

ORIGINAL ARTICLE

Particle-size distributions and seasonal diversity of allergenic and pathogenic fungi in outdoor air

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Fungi are ubiquitous in outdoor air, and their concentration, aerodynamic diameters and taxonomic composition have potentially important implications for human health. Although exposure to fungal allergens is considered a strong risk factor for asthma prevalence and severity, limitations in tracking fungal diversity in air have thus far prevented a clear understanding of their human pathogenic properties. This study used a cascade impactor for sampling, and quantitative real-time PCR plus 454 pyrosequencing for analysis to investigate seasonal, size-resolved fungal communities in outdoor air in an urban setting in the northeastern United States. From the 20 libraries produced with an average of ~800 internal transcribed spacer (ITS) sequences (total 15 326 reads), 12 864 and 11 280 sequences were determined to the genus and species levels, respectively, and 558 different genera and 1172 different species were identified, including allergens and infectious pathogens. These analyses revealed strong relationships between fungal aerodynamic diameters and features of taxonomic compositions. The relative abundance of airborne allergenic fungi ranged from 2.8% to 10.7% of total airborne fungal taxa, peaked in the fall, and increased with increasing aerodynamic diameter. Fungi that can cause invasive fungal infections peaked in the spring, comprised 0.1–1.6% of fungal taxa and typically increased in relative abundance with decreasing aerodynamic diameter. Atmospheric fungal ecology is a strong function of aerodynamic diameter, whereby through physical processes, the size influences the diversity of airborne fungi that deposit in human airways and the efficiencies with which specific groups of fungi partition from outdoor air to indoor environments.

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Introduction

An estimated 28–50 Tg of fungal materials are emitted into the earth's atmosphere each year, and in some regions, fungal particles may constitute >20% of the primary emissions of organic aerosol (Elbert *et al.*, 2007; Heald and Spracklen, 2009). Airborne fungal material is an important contributor to human diseases. In industrialized countries, an estimated 20–30% of the population is affected by allergic diseases, and at least 3–10% of the general population are sensitized to fungal allergens

(Crameri *et al.*, 2006). Exposure to fungal allergens is considered to be a strong risk factor for asthma symptom prevalence and severity, and there is robust evidence to support a relationship between elevated environmental fungal spore counts and asthma attacks (Crameri *et al.*, 2006; Denning *et al.*, 2006). Although less common than allergic disease, invasive fungal infections occur in both immunocompetent and immunocompromised hosts (Pitman *et al.*, 2011). Invasive fungal infections from *Candidia*, *Aspergillus* and *Cryptococcus* spp. are associated with high mortality in immunocompromised individuals (Lin *et al.*, 2001).

Aerodynamic diameter and species diversity are master variables that determine personal exposures and subsequent human health impacts. For a particle of arbitrary shape, size and density, its aerodynamic diameter is uniquely defined as the diameter of a sphere of unit density (that is, 1 g cm^{-3}) that experiences the same gravitational settling velocity. The size of fungal material controls

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the rate and extent of physical processes that are important for human exposure. These include settling and deposition onto environmental surfaces, deposition in the human respiratory tract, penetration into buildings, resuspension into air, long-range transport and the efficiency of engineered removal systems (Thatcher and Layton, 1995; Yeh *et al.*, 1996; Riley *et al.*, 2002; Nazaroff, 2004; Prospero *et al.*, 2005; Ariya *et al.*, 2009). Large hyphal fragments and multicellular spores that are $>10\ \mu\text{m}$ in size, unicellular spores with diameters of $1\text{--}10\ \mu\text{m}$ and fragments $<1\ \mu\text{m}$ combine to permit a broad size distribution of airborne fungal material. The size distribution can also be influenced by the attachment of fungal particles to other particulate matter. Overall, the inherent diversity in fungal particle size, coupled with the strong influence of aerodynamic diameter on the fate, transport and source strength of fungal particles, indicate that size is an important determinant of how taxonomic compositions of airborne fungi are assembled in the atmosphere and in indoor air.

