred genera showing distinct seasonal patterns. The seasonal patterns could be explained by fungal life cycles or environmental tolerances.

• The communities were hyperdiverse and differed between the urban and nonurban stands, albeit not consistently across the growing season. Foliar macronutrients (nitrogen (N), potassium (K) and sulfur (S)), micronutrients (boron (B), manganese (Mn) and selenium (Se)) and trace elements (cadmium (Cd), lead (Pb) and zinc (Zn)) were enriched in the urban trees, probably as a result of anthropogenic activities. Because of correlations with the experimental layout, these chemical elements should not be considered as community drivers without further empirical studies.

• We suggest that a combination of mechanisms leads to differences between urban and nonurban communities. Among those are stand isolation and size, nutrient and pollutant accumulation plus stand management, including fertilization and litter removal.

Introduction

The human population in urban environments is predicted to approach 5×10^9 by 2030 (Ash *et al.*, 2008). Coinciding with this population increase, urban land area will increase (Kareiva *et al.*, 2007; Ash *et al.*, 2008). Although urban areas are often thought to retain only limited biodiversity (Blair, 1999; Cincotta & Engelman, 2000; McKinney, 2002), some empirical studies suggest that they can support considerably diverse organismal assemblages (Kühn *et al.*, 2004; Wania *et al.*, 2006). However, these urban communities are usually distinct from those in the surrounding areas and characterized by a high frequency of exotic species (Blair, 2004; Hope *et al.*, 2006; Kareiva *et al.*, 2007). The observed contrasts in diversity and species richness between urban and nonurban environments are a result of a combination of many interlinked and correlated drivers, including systematic selection of plants and animals that are favored by humans in the densely populated areas (McKinney, 2002), as well as active urban land management that removes the unfavorable or poorly performing species.

In contrast to the active manipulation of the macroorganism assemblies in urban greeneries, the microbial communities cannot be selected directly by preferentially choosing specific community components over others (McKinney & Lockwood, 1999). However, urban microbial communities are directly and indirectly affected by human land management. For example, removal of plant

debris can eliminate substrates that some microbial communities may depend on. The microbial communities may also be strongly modified by the biogeochemical properties of their substrates. Substrates grown in the urban environments are often biochemically distinct from those in the surrounding areas and characterized by heavy metal and nutrient enrichment (Pouyat & McDonnell, 1991; Kaye *et al.*, 2006) which may lead to novel communities in urban areas (Jumpponen & Jones, 2009).

Microbial communities contain near-innumerable diversity (Sogin *et al.*, 2006; Roesch *et al.*, 2007). This is also true for foliar microbes which are ubiquitous in many environments (e.g. Lambais *et al.*, 2006; Arnold & Lutzoni, 2007). The phyllosphere hosts diverse fungal communities that occupy leaf surfaces (epiphytes) as well as intra- and intercellular spaces (endophytes) (Carroll *et al.*, 1977; Santamaria & Bayman, 2005). Plants harbor diverse endophytic fungal associates (see reviews by Saikkonen *et al.*, 1998 and Rodrigues *et al.*, 2009) that comprise hyperdiverse communities (Arnold *et al.*, 2000; Arnold, 2007; Arnold & Lutzoni, 2007). The epiphytes add to this diversity, emphasizing the foliage as a habitat for hyperdiverse microbial communities (Santamaria & Bayman, 2005; Osono, 2008).

The fungal communities in the phyllosphere are not temporally stable but vary as a result of fungal lifecycles that dictate when leaf colonization occurs (e.g. Suto, 1999). Additionally, leaf age as well as seasonally prevailing conditions that may favor either growth or propagule production lead to seasonally dynamic abundances among common phyllosphere inhabitants (Wilson & Carroll, 1994; Hata *et al.*, 1998; Kaneko *et al.*, 2003; Osono, 2008). While phyllospheric taxon richness may be low at the time of leaf emergence, the epiphytic fungi in particular appear to colonize the young leaves soon after their emergence (Osono, 2008), if not before (Kaneko & Kaneko, 2004). The endophyte communities are likely to establish more slowly and with some lag time (Stone, 1987; Hata *et al.*, 1998; Osono, 2008).

Our previous study (Jumpponen & Jones, 2009) showed that phyllosphere fungal communities in temperate *Quercus macrocarpa* are diverse and appear distinct between trees in urban and nonurban environments. In this study, we 454-pyrosequenced (Margulies *et al.*, 2005) DNA-tagged (Hamady *et al.*, 2008; Meyer *et al.*, 2008) PCR amplicons to confirm our previous results in a broader sampling across a growing season. In addition to testing hypotheses on phyllosphere fungal communities in urban and nonurban trees, we aimed to elucidate seasonal patterns in these communities and identify taxa that show seasonal trends. Furthermore, we collected a broad environmental data set to determine whether or not the foliar chemistry is distinct between urban and nonurban trees, as well as to identify environmental correlates for foliar communities.

Materials and Methods

Study site descriptions

The City of Manhattan in the Flint Hills, northeastern Kansas, USA has *c.* 50 000 residents and an additional student population that exceeds 20 000, representing a temporally variable transient population. *Quercus macrocarpa* Michx. (bur oak) is a common native tree that is also used as an ornamental. We selected three discrete planted and managed small stands within the Manhattan city limits (urban) and three native or hedgerow stands (nonurban) without regular management regimes (Table 1) located in an undisturbed prairie within the Konza Pairie Long-Term Ecological Research site (http://www.konza.ksu.edu/). Each of the stands was comprised of a small number of tightly clustered trees and the stand characteristics were matched as closely as possible between the two land-use types. As a result, the nonurban stands represented hedgerows and small clusters of trees. However, all selected stands were discrete and separated by a minimum distance of 300 m in both urban and nonurban environments.

The urban trees sampled for this study included those sampled in our previous contribution (Jumpponen & Jones, 2009). However, we relocated two of the nonurban sites in order to increase their isolation from adjacent, larger stands and to thus make them more closely comparable. The age of the sampled trees remains unknown, as we were unable to obtain cores to estimate the tree ages. However, the tree sizes were similar among the sites (Table 1), suggesting that they are, on decadal scales, approximately of equal ages. Additionally, two of the nonurban sites selected for the present study are hedgerows, planted to serve as wind barriers. Although we were unable to find historical records to identify the seed sources for the urban and nonurban planted trees, we believe them all to represent local seed sources.

Sampling, DNA extraction, PCR amplification and sequencing

Two trees per stand (n = 12) were selected and these trees sampled repeatedly on 28 May, 11 June, 7 July, 1 August, 24 August and 17 September 2008. The first sampling was determined based on the leaf emergence and scheduled 2 wk after bud burst. The leaves emerged earlier in the urban than in the nonurban sites, potentially indicating an urban heat island (UHI) effect (Roetzer *et al.*, 2000; White *et al.*, 2002; Zhang *et al.*, 2004). This resulted in a 4-d shorter leaf exposure in the nonurban areas. From the inner canopy, three random shade leaves were collected from distinct 60°C sectors within each focal tree. The excised leaves were stored on ice in a plastic bag until they were

Table 1 Properties of the six stands sampled for *Quercus macrocarpa* phyllosphere fungal communities

	City park	Ahearn, KSU	Beach Museum, KSU	Kings Creek, KZ	Shane Creek, KZ	Nature trail, KZ
Land-use	Urban	Urban	Urban	Rural	Rural	Rural
Location	N39°10′58″ W96°35′25″	N39°11′16″ W96°34′42″	N39°11′46″ W96°34′53″	N39°06′21″ W96°36′01″	N39°06′42″ W96°33′49″	N39°06′21″ W96°34′27″
Elevation (m asl)	342	331	329	337	354	333
Stand structure	Street side row	Street side row	Clustered trees	Hedgerow	Hedgerow	Clustered trees
Ground vegetation	Lawn	None or lawn	None or lawn	Mixed prairie	Mixed prairie	Mixed prairie
DBH (cm)	68.9 ± 14.2	73.5 ± 22.5	78.8 ± 9.7	56.7 ± 3.4	42.2 ± 16.9	56.7 ± 12.2

KSU, Kansas State University Campus; KZ, Konza Prairie Long-Term Ecological Research (LTER) site. Note that the tree diameter at breast height (DBH) does not differ among sites (one-way ANOVA: $F_{5,11} = 1.91$, $P = 0.2273$).

transported to the laboratory, where the whole leaves were agitated in 0.1% Triton-X for 30 s and rinsed three times in sterile H_2O to remove nonadhering fungal spores and hyphal fragments that probably represent recently deposited fungal particles on the leaf surfaces. The leaves were dried with paper towels and, avoiding necrotic or insect-damaged areas, three discs per leaf were removed with a 1-cm-diameter cork borer for a total surface area of *c.* 2.4 cm^2 per leaf. It is of note that superficial visual inspection of the leaves for necrosis and damage indicated neither an increasing trend over the sampling period nor observable and consistent differences between the land-use types. The leaf necrosis appeared stochastic, probably indicating patterns dictated by leaf turnover rather than increasing damage over the growing season or true differences between the land-use types. Remaining tissues were dried at 50°C for 48 h and preserved for isotopic and chemical analyses. The nine excised discs from each tree were pooled into MoBio bead solution (UltraClean Soil DNA, MoBio Laboratories, Carlsbad, CA, USA) amended with two 2.4-mm zirconia beads and homogenized in a FastPrep instrument (Qbiogene, Carlsbad, CA, USA) at maximum speed (setting 6.0) for 40 s. Two samples from the second sampling (11 June) were lost as a result of shattered bead tubes, resulting in a grand total of 70 samples and elimination of the 11 June sampling event from the repeated measures ANOVAs (see section 'Statistical analyses' below). From the foliar homogenate, total DNA was extracted and eluted in 100 μl of the buffer S5. The templates were quantified with an ND1000 spectrometer (NanoDrop Technologies, Wilmington, DE, USA) and adjusted to a final concentration of 2.5 ng μl^{-1}.

For direct 454-sequencing of the fungal internal transcribed spacer 2 (ITS2) amplicons using the massively parallel sequencing (MPS), we synthesized primer constructs that incorporated the MPS primers (Margulies *et al.*, 2005), DNA tags and ITS1F or ITS4 primers (Gardes & Bruns, 1993) as described in Jumpponen & Jones (2009). Each sample was amplified in three separate 25-μl PCR reactions that contained final concentrations or absolute amounts of reagents as follows: 200 nM of each of the forward and reverse primers, 5 ng of the template DNA, 200 μM of each deoxynucleotide triphosphate, 2.5 mM $MgCl_2$, 1 U of GoTaq Hot Start DNA polymerase (Promega, Madison, WI, USA), and 5 μl of PCR buffer. 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, Hamburg, Germany). Possible contamination was assessed using a blank sample run through the extraction and a negative PCR control in which the template DNA was replaced with sterile H_2O. These remained free of PCR amplicons.

A total of 15 μl of each of the three amplicons for each sample was pooled, purified using an AmPure SPRI (AgenCourt Bioscience, Beverly, MA, USA) magnetic PCR clean-up and quantified (ND1000 spectrometer). For each sample, 75 ng was combined for sequencing and this pool adjusted to 10 ng μl^{-1}. The pooled products were sequenced in a 1/4th region of a reaction of a GS FLX sequencer (454 Life Sciences, Branford, CT, USA) at the Interdisciplinary Center for Biotechnology Research at University of Florida. We expected an average sequencing depth of *c.* 570 reads for each of the 70 samples assuming a 40 000 sequence yield. The run data are archived at the Short Read Archive (http://www.ncbi.nlm.nih.gov/Traces/sra/) under accession SRA009357.

Bioinformatics and operational taxonomic unit (OTU) designation

The sequences were searched for the DNA tag preceding the ITS4 primer. Sequences shorter than 200 bp, without primer sequence or DNA tag, or with ambiguous bases, were omitted. Where present, the DNA tag was replaced with a sample designation, and sequences were aligned with CAP3 (Huang & Madan, 1999) and assigned to OTUs at 69–99% similarity at 2% intervals using a minimum overlap of 100 bp, with other parameters (including those for matches, mismatches and gap penalties) left at defaults. All singletons as well as example sequences for each OTU at 99% similarity are available at GenBank (accessions GQ508255–GQ527072). The data were parsed by sample to calculate the OTU frequencies for each sample. From this output, SAS (SAS Institute Inc., Cary, NC, USA) was used to calculate richness and diversity indices.

Diversity indices

Overall OTU richness (S) was calculated by summing the number of OTUs, including singletons, within each sample. Simpson's dominance ($D = \sum p_i^2$), Simpson's diversity ($1 - \sum p_i^2$ and $1/\sum p_i^2$), and Shannon's diversity ($H' = -\sum p_i(\log_e(p_i))$) were calculated for each sample, where p_i is the frequency of occurrence of each OTU. Evenness was calculated as the ratio of Shannon's diversity and richness ($H'/\log_e(S)$). A final index of diversity, Fisher's α log-series (Fisher *et al.*, 1943), was calculated by iterating the equation $S/N = [(1 - x)/x][-\log_e(1 - x)]$, where S is richness and N is the total number of sequences within the sample. To explore and compare organismal coverage between the land-use types, species accumulation (rarefaction) curves and extrapolative richness estimators were generated using EstimateS (version 8; Colwell, 2006) for both urban and nonurban trees at each sampling event.

OTU frequency

OTU frequencies were analyzed for OTUs assigned at 95% sequence identity to provide a conservative estimate of the richness and to minimize the impacts of sequencing errors on the GS-FLX platform (see Quince *et al.*, 2009; Reeder & Knight, 2009). We observed that the OTU richness was stable up to 95% sequence identity, but grew near-exponentially at thresholds greater than 95% (Supporting Information Fig. S1). We used two different strategies to identify taxa that differed between the land-use types. First, all OTUs that occurred in > 20% of the samples (14 occurrences among the 70 samples across the entire experiment) and were represented by more than 100 reads were analyzed for effects of land-use or temporal patterns using JMP 7.0.1 (SAS Institute). Secondly, a representative of each nonsingleton OTU was assigned to a genus, family and order based on BLAST matches (Zhang *et al.*, 2000) and the lineage information available for each accession. To avoid the overwhelmingly large numbers of environmental and unannotated reads that populate the public databases, we aimed to filter out accessions that were annotated as environmental sequences or as unculturable fungi. The best BLAST matches for both filtered and unfiltered queries are shown in Table S1. OTU frequencies for each taxon with adequate lineage information were summed across each experimental unit and genus or family and analyzed for effects of land-use or temporal patterns. It is important to note that these taxon assignments are sensitive to annotation errors that occur commonly (Nilsson *et al.*, 2006; Arnold *et al.*, 2007). Additionally, the separate accessioning of teleomorph and anamorph genera presents potential problems. Nonetheless, even with these potential shortcomings, the assignments provide a taxonomic framework beyond simple OTU level. To account for the effect of poor BLAST matches, we removed data points whose assignment was based on short overlap (< 80% coverage) or low sequence identity (< 90% similarity) from these analyses.

Foliar chemistry and isotope analyses

Tissues from three sampling occasions (11 June, 1 Aug and 17 Sept 2008) were selected for isotopic and chemical analyses. The dried leaves were combined into one sample per tree and homogenized. A subsample was analyzed for $\delta^{13}C$ and $\delta^{15}N$ (δ ‰ = $((R_{sample}/R_{standard}) - 1) \times 1000$, where R is the molar ratio of the heavy (^{13}C or ^{15}N) and light (^{12}C or ^{14}N) isotopes in the samples and international standards) as well as carbon (C) and nitrogen (N) concentrations using a ThermoFinnigan Delta Plus (Thermo Fisher Scientific, Waltham, MA, USA) mass spectrometer with a CE 1110 (CE Instruments, Rodano, Italy) elemental analyzer for solid sample combustion and a Conflo II interface (Thermo Fisher Scientific, Waltham, MA, USA) at the Stable Isotope

Mass Spectrometry Laboratory in the Division of Biology at Kansas State University, KS, USA. The standard deviation was 0.04 for $\delta^{13}C$ and 0.12 for $\delta^{15}N$ based on internal references.

The remaining foliar homogenates (0.5 g) were analyzed for a full suite of 53 elements using ultratrace inductively coupled plasma mass spectrometry (ICP-MS) after Aqua Regia digestion for low and ultra-low elemental determination at Acme Analytical Laboratories (Vancouver, BC, Canada). ICP-MS is a highly sensitive type of spectrometry that is capable of the determination of a range of metals and nonmetals at parts per billion (ppb) concentrations. However, only 30 of the 53 elements were present in concentrations above the minimum ICP-MS detection concentration. The derived data were combined with other environmental data (isotopic, stand characterization etc.) and used to test for differences between the land-use types within this environmental data matrix in ordination analyses as well as to seek potential environmental correlates for observed community compositions (see Statistical analyses).

Statistical analyses

Each tree represents a repeatedly sampled experimental unit (subject). The loss of two samples from the second sampling event reduced the number of sampling events that could be used in the repeated measures analyses; we omitted the samples from 11 June from the repeated measures analyses. For the remaining sampling events, we analyzed the responses using repeated-measures ANOVA in a nested model with independent 'between subject' variables 'land-use' and 'site (land-use)' to account for both site and land-use effects. The repeated measures ANOVA tests between subject effects using the average across the repeated samples. However, as seasonal dynamics also were a focus of the repeated sampling, we examined the temporal or the 'within subject' terms and their interactions with the temporal component (sampling events). We also tested whether or not the diversity and summary statistics would differ within each sampling occasion and used a nested ANOVA model with independent variables 'land-use' and 'site (land-use)' in these comparisons (n = 12, except for the 11 June sampling occasion where n = 8 after omission of sites without replication). All these analyses were carried out in JMP 7.0.1 (SAS Institute).

The phyllosphere communities and the foliar chemistry data were analyzed in PC-ORD (v. 4.1; McCune & Mefford, 1999) to examine differences in fungal communities between the land-use types as well as to summarize the environmental parameter matrix. Pairwise distances were estimated using the Sørensen (Bray-Curtis) index and analyzed using nonmetric multidimensional scaling (NMS; Mather, 1976) multivariate analysis to avoid issues stemming from potential nonnormality in either data set. The optimal number of dimensions (k) was selected based on a Monte Carlo test of significance at each level of dimensionality comparing 40 runs with empirical data against 50 randomized runs, with a step-down in dimensionality from 6 to 1 and a random seed starting value. For the environmental (foliar chemistry) data, the $k \geq 3$ dimensional solutions yielded similar results and produced solutions with stress values smaller than those in randomized runs (P = 0.0196). Accordingly, the three-dimensional solution was selected and the data re-ordinated with a k = 3 configuration. The number of optimal dimensions varied among the different taxonomic levels (k = 2 for OTU level and k = 3 for generic and familial levels); the analyses for each level were conducted on these optimal levels. To determine differences, the NMS scores were analyzed using repeated measures ANOVA as described above.

Results

Foliar chemistry

The foliar chemistry data indicated clear and distinct differences between land-use types and among sites (Fig. 1, Table S2). Land-use and sites within a land-use type were separated on the first axis which represented 55.7% of the variability. Several elements (strontium (Sr), cerium (Ce), manganese (Mn), lead (Pb), yttrium (Y), boron (B) and

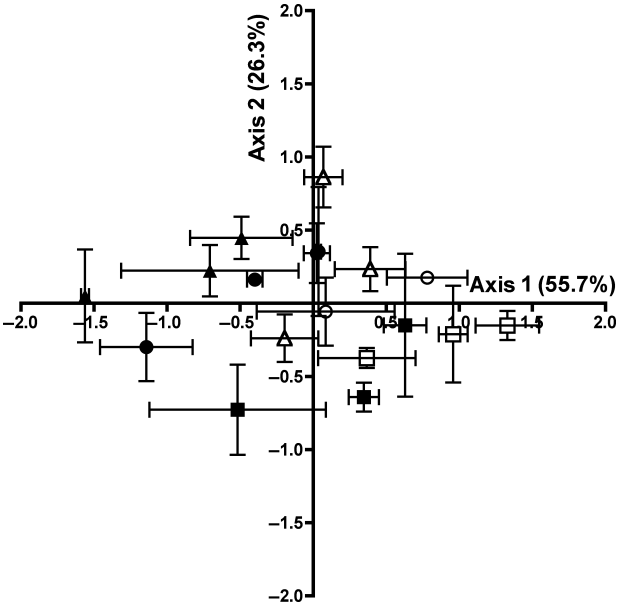

Fig. 1 Nonparametric multidimensional scaling (NMS) of the environmental data. The first two of the three (k = 3) axes represent 82% of the variability. The urban and nonurban land-use types as well as the sites are separated on the first axis, whereas the seasonal samples are separated on the first and second axes (Table 2). Closed symbols, urban sites; open symbols, nonurban sites. Squares, 11 June; circles, 1 August; triangles, 17 September.

($\delta^{15}N$) were strongly and negatively correlated with the first NMS axis, indicating their association with the urban environment (Table S3). By contrast, none of the measured foliar parameters was strongly and positively correlated with this axis. The concentration of foliar C, the only chemical parameter that tended to be elevated in the nonurban sites, had an $r^2 < 0.20$.

The observed differences in foliar chemistry between the urban and nonurban land-use types were largely attributable to macronutrient enrichment and heavy metal or trace element accumulation (Table S4). The urban sites were enriched in N as well as in the heavy ^{15}N isotope compared with the nonurban sites, indicating differences not only in N availability but also in N sources. Similarly, foliage in the urban sites was enriched in potassium (K) and sulfur (S), but not in phosphorus (P), compared with the nonurban sites. Additionally, B, cadmium (Cd), Ce, Mn, Pb, selenium (Se), Sr, Y and zinc (Zn) – trace elements or heavy metals – were higher in the urban foliage.

To also examine the temporal variability in foliar chemistry, the foliage obtained on three sampling occasions at *c.* 6-wk intervals was analyzed. The NMS analyses indicated some strong seasonal patterns, and scores on the first axis tended to decrease over the growing season and those on the second axis tended to increase (Fig. 1). A number of chemical elements analyzed here showed inclining (gold (Au), B, barium (Ba), calcium (Ca), Ce, lithium (Li), sodium (Na), Pb, Sr and Y) or declining (silver (Ag), copper (Cu), K, N, rubidium (Rb), S, Zn, $\delta^{13}C$ and $\delta^{15}N$) seasonal trends (Table S2).

General sequence data characterization

To characterize the fungal communities in the *Q. macrocarpa* phyllosphere, we 454-sequenced 105 838 amplicons. During the quality control, 20 882 sequences were removed. The resulting data set contained 84 956 sequences averaging 268 ± 19 bp in length and providing a sequencing depth of 1259 ± 339 reads per sample (mean ± 1 SD). The number of sequences was invariable between land-use types (nested ANOVA, $F_{1,67} = 0.91$; $P = 0.3436$) and among sites within land-use types (nested ANOVA, $F_{4,67} = 0.45$; $P = 0.7754$), indicating that the samples were relatively accurately pooled and that the diversity- or richness-related conclusions were unlikely to be driven by unequal sampling effort.

The data were distributed among three kingdoms: Fungi (83 554 sequences; 98.3%), Plantae (14 sequences; $2 \times 10^{-4\%}$), and Animalia (16 sequences; $2 \times 10^{-4\%}$). Phylum level assignment remained unclear for 704 sequences (0.8%). Those few reads that represented nontargets (Viridiplantae, Metazoa) were distributed across seven OTUs. Some of these nontargets are probably erroneously accessioned in GenBank (OTUs 199, 387 and 537). For example, OTUs 199 and 537 show highest affinity to *Oxalis corniculata* (Oxalidales; accession DQ1515552) but appear among mainly fungal accessions (closest representative after the *Oxalis* accessions is *Erysiphe pisi* (Erysiphales), accession FJ375579). By contrast, many others (OTUs 597, 704, 740 and 742) may represent truly novel organisms because, beyond this data set and that reported by Jumpponen & Jones (2009), there are no accessions with sequence coverage greater than 40%. However, we cannot exclude the possibility of PCR or sequencing artifacts, as recent evidence indicates that 454-sequencing is prone to overestimate richness and to produce artifacts (Quince *et al.*, 2009; Reeder & Knight, 2009).

To improve the reliability of the BLAST-based taxon assignments, we set thresholds to account for coverage (≥ 80%) and sequence identity (≥ 90%). Based on the filtered data (70 426 sequences), the phyllosphere samples were strongly dominated by Ascomycota (95.5% of sequences) followed by Basidiomycota (4.5%). Additionally, two sequences were placed in the Spizellomycetales (Chytridiomycota) and all nonfungal data were filtered. Among the 39 observed orders, Pleosporales (34 587 sequences; 49.1%) were most abundant followed by Capnodiales (11 311 sequences, 16.1%) and taxa not presently assigned to orders (9089 sequences; 12.9%). On a family level, the 55 assigned families were dominated by Pleosporaceae (Pleosporales; 30 407 sequences; 43.2%), Davidiellaceae (Capnodiales; 8436 sequences; 12.0%) and Dothioraceae (Dothideales; 8166 sequences; 11.6%). Finally, among the 165 assigned genera, *Alternaria* (17 807 sequences; 25.3%), *Phoma* (10 589 sequences; 15.0%) and *Aureobasidium* (8106 sequences; 11.6%) were most common. Additional putative, BLAST-assigned affinities for the most common OTUs are listed in Fig. 2.

To estimate species level coverage in GenBank, we also estimated how many of our sequences would find a distinctly named species in GenBank. We chose 95% sequence identity as a proxy for species level assignment and screened the filtered BLAST data for those occurrences. Of the total of 744 distinct nonsingleton OTUs, 492 were assigned to taxa that did not contain a complete specific epithet, resulting in an approximate ratio of two unnamed accessions to one with a complete species level annotation.