The study reported here investigates the size-resolved, seasonal diversity of fungi in the atmosphere at one location in the north eastern United States. We use barcoded DNA pyrosequencing technology applied to a fungal genetic marker (Amend *et al.*, 2010) to provide in-depth descriptions of seasonal aerosol fungal diversity collected for eight different size ranges between $0.4\ \mu\text{m}$ to $>9\ \mu\text{m}$ in aerodynamic diameter. Genus-level and species analyses were performed to document the size-distributed compositions and abundances of allergenic and pathogenic fungi whereas selected allergenic and pathogenic fungal species were enumerated by quantitative PCR (qPCR) to characterize their absolute concentrations and particle-size distributions. Coupling size-resolved aerosol sampling with next generation DNA sequencing approaches allows for an unprecedented description of atmospheric fungal ecology. The results provide important insights into how fungal aerodynamic diameter affects taxonomic composition, and improve our understanding of human inhalation exposure to atmospheric fungal allergens and pathogens.

Materials and methods

Air sampling

Air sampling was conducted for four different seasons in 2009–2010 on the rooftop of a five-story building in New Haven, CT, USA ($41^{\circ}18'29''\text{N}$ $72^{\circ}55'43''\text{W}$). Sampling for the four seasons occurred on the following days: spring 13 May to 10 June 2009; summer 11 August to 8 September 2009; fall 15 October to 12 November 2009; and winter 14 January to 11 February 2010. Two eight-stage non-viable Andersen samplers (aerodynamic diameter (d_a) = $0.4\text{--}0.7$, $0.7\text{--}1.1$, $1.1\text{--}2.1$, $2.1\text{--}3.3$,

$3.3\text{--}4.7$, $4.7\text{--}5.8$, $5.8\text{--}9.0$ and $>9.0\ \mu\text{m}$; New Star Environmental, Roswell, GA, USA) were deployed to collect airborne particles on glass fiber substrates and operated continuously for the ca. 4-week durations specified above. The cutoff diameters described here are the particle diameters having 50% collection efficiency. Aerosol samples were collected at an air flow rate of $28.3\ \text{l min}^{-1}$, which were calibrated twice weekly. After collection, the filters were stored at $-20\ ^{\circ}\text{C}$ until subsequent analyses. Outdoor temperature, wind speed and precipitation during the sampling periods are presented in Supplementary Table 1.

DNA extraction

One-eighth section of the loaded glass fiber filter was used for DNA extraction. Extraction was performed using the PowerMax Soil DNA Isolation Kit (Mbio Laboratory, Carlsbad, CA, USA) with the following modifications. The filter section was enclosed in a 2-ml microcentrifuge tube along with the kit's power beads (1.0 g), kit's extraction solution ($750\ \mu\text{l}$), $0.1\ \text{mm}$ diameter glass beads (300 mg) and $0.5\ \text{mm}$ diameter glass beads (100 mg). The samples were then homogenized for 5 min by a bead beater (Model 607; BioSpec Products, Bartlesville, OK, USA). After bead beating, the extracted nucleic acids were purified in accordance with the kit protocol and purified DNA was eluted with $50\ \mu\text{l}$ of TE buffer ($10\ \text{mM}$ Tris-HCl, $1\ \text{mM}$ EDTA, $\text{pH} = 8.0$).

Pyrosequencing of the internal transcribed spacer (ITS) region of rDNA

Sequencing was performed on 20 impactor samples, comprising the four seasons, each with five particle-size stages ($d_a > 2.1\ \mu\text{m}$). Samples with $d_a < 2.1\ \mu\text{m}$ were not included for the analyses owing to no or weak PCR amplification. Universal fungal primers ITS1F and ITS4 (Larena *et al.*, 1999; Manter and Vivanco, 2007) along with adaptor, key and multiplex identifier sequences were used to amplify the ITS region of fungal rDNA (Supplementary Table 2). The $50\ \mu\text{l}$ reaction mixture contained the template DNA ($2\ \mu\text{l}$ sample extract), $1\times$ PCR Master Mix (Roche Applied Science, Indianapolis, IN, USA) and $0.3\ \mu\text{M}$ of each primer. Cycling conditions were: $95\ ^{\circ}\text{C}$ for 5 min of initial denaturation and 35 cycles of $95\ ^{\circ}\text{C}$ for 30 s of dissociation, $55\ ^{\circ}\text{C}$ for 30 s of annealing, $72\ ^{\circ}\text{C}$ for 1 min for each cycle extension and $72\ ^{\circ}\text{C}$ for 10 min of final extension step. PCR amplicons were purified and concentrations normalized with SequalPrep Normalization Plate (96 well) Kit (Invitrogen, Grand Island, NY, USA). The normalized amplicons were pooled and then further purified by agarose gel extraction (QIAquick Gel Extraction Kit; Qiagen, Valencia, CA, USA). Pyrosequencing of amplicons was performed using the 454 GS FLX Titanium Platform (454 Life Sciences) at the Yale University Center for Genome Analysis.