Fungal richness and diversity in the phyllosphere

In all, the sampled stands hosted a substantial organismal richness. At 95% sequence identity, we detected 744 nonsingleton OTUs and 491 singletons for a total of 1232 potential OTUs. Richness (number of OTUs), diversity (Fisher's α, Simpson's $1/D$ or $1 - D$, and Shannon's H') and evenness tended to be lower in the urban than in the nonurban sites (Fig. 3; Table 2). Although the repeated

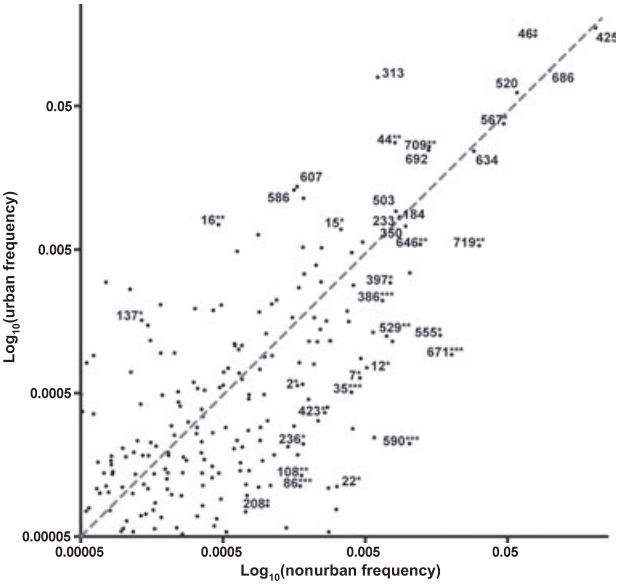

Fig. 2 The frequencies of operational taxonomic units (OTUs) in nonurban (*x*-axis) and urban (*y*-axis) sites. OTUs with frequencies $> 0.5 \times 10^{-4}$ are shown. The axes are $\log_{10}$-transformed. The dashed line indicates an isocline of equal frequencies in nonurban and urban sites. OTUs below this line are more frequent in the nonurban environments and OTUs above it are more frequent in the urban environments. The most abundant OTUs are shown and listed here along with their closest generic level BLAST matches in order of abundance: (1) 425 *Alternaria* sp., (2) 46 *Phoma* sp., (3) 686 *Aureobasidium* sp., (4) 520 *Davidiella* sp., (5) 313 *Oidium* sp., (6) 567 *Cladosporium* sp., (7) 634 *Epicoccum* sp., (8) 709 *Didymella* sp., (9) 692 *Neofabraea* sp., (10) 719 *Discula* sp., (11) 44 *Cladosporium* sp., (12) 671 *Mycosphaerella* sp., (13) 555 *Pestalotiopsis* sp., (14) 646 *Mycosphaerella* sp., (15) 503 *Alternaria* sp., (16) 184 Leptosphaeriaceae sp., (17) 233 *Leptosphaerulina* sp., (18) 350 *Sporobolomyces* sp., (19) 607 *Pleiochaeta* sp., and (20) 586 *Erysiphe* sp. Asterisks identify OTUs whose frequencies differ between nonurban and urban sites in repeated measures ANOVA (Table 3) – their BLAST-inferred taxon affinities are available in the Supporting Information Table S1. *, $0.01 \le P < 0.05$; **, $0.001 \le P < 0.01$; ***, $P < 0.001$.

measures ANOVA indicated lower richness and diversity in the urban environments (Table 2), the results were variable through the season and among samplings: only up to three of the six samplings provided support for lower richness or diversity in the urban settings (Fig. 3).

We explored the coverage of organismal richness and diversity in the phyllosphere using rarefaction (Fig. 4) and extrapolated richness estimators (Table S5). The rarefaction analyses suggest that the total richness in the phyllosphere fungal communities was inadequately covered despite an average of >1200 sequences per sample or 7000 sequences per land-use type and sampling. The extrapolative richness estimators corroborated and exceeded the observed richness (Table S5). Similarly, singletons – OTUs that occur only once in a sample – comprised on average 30% of the total OTUs.

Fungal communities in the phyllosphere

To summarize and visualize the compositional differences among the phyllosphere communities, we used NMS multivariate analyses. Regardless of the taxonomic rank (OTUs as proxies for species; or BLAST-assigned genera, families, and orders) or removal of the infrequent data points, the repeated measures ANOVA of the NMS scores invariably indicated that the communities were distinct between the land-use types (Table 2). The urban and nonurban communities were separated by one or two of the primary NMS axes. Similarly, these NMS analyses indicated strong, site-related effects as well as seasonal variability in the phyllosphere fungal communities. For clarity and to minimize redundancy, we present OTU level results summarized across the land-use types and sampling events and display the NMS axis scores (insets) to emphasize the seasonal trends on the second NMS axis (Fig. 5).

Among the 744 nonsingleton OTUs, 26 (3.5%) differed in their frequencies between the two land-use types when analyzed with the repeated measures ANOVA. Although many OTUs showed significant variability among sites within land-use type (Table 3), we focus here on the effects of land-use. A total of six of the 26 OTUs occurred at greater frequencies in the urban sites, whereas 20 occurred at greater frequencies in the nonurban sites (Fig. 2). To provide a broader taxonomic context, we also analyzed these data at generic and familial levels after the OTUs had been assigned to taxa based on BLAST affinities. To decrease erroneous assignments based on poor coverage or sequence identity queries, we filtered those data points that did not meet our thresholds. Even after threshold filtering such as ours here, we emphasize that all the taxon affinities discussed here are only as good as are the annotations provided for them in the databases and, therefore, should be considered cautiously. Among the detected genera, 17 (*Articulospora*, *Bipolaris*, *Capnobotryella*, *Cladophialophora*, *Dioszegia*, *Discula*, *Dissoconium*, *Elsinoe*, *Gnomonia*, *Lalaria*, *Mycosphaerella*, *Pestalotiopsis*, *Rhinocladiella*, *Sarcostroma*, *Selenophoma*, *Taphrina*, and *Tumularia*) were more frequent in the nonurban sites, whereas only five (*Bartalinia*, *Candida*, *Cladosporium*, *Didymella* and *Phoma*) were more frequent in the urban sites. On a family level, seven families (Amphispaeriaceae, Elsinoaceae, Filobasidiaceae, Gnomoniaceae, Helotiaceae, Mycosphaerellaceae, and Taphrinaceae) were all more frequent in the nonurban areas whereas no families occurred more frequently in the urban environment, a likely result of the overall lower OTU richness.

Seasonal trends in the phyllosphere communities

The phyllosphere accumulated diverse and species-rich fungal assemblies soon after bud burst. Assuming low abundance or near-absence of fungal colonization in the buds

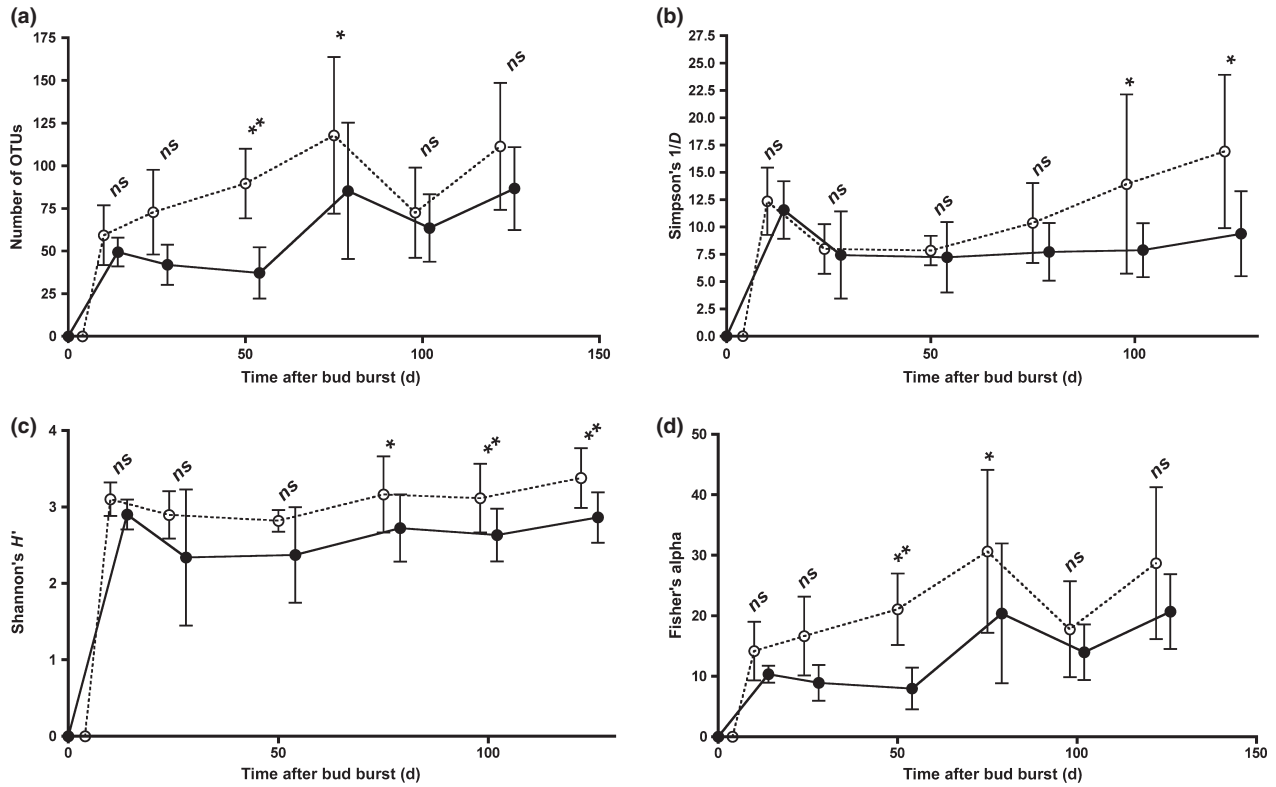

Fig. 3 Richness and diversity of phyllosphere fungi in nonurban and urban environments across the growing season in 2008. Note that bud burst occurred 4 d earlier within the city limits than outside, potentially indicating an urban heat island (UHI) effect. (a) Operational taxonomic unit (OTU) richness (*S*), (b) Simpson's diversity (1/*D*), (c) Shannon's diversity (*H'*), and (d) Fisher's alpha diversity (α). Results from the nested ANOVA for land-use are displayed for each sampling to show the comparisons between the nonurban and urban sites. *, $0.01 \leq P < 0.05$; **, $0.001 \leq P < 0.01$; ***$P < 0.001$; ns, not significant. Dashed lines and open circles, nonurban; solid lines and closed circles, urban.

before leaf emergence (see Johnson & Whitney, 1992; Kaneko & Kaneko, 2004; Osono & Mori, 2005; Osono, 2008), the rate of taxon accumulation was remarkable. Within 2 wk of the leaf emergence, the foliar samples contained an average of 50 ± 19 OTUs (mean ± 1 SD). The richness and diversity estimators varied across the season, but time × land-use interactions were rare (Table 2), suggesting parallel seasonal patterns between the nonurban and urban trees (Fig. 5). The richness peaked in early August (102 ± 44 OTUs) and at the end of the growing season (99 ± 33 OTUs), but did not show simple linear accumulation of OTUs throughout the season (Fig. 3).

The community-level seasonal turnover was corroborated by dynamics in the OTU and taxon frequencies: many OTUs and taxa fluctuated seasonally as well as showed significant interactions between the sampling events and land-use or sites within land-use (within-subjects terms; Tables 3 and 4). On a family level, Botryosphaeriaceae and Elsinoaceae peaked in August; Filobasidiaceae, Mycosphaerellaceae, and Taphrinaceae tended to increase their frequencies at the end of the growing season; and, by contrast, Gnomoniaceae and Helotiaceae tended to decline towards the end of the growing season. On a genus level, *Alternaria*, *Articulospora*, *Bartalinia*, *Taphrina*, and *Tumularia* peaked early to mid-season; *Aureobasidium*, *Erysiphe*, and *Teratosphaeria* peaked mid- to late season; *Capnobotryella*, *Mycosphaerella*, and *Scleroconidioma* frequencies increased through the growing season; and *Davidiella* as well as *Discula* frequencies decreased through the growing season. Many of these general trends varied between the urban and nonurban stands as well as among the sites, as indicated by the interaction terms in Table 4. To exemplify clear seasonal trends, Fig. 6 displays two well-recognized and common genera, whose seasonal variability is described above. Among the eight exemplified BLAST-assigned genera, five (*Alternaria*, *Capnobotryella*, *Discula*, *Mycosphaerella*, and *Taphrina*) showed significant 'time × land-use' and three (*Alternaria*, *Mycosphaerella*, and *Taphrina*) significant 'time × site (land-use)' interaction terms (Table 4). These interaction terms probably indicate the spatial variability in the observed temporal patterns. For example, in the case of *Mycosphaerella*, which increased in its abundance throughout the growing season, these interaction terms stem from its slower increase in the urban environment and its variable occurrence in the urban sites.

Table 2 Repeated measures ANOVA table of foliar diversity and richness, and nonmetric multidimensional scaling (NMS) scores for operational taxonomic units (OTUs), genera, families and orders before and after removal of low-abundance observations

	Between subjects		Within subjects		
Response	Use $F_{1,6}^{P}$	Site (use) $F_{4,6}^{P}$	Time $F_{4,3}^{P}$	Time × use $F_{4,3}^{P}$	Time × site (use) $F_{16,9.8}^{P}$
Fisher's α	**54.31$^{0.0003}$⇓**	**6.23$^{0.0249}$**	**17.12$^{0.0209}$⇑**	0.38$^{0.8126}$	1.92$^{0.1503}$
Richness (*S*)	**48.74$^{0.0002}$⇓**	**5.20$^{0.0373}$**	**24.15$^{0.0128}$⇑**	0.55$^{0.7140}$	1.65$^{0.2163}$
Singletons	**7.11$^{0.0372}$⇓**	0.84$^{0.5478}$	4.06$^{0.1394}$	1.66$^{0.3537}$	1.23$^{0.3787}$
Simpson's *D*	**13.15$^{0.0110}$⇑**	3.08$^{0.1062}$	**10.48$^{0.0414}$⇓**	8.64$^{0.0538}$	**3.67$^{0.0220}$**
Simpson's 1/*D*	**32.08$^{0.0013}$⇓**	**6.21$^{0.0252}$**	**27.38$^{0.0107}$⇑**	**13.51$^{0.0292}$**	**3.49$^{0.0262}$**
Shannon's *H′*	**33.56$^{0.0012}$⇓**	**5.02$^{0.0403}$**	**22.78$^{0.0140}$⇑**	1.98$^{0.3009}$	**3.30$^{0.0317}$**
Evenness (*H′*/ln(*S*))	**6.57$^{0.0428}$⇓**	2.97$^{0.1131}$	6.62$^{0.0762}$	1.11$^{0.4841}$	0.59$^{0.8290}$
OTUs (744)[a]					
NMS1 (49.7%)	**32.43$^{0.0013}$⇓**	**4.96$^{0.0413}$**	4.29$^{0.1305}$	0.71$^{0.6358}$	1.46$^{0.2796}$
NMS2 (25.5%)	5.43$^{0.0586}$	3.46$^{0.0857}$	**116.56$^{0.0013}$⇑**	2.34$^{0.2557}$	**3.31$^{0.0312}$**
OTUs (194)[b]					
NMS1 (52.6%)	**41.67$^{0.0007}$⇓**	3.83$^{0.0705}$	**36.03$^{0.0072}$⇑**	0.69$^{0.6482}$	1.18$^{0.4090}$
NMS2 (24.0%)	0.09$^{0.7802}$	4.21$^{0.0582}$	**84.51$^{0.0021}$⇑**	1.42$^{0.2557}$	2.79$^{0.0538}$
Genera (160)[a]					
NMS1 (43.7%)	**12.31$^{0.0127}$⇑**	1.52$^{0.3080}$	**174.45$^{0.0007}$⇓**	1.83$^{0.3238}$	2.20$^{0.1060}$
NMS2 (18.6%)	**13.43$^{0.0105}$⇑**	**12.52$^{0.0045}$**	**12.24$^{0.0335}$⇓**	**16.84$^{0.0214}$**	**2.88$^{0.0490}$**
NMS3 (23.5%)	2.96$^{0.1362}$	**4.98$^{0.0411}$**	**33.13$^{0.0081}$⇑**	0.42$^{0.7887}$	1.34$^{0.3269}$
Genera (74)[b]					
NMS1 (16.7%)	**26.31$^{0.0022}$⇓**	**14.12$^{0.0033}$**	**23.34$^{0.0135}$⇑**	**14.38$^{0.0268}$**	**4.62$^{0.0098}$**
NMS2 (18.7%)	3.36$^{0.1168}$	**6.16$^{0.0256}$**	**57.89$^{0.0036}$⇑**	1.22$^{0.4530}$	0.98$^{0.5304}$
NMS3 (50.4%)	**10.68$^{0.0171}$⇑**	1.58$^{0.2935}$	**146.39$^{0.0009}$⇓**	0.53$^{0.7276}$	**3.08$^{0.0395}$**
Family (55)[a]					
NMS1 (7.5%)	**16.69$^{0.0065}$⇑**	**10.66$^{0.0068}$**	**18.15$^{0.0193}$⇓**	4.79$^{0.1144}$	**3.52$^{0.0255}$**
NMS2 (21.2%)	4.65$^{0.0745}$	**8.65$^{0.0113}$**	**56.55$^{0.0037}$⇓**	5.47$^{0.0970}$	1.22$^{0.3860}$
NMS3 (61.4%)	4.65$^{0.0744}$	1.46$^{0.3219}$	2.62$^{0.2274}$	0.50$^{0.7457}$	**2.99$^{0.0432}$**
Family (27)[b]					
NMS1 (17.6%)	**6.53$^{0.0432}$⇑**	**10.25$^{0.0075}$**	**62.11$^{0.0032}$⇑**	5.58$^{0.0947}$	1.17$^{0.4155}$
NMS2 (11.1%)	**14.82$^{0.0085}$⇓**	**8.72$^{0.0112}$**	**20.37$^{0.0164}$⇑**	3.52$^{0.1646}$	**3.89$^{0.0181}$**
NMS3 (61.8%)	5.24$^{0.0620}$	1.42$^{0.3322}$	0.87$^{0.5688}$	0.42$^{0.7910}$	1.40$^{0.3018}$
Order (39)[a]					
NMS1 (52.3%)	4.27$^{0.0843}$	1.24$^{0.3866}$	1.41$^{0.4048}$	0.49$^{0.7502}$	0.95$^{0.5568}$
NMS2 (33.8%)	**13.58$^{0.0426}$⇓**	**4.89$^{0.0426}$**	2.39$^{0.2497}$	1.84$^{0.3216}$	0.47$^{0.9109}$
Order (23)[b]					
NMS1 (53.9%)	4.85$^{0.0698}$	1.44$^{0.3280}$	3.20$^{0.1832}$	0.31$^{0.8590}$	1.29$^{0.3521}$
NMS2 (32.5%)	**11.91$^{0.0136}$⇓**	4.48$^{0.0514}$	2.37$^{0.2524}$	2.15$^{0.2772}$	0.56$^{0.8538}$

[a]Full nonsingleton frequency data matrix.
[b]Includes only those OTUs or taxa that occur in > 6 samples.
'Use' refers to the urban vs nonurban comparison, 'site' to sites within urban or nonurban land-use types (Table 1), and 'time' to comparisons among the seasonal samples and its interactions with the between-subject effects. Between-subject terms test the overall effects of land-use and site across the sampling events, whereas the within-subject terms allow an assessment of the time-related effects and their interactions with the explanatory design variables. *F*-test variables are exact values for all parameters except for the term 'sampling × site (use)' where *F* is approximated using Wilk's lambda. The second sampling event (11 June) was omitted because of two missing samples. Numbers in parentheses indicate the number of OTUs or taxa that were included in the NMS analyses or the proportion of variation represented by the NMS axes. Significant *F*-values are highlighted in bold for emphasis. The arrows indicate direction of difference. Upward arrows in the 'use' column indicate greater means within the urban center and those in the 'time' column indicate greater means later in the season.

Discussion

We combined massively parallel 454-sequencing and DNA tagging to characterize fungal communities through one growing season in urban and nonurban environments. It is important to bear in mind that we are unable to distinguish between endophytes and epiphytes but consider the phyllospheric fungal community in its entirety. In these phyllosphere communities, we detected over 1200 OTUs, more than a third of which occurred only once in our data set. Rarefaction analyses and extrapolative richness estimators indicate that much of the total diversity was not observed at any of the sampling events. However, sequencing artifacts in a 454-sequencing may lead to overestimation of the resident diversity (Quince *et al.*, 2009; Reeder & Knight, 2009) and one must be cautious in interpreting singleton or rare taxon occurrences in 454-sequencing assays. Even so, our data contribute to the growing body of observations

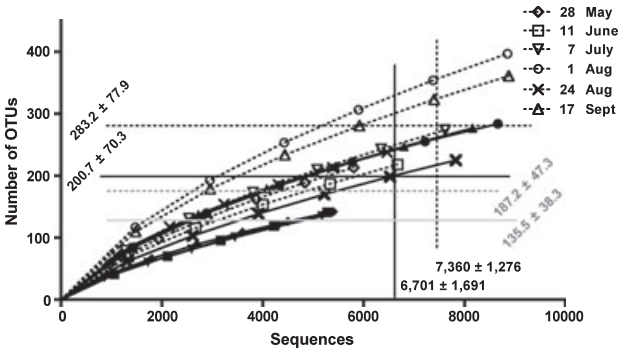

Fig. 4 Rarefaction curves of the phyllosphere fungi in nonurban (dashed lines) and urban sites (solid lines) for each of the six samplings. The horizontal lines indicate the number of unique operational taxonomic units (OTUs) (grey lines) and the number of all OTUs (black lines) in urban and nonurban sites averaged across sites at each seasonal sampling (mean ± 1 SD); the vertical lines show overall sampling effort (mean ± 1 SD).

that tree foliage is inhabited by hyperdiverse fungal communities (Arnold *et al.*, 2000; Santamaria & Bayman, 2005; Arnold, 2007, 2008). While failing to saturate the organismal richness in the phyllosphere fungal communities, our compromise between number of samples (experimental units) and sequencing depth allowed successful testing of hypotheses on the effects of land-use and on the seasonal dynamics in these communities.

As in the commentary by Hibbett *et al.* (2009), we aimed to provide a conservative estimate of the ratio of fungal species that are known and annotated with a species-level affinity. Our conservative estimates that exclude the singletons show that perhaps two unannotated fungal species are available for each annotated taxon. Although Hibbett *et al.* (2009) discuss this in the context of hidden biodiversity and potentially undiscovered species, as well as indicating a need for further taxonomic effort, we consider this to primarily indicate poor sampling across all fungal taxa and, as such, to underline the importance of continuous taxonomic research to provide baseline material for improved taxon assignments based on publicly available sequence data.

Our primary goal was to evaluate whether or not conspecific trees in a small urban center maintain fungal richness and diversity similar to that hosted in an adjacent nonurban environment. Our results overall strongly indicate that urban trees, even in a small urban center, host distinct fungal communities that are lower in diversity and richness than those in surrounding nonurban areas. These results corroborate studies on other organisms that have suggested declines in aquatic and terrestrial species richness within cities (e.g. Paul & Meyer, 2001; McKinney, 2002)

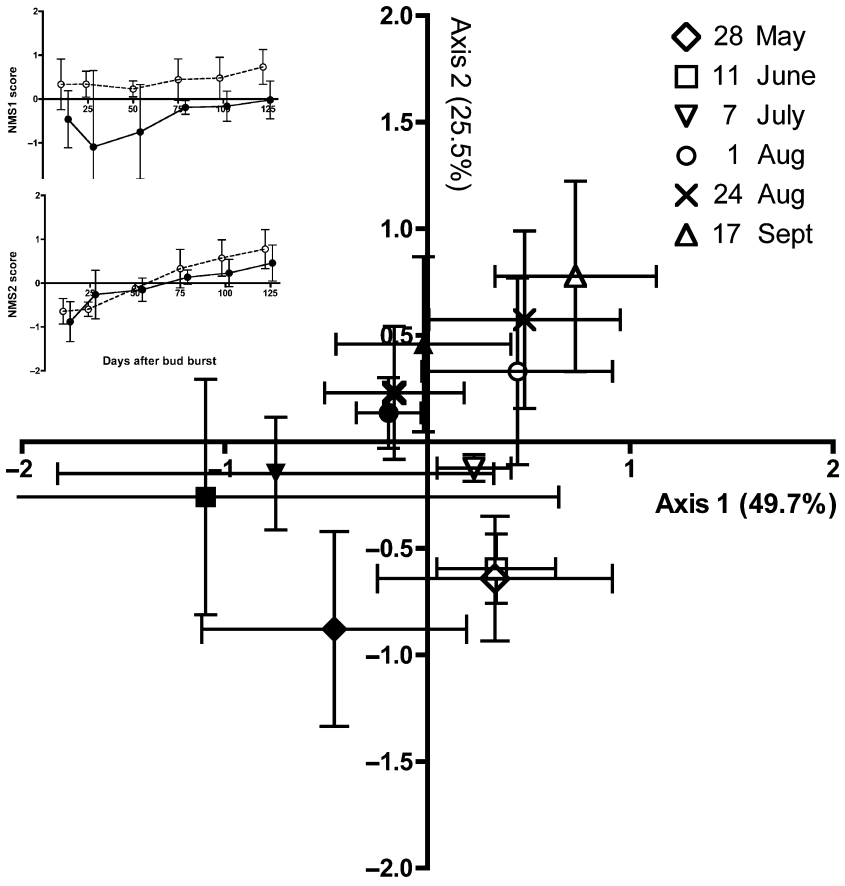

Fig. 5 Nonparametric multidimensional scaling (NMS) of the operational taxonomic units (OTUs) detected in the *Quercus macrocarpa* phyllosphere in nonurban (open symbols) and urban (closed symbols) environments. To emphasize the seasonal trends in the fungal communities the axis scores are shown in the insets. Note the parallel trends of nonurban and urban communities on axis 2.