DNA sequence processing and analyses

Sequence FASTA and QUAL files were extracted from the machine output file, trimmed and parsed by sequence tag using the Ribosomal Database Project pyrosequencing pipeline (Cole *et al.*, 2005). Trimming removed primers, sequences with one or more undefined base, sequences below a minimum machine quality score of 20 and sequences with a trimmed read length of fewer than 300 bp. Trimmed sequences were taxonomically placed using BLASTn ver. 2.2.19 (Altschul *et al.*, 1990) and a maximum E-value of 10^{-5} using a database that contained only named fungal ITS sequences (Nilsson *et al.*, 2009). Taxonomic placement was assigned when that species was the sole top hit. In cases of multiple top hits, identity was assigned to the taxonomic level that could be unambiguously identified. Taxonomic placement of fungal genera in their respective phyla, classes and orders was adopted from the Index Fungorum (<http://www.indexfungorum.org/names/names.asp>). Allergenic and human pathogenic (infectious) fungi were identified through literature searches (Makimura, 2001; Simon-Nobbe *et al.*, 2008) and are listed in Supplementary Tables 3 and 4, respectively. Based on the above classifications and assembled lists in Supplementary Tables 3 and 4, the relative abundance allergens and pathogens were determined. The method reproducibility based on relative differences of field replicates was generally <20% for characterizing relative abundances of fungal species with >100 sequences detected per sample. Raw sequencing data have been deposited in the MG-RAST (metagenomics.anl.gov) archive under accession number 4476444.3.

Diversity analyses

UCLUST ver. 1.2.22 (<http://www.drive5.com/uclust>) with default settings was used for cluster analysis. Before diversity analyses, the sequence libraries were split within QIIME 1.3.0 (Caporaso *et al.*, 2010) and denoised (Quince *et al.*, 2009; Reeder and Knight, 2009; Quince *et al.*, 2011) to avoid diversity overestimation caused by sequencing errors. Following denoising, the operational taxonomic units (OTUs) were defined based on 97% similarity (O'Brien *et al.*, 2005; Buée *et al.*, 2009), and the samples were rarified through the QIIME alpha-diversity workflow script. These OTUs were then used as a basis for calculating alpha-diversity metrics, including Chao1 and Shannon indices and rarefaction curves.

Real-time qPCR

Detailed method descriptions for qPCR of fungal genomes in environmental samples have been published (Yamamoto *et al.*, 2011). The TaqMan method was used to quantify specific allergenic fungi based on well described primer and probe

sequences (Haugland and Vesper, 2002). Sequences of primers and probes and the fungal taxa included are listed in Supplementary Tables 2. For the qPCR reaction, 50 μ l reaction mixtures contained 2 μ l of template DNA, 1 \times TaqMan Universal PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA), 1 μ M of each primer, and 0.08 μ M of the probe. Amplification was performed in a real-time PCR system (ABI 7500 Fast Real-time PCR System; Applied Biosystems) at the following cycling conditions for all PCR assays: 50 °C for 2 min, 95 °C for 15 min of initial denaturation, and 45 cycles of 95 °C for 15 s of dissociation and 60 °C for 1 min of annealing and extension. Cycle thresholds were calculated using the auto function in the ABI 7500 software (Applied Biosystems) and all samples were run in triplicate. To test for PCR inhibition, seven randomly selected extracts from air samples collected in this study were added to subsets of diluted *Epilobium nigrum* DNA standard and standard curves were produced. No significant inhibition was observed.

To generate qPCR standards for *Aspergillus fumigatus* (ATCC 34506), *Cladosporium cladosporioides* (ATCC 16022) and *Penicillium chrysogenum* (CAES PC1), the spore samples harvested from pure cultures were enumerated by direct microscopy. For *Alternaria alternata* (PEM 01043) and *Epilobium nigrum* (TU BL-3), tissues of the pure-cultured colonies were used to prepare the DNA standards and the original cell counts were back-calculated based on recovered DNA quantities and genome sizes. The accuracy, precision and method detection limits of this methodology are reported elsewhere (Hospodsky *et al.*, 2010; Yamamoto *et al.*, 2011).