Table 3 Repeated measures ANOVA table of operational taxonomic unit (OTU) responses

	Between subjects		Within subjects		
OTU[a]	Use $F_{1,6}{}^{P}$	Site (use) $F_{4,6}{}^{P}$	Time $F_{4,3}{}^{P}$	Time × use $F_{4,3}{}^{P}$	Time × site (use) $F_{16,9.8}{}^{P}$ [b]
2	**12.66$^{0.0119}$⇓**	1.68$^{0.2704}$	6.66$^{0.0756}$	**20.37$^{0.0164}$**	**4.06$^{0.0156}$**
5	1.78$^{0.2304}$	0.56$^{0.7000}$	1.31$^{0.4283}$	0.73$^{0.6289}$	0.76$^{0.6977}$
7	**8.36$^{0.0277}$⇓**	0.78$^{0.5765}$	**185.58$^{0.0006}$⇑**	**153.75$^{0.0008}$**	**7.21$^{0.0017}$**
9	2.23$^{0.1885}$	1.54$^{0.3030}$	1.98$^{0.3005}$	6.76$^{0.0742}$	1.96$^{0.1435}$
12	**8.23$^{0.0285}$⇓**	**6.51$^{0.0226}$**	8.75$^{0.0529}$	**10.85$^{0.0395}$**	**1.66$^{0.2128}$**
14	0.01$^{0.9156}$	0.59$^{0.6848}$	6.14$^{0.0839}$	**37.86$^{0.0067}$**	**5.44$^{0.0067}$**
15	**10.06$^{0.0193}$⇑**	3.79$^{0.0717}$	2.54$^{0.2350}$	1.98$^{0.3010}$	0.84$^{0.6371}$
16	**14.58$^{0.0088}$⇑**	**8.80$^{0.0110}$**	**369.16$^{0.0002}$⇓⇑**	**362.54$^{0.0002}$**	**9.59$^{0.0005}$**
19	2.72$^{0.1504}$	**4.63$^{0.0478}$**	**34.24$^{0.0077}$⇑⇓**	7.15$^{0.0689}$	**4.56$^{0.0102}$**
20	3.20$^{0.1237}$	0.32$^{0.8531}$	1.39$^{0.4107}$	1.66$^{0.3536}$	1.62$^{0.2251}$
21	3.21$^{0.1235}$	**4.54$^{0.0499}$**	**576.18$^{0.0001}$⇓**	**438.35$^{0.0002}$**	**41.66$^{0.0001}$**
22	**8.61$^{0.0261}$⇓**	1.70$^{0.2679}$	2.25$^{0.2650}$	1.68$^{0.3501}$	0.84$^{0.6365}$
24	3.26$^{0.1212}$	0.38$^{0.8151}$	1.16$^{0.4691}$	0.53$^{0.7278}$	1.11$^{0.4465}$
28	3.51$^{0.1103}$	0.93$^{0.5036}$	2.25$^{0.2655}$	2.43$^{0.2459}$	1.17$^{0.4105}$
29	0.27$^{0.6198}$	0.89$^{0.5256}$	7.75$^{0.0620}$	0.81$^{0.5927}$	0.59$^{0.8325}$
34	0.41$^{0.5416}$	2.90$^{0.1182}$	**52.07$^{0.0042}$⇑**	2.99$^{0.1973}$	**2.89$^{0.0482}$**
35	**103.59$^{0.0001}$⇓**	**26.61$^{0.0006}$**	**11.58$^{0.0361}$⇑⇓⇑**	**9.76$^{0.0456}$**	**3.33$^{0.0.308}$**
37	0.25$^{0.6371}$	0.72$^{0.6096}$	4.34$^{0.1290}$	0.21$^{0.9161}$	0.58$^{0.8370}$
43	3.21$^{0.1233}$	3.05$^{0.1076}$	2.71$^{0.2195}$	0.66$^{0.6627}$	0.64$^{0.7952}$
44	**16.52$^{0.0066}$⇑**	3.45$^{0.0861}$	5.40$^{0.0987}$	3.22$^{0.1819}$	1.03$^{0.4967}$
45	2.80$^{0.1453}$	0.49$^{0.7422}$	1.65$^{0.3539}$	0.72$^{0.6324}$	0.60$^{0.8271}$
46	**8.13$^{0.0291}$⇑**	1.47$^{0.3201}$	2.57$^{0.2324}$	**9.46$^{0.0476}$**	0.94$^{0.5617}$
49	5.64$^{0.0552}$	**16.13$^{0.0023}$**	1.65$^{0.3542}$	2.95$^{0.2001}$	0.87$^{0.6101}$
51	1.67$^{0.2426}$	0.17$^{0.9445}$	3.99$^{0.1423}$	0.65$^{0.6660}$	1.38$^{0.3104}$
62	1.83$^{0.2246}$	0.27$^{0.8850}$	3.99$^{0.1425}$	0.69$^{0.6455}$	0.72$^{0.7308}$
63	0.61$^{0.4641}$	0.89$^{0.5250}$	**11.79$^{0.0353}$⇑⇓**	0.11$^{0.9721}$	1.80$^{0.1752}$
65	1.52$^{0.2633}$	0.93$^{0.5034}$	0.77$^{0.6079}$	0.53$^{0.7267}$	0.50$^{0.8930}$
68	1.26$^{0.3038}$	1.12$^{0.4282}$	2.25$^{0.2656}$	4.71$^{0.1169}$	1.55$^{0.2471}$
73	1.41$^{0.2800}$	1.25$^{0.3834}$	1.61$^{0.3631}$	0.30$^{0.8606}$	0.38$^{0.9569}$
82	2.64$^{0.1551}$	0.84$^{0.5468}$	4.52$^{0.1229}$	2.70$^{0.2200}$	1.01$^{0.5113}$
84	4.04$^{0.0912}$	**14.24$^{0.0032}$**	**1117.59$^{0.0001}$⇑**	**251.53$^{0.0004}$**	**23.28$^{0.0001}$**
124	0.27$^{0.6247}$	0.43$^{0.7851}$	1.63$^{0.3582}$	0.34$^{0.8374}$	0.68$^{0.7613}$
127	2.19$^{0.1893}$	0.47$^{0.7561}$	9.08$^{0.0503}$	1.08$^{0.4956}$	1.37$^{0.3129}$
130	2.24$^{0.1852}$	0.15$^{0.9555}$	1.26$^{0.4431}$	1.25$^{0.4454}$	0.50$^{0.8954}$
184	0.06$^{0.8123}$	0.54$^{0.7144}$	3.27$^{0.1791}$	0.44$^{0.7787}$	0.62$^{0.8058}$
233	0.00$^{0.9567}$	2.26$^{0.1778}$	2.46$^{0.2430}$	1.14$^{0.4775}$	0.45$^{0.9270}$
258	5.20$^{0.0628}$	0.87$^{0.5307}$	1.35$^{0.4194}$	1.31$^{0.4292}$	1.04$^{0.4887}$
313	1.75$^{0.2343}$	0.65$^{0.6464}$	2.47$^{0.2420}$	7.02$^{0.0706}$	1.76$^{0.1865}$
314	0.26$^{0.6305}$	1.82$^{0.2442}$	1.52$^{0.3812}$	3.03$^{0.1945}$	0.81$^{0.6557}$
350	0.50$^{0.5053}$	0.69$^{0.6246}$	1.59$^{0.3654}$	0.28$^{0.8764}$	1.13$^{0.4333}$
376	3.72$^{0.1020}$	2.29$^{0.1742}$	0.78$^{0.6053}$	4.95$^{0.1098}$	0.95$^{0.5486}$
378	0.03$^{0.8777}$	3.21$^{0.0982}$	4.94$^{0.1101}$	3.82$^{0.1498}$	**3.00$^{0.0431}$**
386	**50.98$^{0.0004}$⇓**	**9.34$^{0.0095}$**	8.80$^{0.0524}$	4.08$^{0.1387}$	1.30$^{0.3470}$
397	**6.15$^{0.0478}$⇓**	1.29$^{0.3716}$	**26.64$^{0.0111}$⇑**	0.57$^{0.7067}$	**3.54$^{0.0251}$**
423	**11.52$^{0.0146}$⇓**	0.77$^{0.5804}$	**15.27$^{0.0246}$⇓**	**13.75$^{0.0285}$**	1.79$^{0.1796}$
425	0.06$^{0.8185}$	1.73$^{0.2615}$	**20.40$^{0.0163}$**	**10.59$^{0.0408}$**	**3.09$^{0.0392}$**
503	0.26$^{0.6275}$	0.82$^{0.5558}$	3.03$^{0.1945}$	0.86$^{0.5736}$	0.44$^{0.9282}$
520	1.13$^{0.3296}$	**6.29$^{0.0244}$**	**24.47$^{0.0126}$⇑⇓**	1.24$^{0.4483}$	0.61$^{0.8199}$
529	**15.27$^{0.0079}$⇓**	**7.92$^{0.0142}$**	5.94$^{0.0875}$	3.84$^{0.1487}$	2.21$^{0.1044}$
534	0.92$^{0.3738}$	0.69$^{0.6249}$	0.75$^{0.6192}$	0.75$^{0.6193}$	0.61$^{0.8199}$
555	**11.10$^{0.0158}$⇓**	1.77$^{0.2526}$	4.42$^{0.1260}$	5.12$^{0.1055}$	1.57$^{0.2395}$
567	**12.78$^{0.0117}$⇓**	**11.55$^{0.0055}$**	**9.32$^{0.0485}$⇑⇓**	1.94$^{0.3066}$	2.11$^{0.1194}$
590	**85.99$^{0.0001}$⇓**	**70.05$^{0.0001}$**	**16792.52$^{0.0001}$⇑**	**16547.92$^{0.0001}$**	**71.76$^{0.0001}$**
607	2.19$^{0.1897}$	0.34$^{0.8448}$	1.75$^{0.3377}$	0.71$^{0.6349}$	0.71$^{0.7424}$
630	3.69$^{0.1033}$	1.23$^{0.3908}$	**65.57$^{0.0030}$⇓⇑**	**54.78$^{0.0039}$**	**7.89$^{0.0012}$**
634	0.11$^{0.7498}$	**7.57$^{0.0159}$**	0.86$^{0.5743}$	1.40$^{0.4063}$	0.69$^{0.7575}$
646	**14.07$^{0.0095}$⇓**	**11.01$^{0.0063}$**	**48.72$^{0.0046}$⇑**	1.32$^{0.4268}$	**3.81$^{0.0194}$**

Table 3 (Continued)

	Between subjects		Within subjects		
OTU[a]	Use $F_{1,6}{}^{P}$	Site (use) $F_{4,6}{}^{P}$	Time $F_{4,3}{}^{P}$	Time × use $F_{4,3}{}^{P}$	Time × site (use) $F_{16,9.8}{}^{P}$ [b]
671	**$63.41^{0.0002}\Downarrow$**	**$19.79^{0.0013}$**	**$289.85^{0.0003}\Uparrow$**	**$254.40^{0.0004}$**	**$15.40^{0.0001}$**
686	$1.68^{0.2429}$	**$5.04^{0.0400}$**	**$9.65^{0.0464}\Uparrow\Downarrow$**	$0.58^{0.6981}$	$0.74^{0.7123}$
692	$2.60^{0.1580}$	$0.82^{0.5548}$	**$15.72^{0.0236}\Uparrow$**	$4.00^{0.1419}$	$1.09^{0.4614}$
709	**$17.46^{0.0058}\Uparrow$**	**$5.82^{0.0292}$**	$2.42^{0.2464}$	$3.09^{0.1902}$	$1.86^{0.1621}$
716	$4.59^{0.0759}$	$0.77^{0.5819}$	$1.34^{0.4225}$	$2.69^{0.2209}$	$1.34^{0.3274}$
719	**$14.17^{0.0093}\Downarrow$**	$0.21^{0.9252}$	**$28.66^{0.0101}\Downarrow$**	**$26.66^{0.0111}$**	$1.90^{0.1550}$

[a]Only those OTUs that occurred in at least 20 samples and were represented by more than 100 reads across the data set were included.
[b]Wilk's lambda, approximate *F*.
OTUs were assigned at 95% similarity of internal transcribed spacer 2 (ITS2) and its flanking regions. 'Use' refers to the urban vs nonurban comparison, 'site' to sites within urban or nonurban land-use types (Table 1), and 'time' to comparisons among the seasonal samples and its interactions with the between-subject effects. Between-subject terms test the overall effects of land-use and site across the sampling events, whereas the within-subject terms allow an assessment of the time-related effects and their interactions with the explanatory design variables. *F*-test variables are exact values for all parameters except for term 'sampling × site (use)' where *F* is approximated using Wilk's lambda. The second sampling event (11 June) was omitted because of two missing samples and only those OTUs that occur in more than 14 samples (20%) or whose occurrence exceeds 100 reads are included. Significant *F*-values are highlighted in bold for emphasis. The arrows indicate direction of difference. Upward arrows in the 'use' column indicate greater means within the urban center and those in the 'time' column indicate greater means later in the season. OTU assignments are available in the Supporting Information Table S1.

as well as our previous results (Jumpponen & Jones, 2009), which, in a more limited sampling, indicated lower phyllosphere richness and diversity in urban areas.

The repeated measures ANOVA showed that richness and diversity varied across the land-use types. However, the analyses of each sampling event separately provide a more detailed picture. None of the richness or diversity estimators was consistently and significantly greater in the nonurban stands throughout the whole growing season. These findings underline and emphasize the importance of accounting for seasonal dynamics in fungal communities. Although we are unable to unequivocally identify the underlying reasons for this variability, it is likely that leaf turnover and/or variable environmental conditions within the growing season contribute to these observations.

Not only the diversity and richness, but also the phyllosphere community compositions were distinct between the nonurban and urban trees, as indicated by our multivariate analyses. A number of the common OTUs, genera and families occurred at different frequencies between the land-use types. Although the differing taxon frequencies are interesting, the underlying reasons are even more so. To provide a broad characterization of the foliar environment that the phyllosphere fungi experience, we utilized ICP-MS and MS. Our analyses indicated that a number of macronutrients (N, K and S) and micronutrients (B, Mn and Se) as well as potentially toxic trace elements (Cd, Pb and Zn) were enriched in the urban environments, as has been reported previously (Pouyat & McDonnell, 1991; Kaye *et al.*, 2006). We also measured stable isotopes ($\delta^{13}C$ and $\delta^{15}N$) to test other environmental stressors. Our results confirmed that water and its availability did not affect $\delta^{13}C$-implied host physiology across the two different land-use types. By contrast, $\delta^{15}N$ natural abundances suggested that the plants utilized different sources of N, a likely result of repeated fertilization in the managed, urban areas. Unfortunately, because all the measured chemical elements are correlated as a result of the anthropogenic enrichment, pinpointing specific mechanisms for the community differences in the absence of empirical tests remains speculative at best. While acknowledging the speculative nature of this discussion, we put forth four components that we consider most likely and propose them as testable hypotheses to identify drivers in the phyllosphere community assembly.

First, a recent study by Helander *et al.* (2007) concluded that the frequency of foliar endophytes of birch (*Betula pubescens*) was dependent on the size of an island (area) as well as its distance (isolation) from the mainland. Those results indicate that the species–area relationships and isolation of patches may control the foliar fungal communities. Although we aimed to select our sites to match the urban and nonurban stands as closely as possible, complete control of the distance to the closest neighboring stand is impossible and a greater and more diverse inoculum pool may be available in the nonurban stands.

Second, our extensive analyses of the foliar chemistry indicated that the urban and nonurban sites are biogeochemically distinct. As also indicated by our data, urban stands are characterized by nutrient enrichment and accumulation of heavy metals (Pouyat & McDonnell, 1991; Kaye *et al.*, 2006). The foliar fungal communities are likely to be sensitive to air pollution. For example, Helander (1995) showed that pine (*Pinus sylvestris*) trees adjacent to a point source hosted less endophyte colonization than trees further away. Some foliar fungi seemed more sensitive than others: *Cenangium ferrucinosum* occurrence was lower near

Table 4 Repeated measures ANOVA table of taxon (genus- and family-level) responses within and outside the city limits in Manhattan, KS, USA

	Between subjects		Within subjects		
Taxon[a]	Use $F_{1,6}{}^{P}$	Site (use) $F_{4,6}{}^{P}$	Time $F_{4,3}{}^{P}$	Time × use $F_{4,3}{}^{P}$	Time × site (use) $F_{16,9.8}{}^{P}$ [b]
Genus					
Alternaria	$0.02^{0.8881}$	$1.76^{0.2555}$	**$29.10^{0.0098}$⇑⇓**	**$19.41^{0.0175}$**	**$4.46^{0.0111}$**
Articulospora	**$19.56^{0.0045}$⇓**	$1.55^{0.2993}$	**$68.39^{0.0028}$⇓**	**$41.66^{0.0058}$**	**$4.80^{0.0085}$**
Aureobasidium	$1.61^{0.2516}$	**$5.02^{0.0404}$**	**$9.71^{0.0459}$⇑⇓**	$0.59^{0.6947}$	$0.75^{0.7060}$
Bartalinia	$3.88^{0.0965}$	$0.64^{0.6548}$	**$13.32^{0.0298}$⇑⇓⇑**	$7.10^{0.0696}$	**$3.51^{0.0256}$**
Candida	**$6.37^{0.0450}$⇑**	$2.71^{0.1329}$	$2.40^{0.2494}$	$1.91^{0.3103}$	$0.85^{0.6274}$
Capnobotryella	**$18.29^{0.0052}$⇓**	**$14.74^{0.0029}$**	**$52.99^{0.0041}$⇑**	**$24.27^{0.0127}$**	$2.73^{0.0573}$
Cladophialophora	**$12.66^{0.0119}$⇓**	$1.68^{0.2704}$	$6.66^{0.0756}$	**$20.37^{0.0164}$**	**$4.06^{0.0156}$**
Cladosporium	**$18.07^{0.0054}$⇓**	**$8.63^{0.0115}$**	$4.38^{0.1275}$	$1.49^{0.3880}$	$1.49^{0.2652}$
Cryptococcus	**$15.07^{0.0081}$⇓**	$3.44^{0.0865}$	$3.86^{0.1661}$	$3.53^{0.1481}$	$2.53^{0.0719}$
Davidiella	$1.12^{0.3303}$	**$6.23^{0.0250}$**	**$24.04^{0.0129}$⇓**	$1.21^{0.4554}$	$0.60^{0.8212}$
Diaporthe	$1.38^{0.2846}$	$1.52^{0.3084}$	$3.59^{0.1608}$	$1.89^{0.3134}$	$0.85^{0.6271}$
Didymella	**$10.69^{0.0170}$⇑**	**$7.11^{0.0184}$**	$1.76^{0.3351}$	$1.19^{0.4618}$	$1.55^{0.2458}$
Discula	**$14.19^{0.0093}$⇓**	$0.20^{0.9267}$	**$29.07^{0.0098}$⇓**	**$27.07^{0.0109}$**	$1.91^{0.1534}$
Dissoconium	**$8.61^{0.0261}$⇓**	$1.70^{0.2679}$	$2.26^{0.2650}$	$1.68^{0.3501}$	$0.84^{0.6365}$
Elsinoe	**$7.35^{0.0351}$⇓**	$2.41^{0.1604}$	**$16.99^{0.0212}$⇓⇑⇓**	**$9.18^{0.0495}$**	$2.26^{0.0994}$
Epicoccum	$0.87^{0.7135}$	**$7.43^{0.0166}$**	$0.89^{0.5692}$	$1.52^{0.3800}$	$0.69^{0.7521}$
Erysiphe	$1.74^{0.2347}$	$1.84^{0.2401}$	**$37.65^{0.0067}$⇑**	**$83.89^{0.0021}$**	**$6.00^{0.0036}$**
Exophiala	$5.64^{0.0552}$	**$16.13^{0.0023}$**	$1.65^{0.3542}$	$2.95^{0.2001}$	$0.87^{0.6101}$
Filobasidium	$4.56^{0.0765}$	$3.54^{0.0818}$	$7.82^{0.0613}$	$8.89^{0.0517}$	$1.63^{0.2197}$
Glomerella	$2.89^{0.1399}$	$0.28^{0.8777}$	$0.68^{0.6488}$	$2.20^{0.2718}$	$1.57^{0.2384}$
Gnomonia	**$18.84^{0.0049}$⇓**	**$6.93^{0.0195}$**	$5.94^{0.0876}$	$7.90^{0.0605}$	$2.14^{0.1141}$
Leptosphaeria	$0.81^{0.4041}$	$2.10^{0.1989}$	$0.91^{0.5523}$	$2.18^{0.2735}$	$0.82^{0.6547}$
Leptosphaerulina	$0.02^{0.8864}$	$2.13^{0.1945}$	$2.95^{0.2020}$	$0.87^{0.5692}$	$0.49^{0.9015}$
Leptospora	$3.77^{0.1001}$	$2.95^{0.1146}$	$2.82^{0.2103}$	**$12.05^{0.0342}$**	**$4.11^{0.0149}$**
Mycosphaerella	**$52.52^{0.0004}$⇓**	**$14.22^{0.0032}$**	**$27.83^{0.0105}$⇑**	**$15.91^{0.0232}$**	**$3.64^{0.0227}$**
Oidium	$1.49^{0.2684}$	$0.65^{0.6467}$	$0.84^{0.5815}$	$0.37^{0.8168}$	$0.62^{0.8106}$
Paraconiothyrium	$1.26^{0.3038}$	$1.12^{0.4282}$	$2.25^{0.2656}$	$4.71^{0.1169}$	$1.55^{0.2471}$
Pestalotiopsis	**$11.20^{0.0155}$⇓**	$1.63^{0.2820}$	$5.22^{0.1030}$	$6.43^{0.0791}$	$1.76^{0.1855}$
Phaeosphaeria	$2.04^{0.2031}$	$0.49^{0.7456}$	$0.46^{0.7638}$	$0.52^{0.7343}$	$0.37^{0.9615}$
Phoma	**$8.09^{0.0294}$⇓**	$1.43^{0.3297}$	$2.57^{0.2317}$	**$9.67^{0.0462}$**	$0.94^{0.5632}$
Pyrenochaeta	$2.95^{0.1365}$	$0.87^{0.5347}$	$1.84^{0.3215}$	$0.73^{0.6275}$	$0.85^{0.6214}$
Rhodotorula	$1.38^{0.2852}$	$2.61^{0.1409}$	$1.31^{0.4289}$	$0.27^{0.8784}$	$1.03^{0.4983}$
Scleroconidioma	$5.46^{0.0582}$	$2.80^{0.1252}$	**$654.53^{0.0001}$⇑**	**$618.15^{0.0001}$**	**$11.62^{0.0002}$**
Selenophoma	**$16.52^{0.0066}$⇓**	**$8.39^{0.0124}$**	$4.24^{0.1326}$	$1.24^{0.4473}$	$1.16^{0.4165}$
Sporobolomyces	$3.04^{0.1321}$	$1.49^{0.3156}$	$6.76^{0.0742}$	$0.81^{0.5948}$	$1.24^{0.3733}$
Stagonospora	$2.13^{0.1944}$	$0.50^{0.7413}$	$1.36^{0.4180}$	$1.10^{0.4869}$	$0.64^{0.7950}$
Taphrina	**$34.23^{0.0011}$⇓**	**$20.74^{0.0012}$**	**$64.59^{0.0031}$⇑⇓**	**$51.77^{0.0042}$**	**$4.42^{0.0114}$**
Teratosphaeria	$1.29^{0.2993}$	$0.57^{0.6995}$	**$14.75^{0.0258}$⇑⇓**	$1.43^{0.4010}$	$1.88^{0.1596}$
Tumularia	**$7.59^{0.0331}$⇓**	$3.66^{0.0768}$	**$74.02^{0.0025}$⇓**	**$74.62^{0.0025}$**	$2.80^{0.0532}$
Family					
Amphispaeriaceae	**$7.04^{0.0378}$⇑**	$1.22^{0.3934}$	$1.65^{0.3557}$	**$42.30^{0.0057}$**	**$5.39^{0.0055}$**
Botryosphaeriaceae	$5.74^{0.0536}$	$4.14^{0.0603}$	**$237.41^{0.0004}$⇓⇑⇓**	**$223.13^{0.0005}$**	**$7.58^{0.0014}$**
Davidiellaceae	$0.73^{0.4268}$	**$7.87^{0.0144}$**	**$17.41^{0.0205}$⇓⇑⇓**	$3.57^{0.1619}$	$1.12^{0.4377}$
Dothioraceae	$0.11^{0.7386}$	$0.34^{0.8466}$	$2.77^{0.0663}$	$0.84^{0.5225}$	$0.74^{0.7445}$
Elsinoaceae	**$7.35^{0.0351}$**	$2.41^{0.1604}$	**$16.99^{0.0212}$**	**$9.18^{0.0495}$**	$2.26^{0.0994}$
Erysiphaceae	$1.54^{0.2613}$	$0.83^{0.5497}$	$1.67^{0.3505}$	$0.58^{0.7023}$	$1.38^{0.3086}$
Filobasidiaceae	**$8.86^{0.0247}$⇓**	$3.57^{0.0807}$	**$25.25^{0.0120}$⇑**	**$38.30^{0.0066}$**	$1.30^{0.3449}$
Gnomoniaceae	**$18.52^{0.0051}$⇓**	$0.38^{0.8141}$	**$27.61^{0.0106}$⇓**	**$25.61^{0.0118}$**	$1.81^{0.1737}$
Helotiaceae	**$19.56^{0.0045}$⇓**	$1.55^{0.2993}$	**$68.39^{0.0028}$⇓**	**$41.66^{0.0058}$**	**$4.80^{0.0085}$**
Herpotrichiellaceae	$0.80^{0.4062}$	**$5.60^{0.0317}$**	$0.91^{0.5526}$	$0.93^{0.5448}$	$0.89^{0.5997}$
Leptosphaeriaceae	$0.24^{0.6408}$	**$7.01^{0.0190}$**	$3.66^{0.1575}$	$7.02^{0.0706}$	$1.21^{0.3922}$
Montagnulaceae	$1.25^{0.3069}$	$1.72^{0.2633}$	$1.35^{0.4190}$	$1.57^{0.3709}$	$0.91^{0.5844}$
Mycosphaerellaceae	**$47.04^{0.0005}$⇓**	**$10.54^{0.0070}$**	**$17.11^{0.0210}$⇑**	**$12.72^{0.0317}$**	**$3.22^{0.0343}$**
Phaeosphaeriaceae	$0.79^{0.4070}$	$0.42^{0.7918}$	$1.09^{0.4904}$	$1.10^{0.4881}$	$0.42^{0.9411}$
Phyllachoraceae	$2.89^{0.1399}$	$0.28^{0.8777}$	$0.68^{0.6488}$	$2.20^{0.2718}$	$1.57^{0.2384}$