Calculations of geometric means and geometric s.d. of aerodynamic diameters

To quantitatively characterize the representative particle-size distributions, geometric means (d_g) and geometric s.d. (σ_g) of aerodynamic diameters were calculated (Hinds, 1999) in accordance to Equation 1 and by assuming a 20 μ m cutoff for the top impactor stage.

$$d_g = \exp\left(\frac{\sum n_i \ln d_i}{N}\right) \quad (1)$$

where n_i is the measured number of cells or genomes in the i th particle-size interval, d_i is the geometric midpoint of the interval, N is the total number of cells or genomes and the summation is carried out over all i . Geometric s.d. was computed in this manner:

$$\ln \sigma_g = \left(\frac{\sum n_i (\ln d_i - \ln d_g)^2}{N}\right)^{1/2} \quad (2)$$

Geometric s.d. were defined to be 'not determined (n.d.)' in case the highest concentration, in terms of $\Delta N/\Delta \log d_a$, was observed for the top impactor stage.

Results and discussion

Richness and diversity of airborne fungi

Globally, fungi are broadly diverse (Mueller and Schmit, 2007), with an estimated 1.5 million unique species (Hawksworth, 2001). Here we used 454 pyrosequencing to provide the most comprehensive survey of taxonomic composition of atmospheric fungi reported to date. From the 20 samples collected during four seasons, a total of 15 326 ITS sequences were obtained after quality filtering. This value represents > 10 times increase from the largest previous sequence-based survey of atmospheric fungi (Fröhlich-Nowoisky *et al.*, 2009). The trimmed sequence lengths ranged from 300 to 749 bp with a median length of 491 bp. Based on taxonomic classification described in 'Materials and methods', 12 864 and 11 280 ITS sequences were determined to the genus and species levels, respectively. ITS diversity between fungal genera and species is high (much higher than 18 S and 28 S sequence diversity), generally allowing species level taxonomic placement (Nilsson *et al.*, 2008). Overall, 558 different genera and 1172 different species were identified (Supplementary Tables 5 and 6).

Fungal richness and diversity in outdoor air were estimated based on OTU with 97% sequence similarity (O'Brien *et al.*, 2005; Buée *et al.*, 2009). The number of OTUs ranged from 173 to 545 for each sample (Supplementary Table 7). Rarefaction curves of observed OTUs continued to rise with increasing numbers of sequences (Supplementary Figure 1), suggesting that further increases in sample size will yield even more species. Chao1 estimator predicted 746–5157 OTUs depending on the samples (Supplementary Table 7), which were similar to the fungal richness recently described in forest soil using 454 pyrosequencing (Buée *et al.*, 2009). The diversity metrics reported here are higher than those previously determined for outdoor air based on Chao1 in conjunction with a cloning and Sanger sequencing method (Fröhlich-Nowoisky *et al.*, 2009). In general, richness variation by particle size and by season was small. There was no statistically significant difference of fungal richness by particle-size class ($P > 0.05$; Friedman's test using seasons as repeated measures) and season ($P > 0.05$; Friedman's test using particle-size classes as repeated measures) although winter samples showed slightly less richness than did other seasons (Supplementary Figure 1 and Supplementary Table 7).

Taxonomic composition of airborne fungi

Taxonomic compositions of airborne fungi, including allergens and pathogens, were dependent on the aerodynamic diameters. Figure 1a illustrates the proportional distributions of *Ascomycota* and *Basidiomycota* phyla in outdoor air. The relative abundance of *Ascomycota* increases with increasing aerodynamic diameter whereas *Basidiomycota* were

more highly enriched in the smaller particle sizes. This observed tendency was likely because of differences of spore sizes. To examine this hypothesis, we searched for spore sizes of the top three most abundant fungal genera belonging to each phylum. The top three most abundant *Ascomycota* genera and their averaged relative abundances were *Leptosphaerulina* (4.9%), *Epicoccum* (4.2%) and *Cladosporium* (3.5%), whereas the top three most abundant *Basidiomycota* genera were *Peniophora* (8.6%), *Exidia* (6.8%) and *Stereum* (4.8%). The reported *Ascomycota* spore sizes (L × W) are: 21–26 × 9.5–13 µm for *Leptosphaerulina* (Roux, 1986), 15–25 µm for *Epicoccum* (Cole and Samson, 1984) and 3–11 × 2–5 µm for *Cladosporium* (Cole and Samson, 1984). The reported *Basidiomycota* spore sizes are: 3–3.5 × 6.5–8 µm for *Peniophora* (Whelden, 1936), 2.5–3.5 × 15–20 µm (basidiospore) and 1 × 2–4 µm (conidia) for *Exidia* (Ingold, 1995), and 6–7 × 2.5 µm for *Stereum* (Burt, 1920). Thus, we found spore sizes of the *Ascomycota* were consistently larger than those of the *Basidiomycota*, suggesting spore sizes as an important determinant for their aerodynamic diameters.