Table 4 (Continued)

Taxon[a]	Between subjects		Within subjects		
	Use $F_{1,6}{}^{P}$	Site (use) $F_{4,6}{}^{P}$	Time $F_{4,3}{}^{P}$	Time × use $F_{4,3}{}^{P}$	Time × site (use) $F_{16,9.8}{}^{P}$ [b]
Pleosporaceae	$0.11^{0.7462}$	$0.06^{0.9923}$	$2.55^{0.0820}$	$2.79^{0.0646}$	$0.58^{0.8837}$
Taphrinaceae	**$134.71^{0.0001}\Downarrow$**	**$104.34^{0.0001}$**	**$61.67^{0.0033}\Uparrow$**	**$63.82^{0.0031}$**	**$8.81^{0.0008}$**
Valsaceae	$0.99^{0.3588}$	$1.10^{0.4338}$	$2.04^{0.2924}$	$2.51^{0.2382}$	$0.75^{0.7070}$

[a]Only those taxa that occurred in at least 20 samples and were represented by >100 reads across the data set were included.
[b]Wilk's lambda, approximate *F*.
The second sampling (11 June) event was omitted because of two missing samples and only those genera and families that occur in >14 samples (20%) are included. Significant *F*-values are highlighted in bold for emphasis. Upward arrows in the 'use' column indicate greater means within the urban center and those in the 'time' column indicate greater means later in the season.

the point sources, whereas *Hormonema* sp. was unaffected (Helander, 1995). Similarly to those results, our analyses of a broad range of fungi from the oak phyllosphere identified taxa that were less frequent in the urban stands and showed that, overall, the phyllosphere richness was lower in the urban stands. Taken together, the findings indicate that air pollution in urban areas may reduce fungal richness in the phyllosphere as well as affect its community composition.

Third, human management of the urban stands ameliorates various environmental stressors via routine landscape maintenance and reduces the potential propagule loads. Watering, pest control and fertilization in urban environments reduce herbivory, water and nutrient stresses (Shochat *et al.*, 2004). The lower nutrient stress in urban stands may therefore result in greater tree resistance against phyllospheric fungi which include a number of plant pathogens. Our environmental data indicate that, while water availability (inferred from $\delta^{13}C$) did not differ between the urban and nonurban trees, several macronutrient concentrations were higher (N, K and S) in the urban stands and they were probably acquired from different sources (inferred from $\delta^{15}N$). It is notable that taxa that differed in their abundances between the urban and nonurban stands included a number of foliar pathogens. Particularly, *Taphrina* – a genus of common leaf curl and witches broom fungi – was near-absent in urban stands (Fig. 6).

Finally, our analyses of *Mycosphaerella* – a genus of common leaf spot fungi – showed a significant interaction between the sampling time and land-use (Table 4) resulting from slower increase in the occurrence of the genus in urban stands compared with the nonurban stands (Fig. 6). This suggests that the propagule loads were lower and/or the propagule arrival was delayed in the urban stands. In addition to the direct and indirect chemical manipulation of the urban stands, other management decisions, such as removal of plant debris from the urban stands, probably shift phyllosphere richness and community composition as a result of reduced propagule availability of the fungi that depend on litter and detrital plant tissues (Sutton, 1992; Herre *et al.*, 2007). These patterns may also be related to the isolation of urban stands. *Mycosphaerella* typically overwinters on fallen leaves and initiates colonization in the spring via ascospores (Suto, 1999). In the absence of resident leaf litter, such fungi may depend more on long-distance dispersal from nonmanaged stands.

Both foliar chemistry and the phyllosphere fungal communities showed seasonal dynamics. The seasonal trends in the foliar chemistry are probably a combination of differing solubilities or availabilities of various elements as a result of temperature and moisture as well as maturation and turnover of foliage during the growing season (e.g. Brekken & Steinnes, 2004; Deram *et al.*, 2006). Although few studies have examined phyllosphere colonization in deciduous trees (Wilson, 2000), differing seasonal patterns among phyllospheric fungal taxa have been described before (e.g. Kaneko & Kaneko, 2004; Osono, 2008). Although foliar chemistry varied across the growing season, we do not consider this a major driver for the observed fungal community dynamics: it is rather unlikely that small shifts in substrate or foliar chemistry would lead to strong seasonal trends such as those that we used to exemplify seasonal declines (*Davidiella* and *Discula*) and inclines (*Capnobotryella* and *Mycosphaerella*) in the frequencies. We speculate that these seasonal shifts are mainly driven by primary and secondary propagule dispersal and/or environmental conditions that favor abundant propagule production or dispersal (see Kaneko & Kaneko, 2004). Many of our observations are consistent with those from studies relying on pure culturing of fungi from foliar tissues. For example, *Discula* – a genus that includes potential causal agents of anthracnose in deciduous trees – declined in abundance through the growing season (Wilson & Carroll, 1994; Sahashi *et al.*, 1999) and *Mycosphaerella* – a genus that includes potential asymptomatic foliar endophytes and leaf spot fungi – increased in occurrence throughout the growing season (Kaneko *et al.*, 2003). The pattern in *Mycosphaerella* probably relates to its overwintering in fallen leaves and primary colonization via ascospores followed by re-dispersal and secondary colonization from established colonies (Suto, 1999). Similarly, the declining pattern of *Discula* is probably associated with its

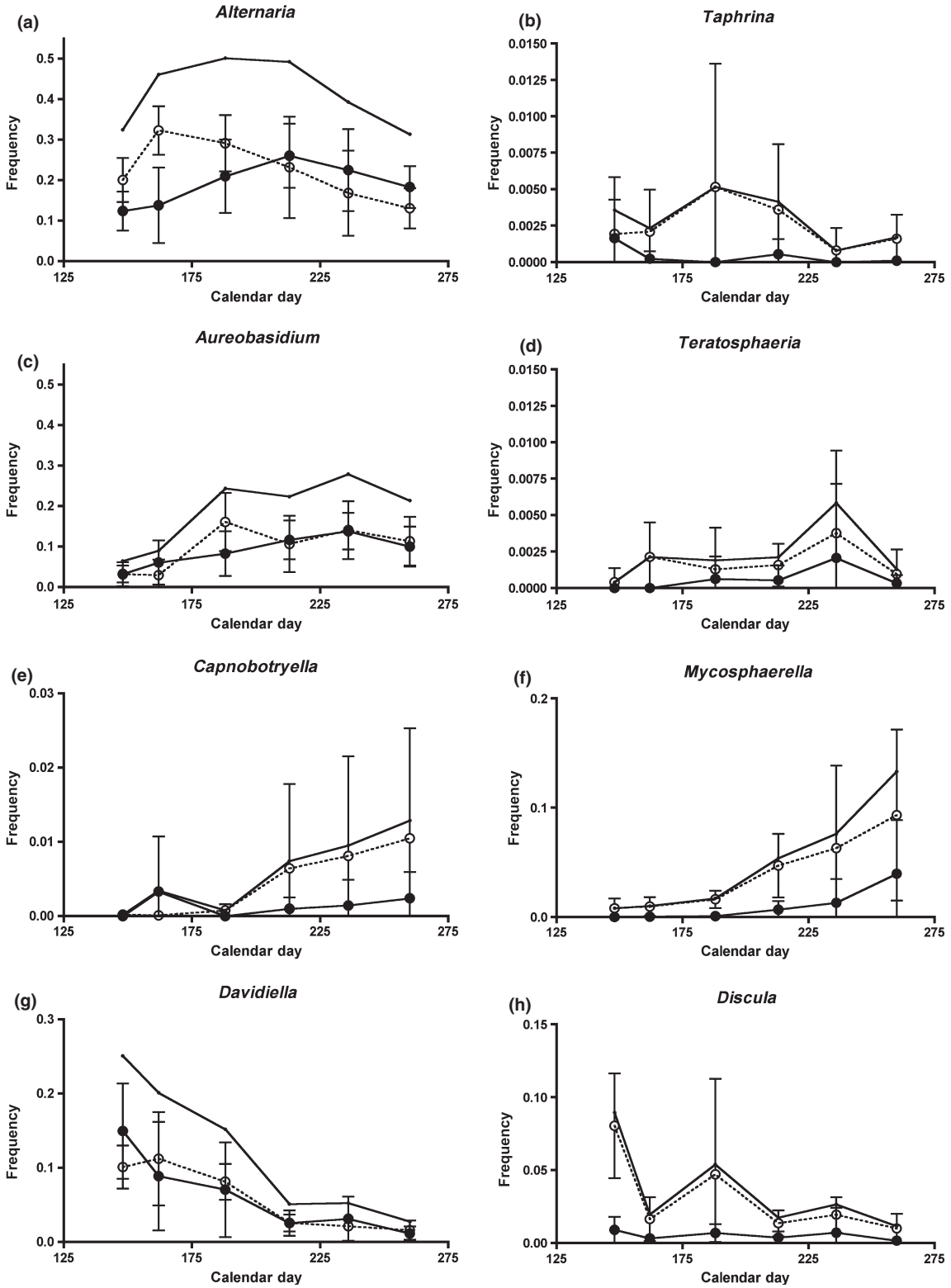

Fig. 6 Examples of genera with identified seasonal trends. (a) *Alternaria* and (b) *Taphrina* with early or mid-season peaks; (c) *Aureobasidium* and (d) *Teratosphaeria* with mid- or late season peaks; (e) *Capnobotryella* and (f) *Mycosphaerella* with increasing frequencies through the growing season; (g) *Davidiella* and (h) *Discula* with declining frequencies through the growing season. Dashed line with large open circles, nonurban; solid line with large closed circles, urban; solid line with closed points, cumulative. Note that *Alternaria*, *Capnobotryella*, *Discula*, *Mycosphaerella*, and *Taphrina* show significant 'time × land-use' interaction terms, indicating differing temporal trends between the urban and nonurban sites (Table 4).

early season preference for younger leaves (Milne & Hudson, 1987) and environmental optima that occur during spring (Neely & Himelick, 1967; Ragazzi *et al.*, 1999).

In this study, we mainly re-sampled stands targeted in our previous research (Jumpponen & Jones, 2009). Although an analysis by Nilsson *et al.* (2009) suggests that OTU assignments using two different regions (ITS1 vs ITS2) may not always be consistent, our results here indicate that sampling across two growing seasons does detect the same common taxa. On an order level, both this and our previous study identified Pleosporales and Capnodiales as the most frequent orders. Similarly, two of the top ranked families (Pleosporaceae and Davidiellaceae, but not Dothioraceae) in this study were among the top five detected previously. Finally, two of the three most common genera (*Alternaria* and *Aureobasidium*, but not *Phoma*) in this study were among the most common genera in the previous study. While the seasonal trends stemming from different timing of the environmental sampling certainly contribute to the observed incongruence between the two discrete data sets, the effect of using two different ITS regions remains uncertain. Notwithstanding, on the higher taxonomic levels, the observed taxa largely overlapped between the data sets.

In conclusion, our 454-sequencing confirmed that urban and adjacent nonurban fungal communities are distinct and vary in their fungal richness and diversity. However, these observations were not consistent but varied across the growing season, emphasizing the importance of understanding the seasonal dynamics in the fungal communities. The community composition was seasonally dynamic and these dynamics were driven by clear shifts in taxon abundances and dominance among the commonly occurring taxa. Encouragingly, the observed seasonal patterns were predictable and supported earlier observations that were based on the fungal life cycles as well as previous, pure-culturing studies. Although the spatial and temporal variability in the phyllosphere communities was obvious, the environmental drivers for these differences beyond the fungal life cycles remain unclear. The broad environmental characterization of the foliar chemistry allowed identification of many correlates for the spatial variability including macro- and micronutrient enrichment as well as heavy metal accumulation in urban environments. However, the causality among these correlates and the observed phyllosphere communities must be established in empirical studies.

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References

Arnold AE. 2007. Understanding the diversity of foliar fungal endophytes: progress, challenges and frontiers. *Fungal Biology Reviews* **21**: 51–66.

Arnold AE. 2008. Endophytic fungi: hidden components of tropical community ecology. In: Schnitzer S, Carson W, eds. *Tropical forest community ecology*. Chichester, UK: John Wiley & Sons, 254–271.

Arnold AE, Henk DA, Eells RL, Lutzoni F, Vilgalys R. 2007. Diversity and phylogenetic affinities of foliar fungal endophytes in loblolly pine inferred by culturing and environmental PCR. *Mycologia* **99**: 185–206.

Arnold AE, Lutzoni F. 2007. Diversity and host range of foliar fungal endophytes: are tropical leaves biodiversity hotspots? *Ecology* **88**: 541–549.

Arnold AE, Maynard Z, Gilbert GS, Coley PD, Kursar TA. 2000. Are tropical endophytes hyperdiverse? *Ecology Letters* **3**: 267–274.

Ash C, Jasny BR, Roberts L, Stone R, Sugden A. 2008. Reimagining cities – introduction. *Science* **319**: 739.

Blair RB. 1999. Birds and butterflies along an urban gradient: surrogate taxa for assessing biodiversity? *Ecological Applications* **9**: 164–170.

Blair RB. 2004. The effects of urban sprawl on birds at multiple levels of biological organization. *Ecology and Society* **9**: 2.

Brekken A, Steinnes E. 2004. Seasonal concentrations of cadmium and zinc in native pasture plants: consequences for grazing animals. *Science of the Total Environment* **326**: 181–195.

Carroll GC, Muller EM, Sutton BC. 1977. Preliminary studies on the incidence of needle endophytes in some European conifers. *Sydowia* **29**: 87–103.

Cincotta RP, Engelman R. 2000. Biodiversity and population growth. *Issues in Science and Technology* **16**: 80–81.

Colwell RK. 2006. *EstimateS: statistical estimation of species richness and shared species from samples. Version 8*. http://purl/oclc.org/estimates

Deram A, Denayer F-O, Petit D, van Haluwyn C. 2006. Seasonal variations of cadmium and zinc in *Arrhenathrerum elatius*, a perennial grass species from highly contaminated soils. *Environmental Pollution* **140**: 62–70.

Fisher RA, Corbet AS, Williams CB. 1943. The relation between the number of species and the number of individuals in a random sample of an animal population. *Journal of Animal Ecology* **12**: 42–58.

Gardes M, Bruns TD. 1993. ITS primers with enhanced specificity for basidiomycetes – application to the identification of mycorrhizae and rusts. *Molecular Ecology* **2**: 113–118.

Hamady M, Walker JJ, Harris JK, Gold NJ, Knight R. 2008. Error-correcting barcoded primers for pyrosequencing hundreds of samples in multiplex. *Nature Methods* **5**: 235–237.

Hata K, Futai K, Tsuda M. 1998. Seasonal and needle age-dependent changes of the endophytic mycobiota in *Pinus thunbergii* and *Pinus densiflora* needles. *Canadian Journal of Botany* **76**: 245–250.

Helander ML. 1995. Responses of pine needle endophytes to air pollution. *New Phytologist* **131**: 223–229.

Helander M, Ahlholm J, Sieber TN, Hinneri S, Saikkonen K. 2007. Fragmented environment affects birch leaf endophytes. *New Phytologist* **175**: 547–553.

Herre EA, Mejia LC, Kyllo DA, Rojas E, Maynard Z, Butler A, van Bael SA. 2007. Ecological implications of anti-pathogen effects of tropical fungal endophytes and mycorrhizae. *Ecology* **88**: 550–558.

Hibbett DS, Ohman A, Kirk PM. 2009. Fungal ecology catches fire. *New Phytologist* **184**: 279–282.

Hope D, Gries C, Casagrande D, Redman CL, Grimm NB, Martin C. 2006. Drivers of spatial variation in plant diversity across the central Arizona - Phoenix ecosystem. *Society & Natural Resources* **19**: 101–116.

Huang XQ, Madan A. 1999. CAP3: a DNA sequence assembly program. *Genome Research* **9**: 868–877.

Johnson JA, Whitney NJ. 1992. Isolation of endophytes from black spruce (*Picea mariana*) dormant buds and needles from New Brunswick, Canada. *Canadian Journal of Botany* **70**: 1754–1757.

Jumpponen A, Jones KL. 2009. Massively parallel 454-sequencing indicates hyperdiverse fungal communities in temperate *Quercus macrocarpa* phyllosphere. *New Phytologist* **184**: 438–448.

Kaneko R, Kakishima M, Tokumasu S. 2003. The seasonal occurrence of endophytic fungus, *Mycosphearella buna*, in Japanese beech, *Fagus crenata*. *Mycoscience* **44**: 277–281.

Kaneko R, Kaneko S. 2004. The effect of bagging branches on levels of endophytic fungal infection in Japanese beech leaves. *Forest Pathology* **34**: 65–78.

Kareiva P, Watts S, McDonald R, Boucher T. 2007. Domesticated nature: shaping landscapes and ecosystems for human welfare. *Science* **316**: 1866–1869.

Kaye JP, Groffman PM, Grimm NB, Baker LA, Pouyat RV. 2006. A distinct urban biogeochemistry? *Trends in Ecology & Evolution* **21**: 192–199.

Kühn I, Brandl R, Klotz S. 2004. The flora of German cities is naturally species rich. *Evolutionary Ecology Research* **6**: 749–764.

Lambais MR, Crowley DE, Cury JC, Bull RC, Rodriques RR. 2006. Bacterial diversity in tree canopies of the Atlantic forest. *Science* **312**: 1917.

Margulies M, Egholm M, Altman WE, Attiya S, Bader JS, Bemben LA, Berka J, Braverman MS, Chen YJ, Chen ZT *et al.* 2005. Genome sequencing in microfabricated high-density picolitre reactors. *Nature* **437**: 376–380.

Mather PM. 1976. *Computational methods of multivariate analysis in physical geography*. London, UK: J Wiley & Sons, 532.

McCune B, Mefford MJ. 1999. *PC-ORD multivariate analysis of ecological data, version 4*. Glendale Beach, OR, USA: MjM Software.

McKinney ML. 2002. Urbanization, biodiversity, and conservation. *BioScience* **52**: 883–890.

McKinney ML, Lockwood JL. 1999. Biotic homogenization: a few winners replacing many losers in the next mass extinction. *Trends in Ecology & Evolution* **14**: 450–453.

Meyer M, Stenzel U, Hofreiter M. 2008. Parallel tagged sequencing on the 454 platform. *Nature Protocols* **3**: 267–278.

Milne KL, Hudson HJ. 1987. Artificial infection of leaves of the London Plane by ascospores and conidia of *Apiognomonia veneta*. *Transactions of the British Mycological Society* **88**: 399–401.

Neely DE, Himelick EB. 1967. Characteristics and nomenclature of the oak anthracnose fungus. *Phytopathology* **57**: 1230–1236.

Nilsson RH, Ryberg M, Abarenkov K, Sjökvist E, Kristiansson E. 2009. The ITS region as a target for characterization of fungal communities using emerging sequencing technologies. *FEMS Microbiology Letters* **296**: 97–101.

Nilsson RH, Ryberg M, Kristiansson E, Abarenkov K, Larsson K-H, Köljalg U. 2006. Taxonomic reliability of DNA sequences in public databases: a fungal perspective. *PLoS ONE* **1**: e59.

Osono T. 2008. Endophytic and epiphytic phyllosphere fungi of *Camellia japonica*: seasonal and leaf age-dependent variations. *Mycologia* **100**: 387–391.

Osono T, Mori A. 2005. Seasonal and leaf age-dependent changes in occurrence of phyllosphere fungi of giant dogwood. *Mycoscience* **46**: 273–279.

Paul MJ, Meyer JL. 2001. Streams in the urban landscape. *Annual Review of Ecology and Systematics* **32**: 333–365.

Pouyat RV, McDonnell MJ. 1991. Heavy metal accumulations in forest soils along an urban-rural gradient in southeastern New York, USA. *Water Air and Soil Pollution* **57–58**: 797–807.

Quince C, Lanzen A, Curtis TP, Davenport RJ, Hall N, Head IM, Read LF, Sloan WT. 2009. Accurate determination of microbial diversity from 454 pyrosequencing data. *Nature Methods* **6**: 639.

Ragazzi A, Moricca S, Capretti P, Dellavalle I. 1999. Infection ability of ascospores and conidia of *Apiognomonia quercina* on oaks. *Journal of Plant Diseases* **106**: 490–494.

Reeder J, Knight R. 2009. The 'rare biosphere': a reality check. *Nature Methods* **6**: 636.

Rodrigues RJ, White JF, Arnold AE, Redman RS. 2009. Fungal endophytes: diversity and functional roles. *New Phytologist* **182**: 314–330.

Roesch LF, Fulthorpe RR, Riva A, Gasella G, Hadwin AKM, Kent AD, Daroub SH, Camargo FAO, Farmerie WG, Triplett EW. 2007. Pyrosequencing enumerates and contrasts soil microbial diversity. *ISME Journal* **1**: 283–290.

Roetzer T, Wittenzeller M, Haeckel H, Nekovar J. 2000. Phenology in central Europe – differences and trends of spring phenophases in urban and rural areas. *International Journal of Biometeorology* **44**: 60–66.

Sahashi N, Kubono T, Miyasawa Y, Ito S. 1999. Temporal variations in isolation frequency of endophytic fungi of Japanese beech. *Canadian Journal of Botany* **77**: 197–202.

Saikkonen K, Faeth SH, Helander M, Sullivan TJ. 1998. Fungal endophytes: a continuum of interactions with host plants. *Annual Review of Ecology and Systematics* **29**: 319–343.

Santamaria J, Bayman P. 2005. Fungal epiphytes and endophytes of coffee leaves (*Coffea arabica*). *Microbial Ecology* **50**: 1–8.

Shochat E, Lerman SB, Katti M, Lewis DB. 2004. Linking optimal foraging behavior to bird community structure in an urban-desert landscape: field experiments with artificial food patches. *American Naturalist* **164**: 232–243.

Sogin ML, Morrison HG, Huber JA, Welch DM, Huse SM, Neal PR, Arrieta JM, Hemdl GJ. 2006. Microbial diversity in the deep sea and the underexplored "rare biosphere". *Proceedings of the National Academy of Science, USA* **103**: 12115–12120.

Stone JK. 1987. Initiation and development of latent infections by *Rhabdocline parkeri* on Douglas-fir. *Canadian Journal of Botany* **57**: 2800–2811.

Suto Y. 1999. *Mycospaerella chaenomelis* sp. nov.: the teleomorph of *Cercosporella* sp., the causal fungus of frosty mildew in *Chaenomeles sinensis*, and its role as the primary infection source. *Mycoscience* **40**: 509–516.

Sutton JC. 1992. Epidemiology of wheat head blight and maize ear rot caused by *Fusarium graminearum*. *Canadian Journal of Plant Pathology* **4**: 195–209.

Wania A, Kuhn I, Klotz S. 2006. Plant richness patterns in agricultural and urban landscapes in central Germany - spatial gradients of species richness. *Landscape and Urban Planning* **75**: 97–110.

White MA, Nemani RR, Thornton PE, Running SW. 2002. Satellite evidence of phenological differences between urbanized and rural areas of the eastern United States deciduous broadleaf forest. *Ecosystems* **5**: 260–273.

Wilson D. 2000. Ecology of woody plant endophytes. In: Bacon CW, White JF Jr, eds. *Microbial endophytes*. New York, NY, USA: Marcel Dekker Inc, 389–420.

Wilson D, Carroll GC. 1994. Infection studies of *Discula quercina*, an endophyte of *Quercus garyana*. *Mycologia* **86**: 635–647.

Zhang XY, Friedl MA, Schaaf CB, Strahler AH, Schneider A. 2004. The footprint of urban climates on vegetation phenology. *Geophysical Research Letters* **31**: L12209.

Zhang Z, Schwartz S, Wagner L, Miller W. 2000. A greedy algorithm for aligning DNA sequences. *Journal of Computational Biology* **7**: 203–214.

Supporting Information

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

Fig. S1 Number of operational taxonomic units (OTUs) as a function of sequence identity.

Table S1 Operational taxonomic unit (OTU) assignments based on BLAST

Table S2 Repeated measures ANOVA tables for foliar chemistry of the 12 trees sampled repeatedly within and outside a small urban center

Table S3 Inductively coupled plasma mass spectrometry (ICP-MS) and mass spectrometry (MS) foliar chemistry correlations with the nonmetric multidimensional scaling (NMS) ordination axes in Fig. 1

Table S4 Mass spectrometry (MS) and inductively coupled plasma mass spectrometry (ICP-MS) results for foliar samples from 11 June, 1 August and 17 September representing trees growing within and outside of a small urban center (Manhattan, KS, USA)

Table S5 Sequence, operational taxonomic unit (OTU), singleton and doubleton information, and richness and diversity estimators for phyllosphere fungal communities in nonurban and urban environments

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