Consistent with a previous study conducted in Germany (Fröhlich-Nowoisky *et al.*, 2009), *Dothideomycetes* and *Sordariomycetes* were the two most abundant fungal classes in airborne *Ascomycota* (Figure 1b). *Dothideomycetes* includes genera associated with allergenic fungi such as *Alternaria*, *Epicoccum*, *Curvularia* and *Cladosporium*. *A. alternata* is a major allergen associated with the development of asthma in children (Halonen *et al.*, 1997) whereas *Cladosporium* spp. are abundant and common fungi both indoors and outdoors (Shelton *et al.*, 2002) and are associated with respiratory allergy (D'Amato *et al.*, 1997).

Large proportions of *Eurotiomycetes* (32% of total *Ascomycota* sequences) were found in the winter samples (Figure 1b). This class contains various clinically important fungi including *Aspergillus* and *Penicillium*. *A. fumigatus* is a major fungal allergen (Nierman *et al.*, 2005), and also causes invasive infections such as allergic bronchopulmonary aspergillosis (Greenberger, 2002). The *Penicillium* genus includes important allergenic species (Schwab *et al.*, 2004).

Agaricomycetes was the most abundant class found in the *Basidiomycota* sequences (> 95%), in which *Russulales* and *Polyporales* were two of the most abundant orders (Figure 1c). *Agaricomycetes* are mushroom-forming fungi (Hibbett *et al.*, 1997), and generally do not contain described allergenic or pathogenic members, with the exception of some *Agaricales* (e.g., *Schizophyllum commune* (Kern and Uecker, 1986)).

Abundance of allergenic and pathogenic fungi

Figure 2 illustrates relative abundances of known allergens and infectious pathogens as well as the 22

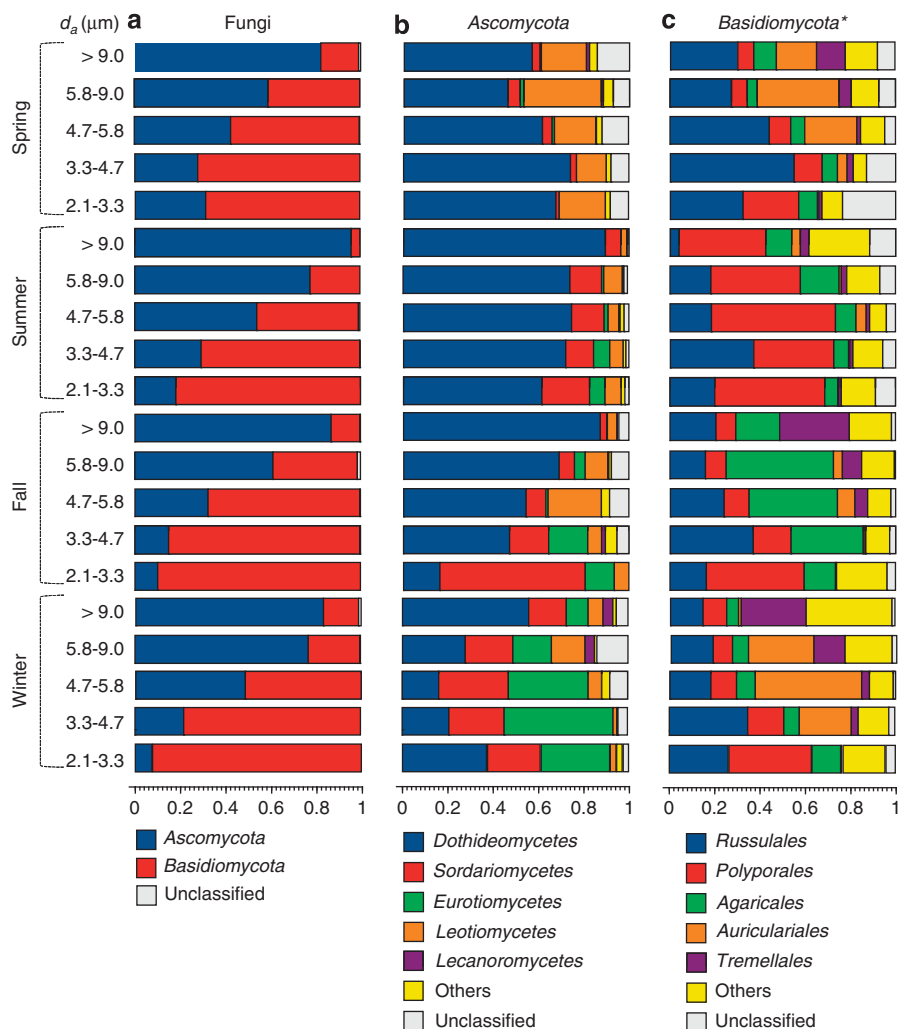


Figure 1 ITS-based proportional distributions of atmospheric fungal phyla (a). Fungal class proportional distribution of the *Ascomycota* phylum (b). Fungal order proportional distribution of the *Basidiomycota* phylum (c). *Greater than 95% of *Basidiomycota* belonged to the *Agaricomycetes* class, thus order-level classification was used to provide additional higher-level taxonomic information.

most abundant fungal genera detected by 454 pyrosequencing. The allergens and pathogens in Figure 2 are based on the lists of currently described allergenic or pathogenic fungal species, which are detailed in Supplementary Tables 3 and 4, respectively (Makimura, 2001; Simon-Nobbe *et al.*, 2008). Several species are both allergens and pathogens, and because not all fungal allergens and pathogens have been explored and documented, some species are not included.

The majority of allergenic and pathogenic fungi were members of the *Ascomycota* phylum (Figure 2). Among them, members of the *Alternaria* and *Epicoccum* genera were the most abundant allergenic fungi, and were found mostly in the larger particle-size ranges ($d_a > 4.7 \mu\text{m}$) (Figure 2). *Cladosporium* was another abundant allergenic fungal genus, distributed evenly across all the particle-size ranges (Figure 2). Although less abundant, *Aspergillus* and *Penicillium* spp. were also detected. Both

Aspergillus and *Penicillium* occurred mostly in the particle-size range of $d_a < 9.0 \mu\text{m}$ (Figure 2).

Medically important members of the *Schizophyllum* and *Cryptococcus* genera were two of the most abundant fungal pathogens found in *Basidiomycota*. *Schizophyllum commune* has been reported as the causative agent of maxillary sinus infection (Kern and Uecker, 1986) and *Cryptococcus neoformans* is a well-studied fungal pathogen (Loftus *et al.*, 2005) that causes life-threatening meningitis in immunocompromised patients (Mitchell and Perfect, 1995). *Schizophyllum* and *Cryptococcus* were concentrated in different particle-size ranges, $d_a < 4.7 \mu\text{m}$ and $d_a > 4.7 \mu\text{m}$, respectively, and *Schizophyllum* was most abundant in the spring (Figure 2).

To date, 150 individual fungal species that contain allergenic proteins in 80 genera have been identified (Simon-Nobbe *et al.*, 2008). In this study, we identified 11 allergenic fungal species in outdoor

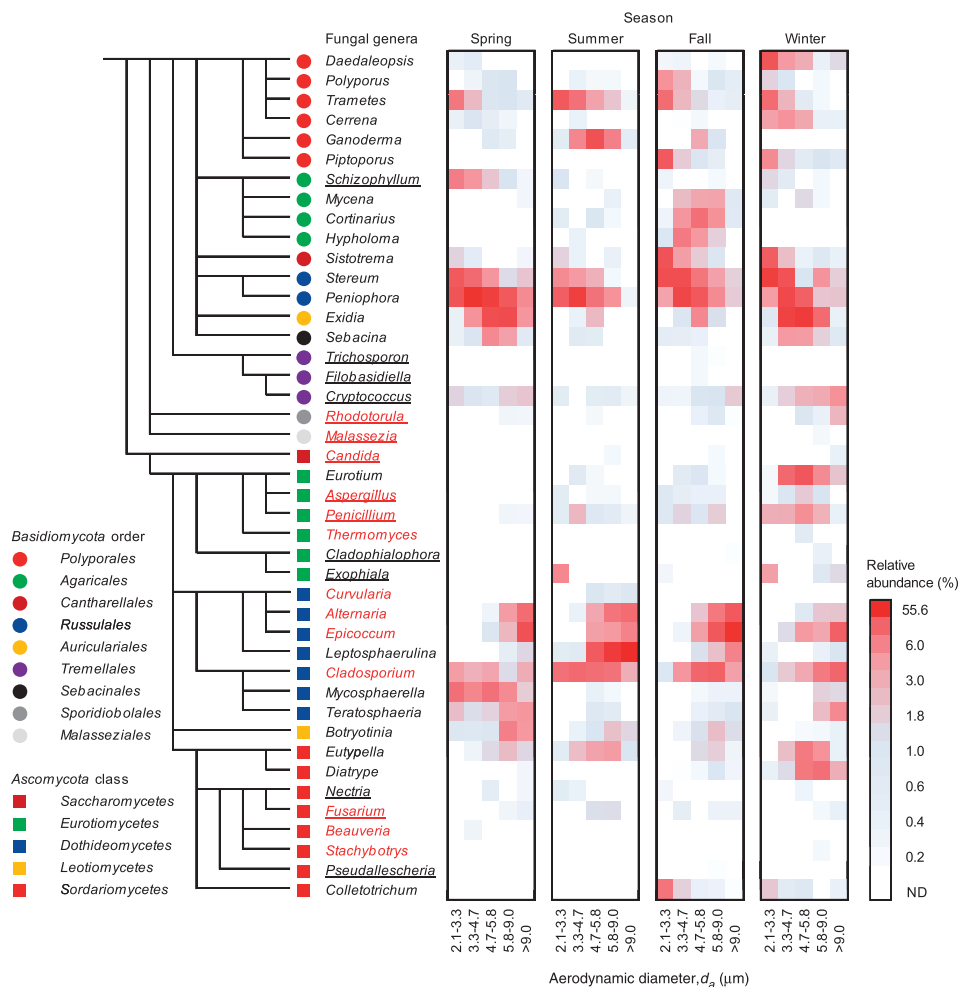


Figure 2 Relative abundances of fungal genera identified in the atmosphere. The most abundant fungal genera, genera containing known allergens (in red) and genera containing infectious pathogenic species (underlined) are listed. A tree was constructed based on taxonomic classifications provided by Index Fungorum. Seven taxonomic levels, that is, phylum, subphylum, class, subclass, order, family and genus, are used to define nodes of the tree. The allergenic and pathogenic genera are defined as genera with allergenic or pathogenic species listed in Supplementary Tables 3 and 4, respectively. Genera shown represent 56% of the outdoor air sequences.

air (Supplementary Figure 2). The top five most abundant allergenic fungi that were identified to the species level and their averaged relative abundances were *Epicoccum nigrum* (4.23%), *Cladosporium cladosporioides* (0.27%), *Alternaria alternata* (0.05%), *Thermomyces lanuginosus* (0.05%) and *Penicillium brevicompactum* (0.03%) (Supplementary Figure 3). Five different pathogenic fungal species were also identified (Supplementary Figure S2) out of sixty-six species of described fungal pathogens in Supplementary Table 4, and the two most abundant were *Schizophyllum commune* (0.46%) and *Aspergillus fumigatus* (0.02%) (Supplementary Figure 3). Overall, allergenic fungi comprised 2.8–10.7% of all taxa in all particle-size ranges. The relative abundance of allergen species increased with increasing aerodynamic diameter (Figure 3). The relative abundance of pathogenic fungi ranged from 0.1% to 1.6% of the sequences, and increased with decreasing aerodynamic

diameter. Over all particle sizes and seasons, the averaged proportions of allergenic and pathogenic fungal species to total number of ITS sequences were 4.7% and 0.5%, respectively (Figure 3).

Absolute concentrations of particle size-resolved allergenic and pathogenic fungi

To circumvent sequencing's inability to identify absolute quantities of atmospheric fungi, selected species and groups from the genera *Alternaria*, *Aspergillus*, *Cladosporium*, *Epicoccum* and *Penicillium* were chosen for qPCR. These groups were commonly detected by pyrosequencing (Figure 2, Supplementary Figures 2 and 3) and are clinically important allergens and pathogens. Table 1 and Supplementary Table 2 summarize the selectivity of each qPCR assay.

Figure 4 displays seasonal concentrations and particle-size distributions of these selected

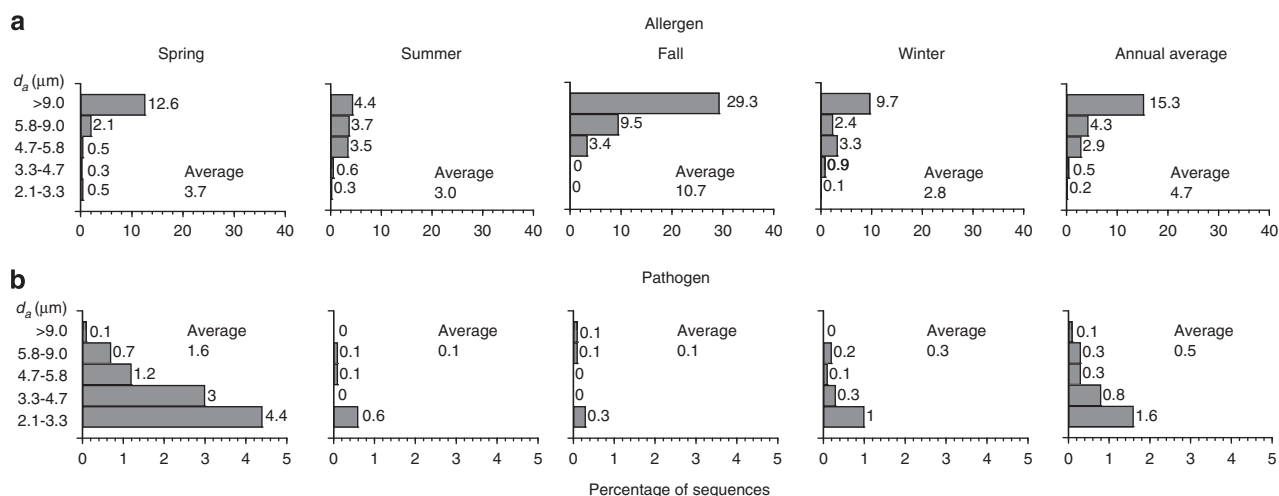


Figure 3 Relative abundances of total allergenic (a) and pathogenic (b) fungal species. The allergenic and pathogenic genera are defined as genera with allergenic or pathogenic species listed in Supplementary Tables 3 and 4, respectively. Average values indicate means of the relative abundances of all particle sizes.

Table 1 Comparison between aerodynamic diameters of airborne fungi and microscopy-based sizes of fungal spores

Assay name	Aerodynamic diameter This study		Species ^a	Aerodynamic diameter Previous pure culture studies		Microscopy-based size			
	d_g (μm) ^b	σ_g ^c		d_g (μm) ^b	σ_g ^c	Method	Reference	$L \times W$ (μm)	Reference
Afumi	4.3	1.6	<i>Aspergillus fumigatus</i>	2.14–2.16	1.11–1.13	Aerodynamic Particle Sizer	Reponen <i>et al.</i> (1996)	2–3.5	Cole and Samson (1984)
			<i>Neosartorya fischeri</i>	n.a.	n.a.	n.a.	n.a.	7–8	Tanaka <i>et al.</i> (2011)
PenGrp3	4.2	1.4	<i>Penicillium chrysogenum</i>	2.6–3.0	n.a.	Aerodynamic Particle Sizer	Madelin and Johnson (1992)	2.5–2.5	Madelin and Johnson (1992)
			<i>P. griseofulvum</i>	n.a.	n.a.	n.a.	n.a.	2.6	Fletcher (1969)
			<i>P. glandicola</i>	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
			<i>P. coprophilum</i>	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
			<i>P. expansum</i>	n.a.	n.a.	n.a.	n.a.	n.a.	3–3.5 × 2.5–3
PenAsp1mgb	4.2	1.4	<i>Eupenicillium</i> spp.	n.a.	n.a.	n.a.	n.a.	2–5	Tanaka <i>et al.</i> (2011)
			Universal	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Cclad2	5.2	1.6	<i>Aspergillus</i> and <i>Penicillium</i> spp.	2.5–2.7	n.a.	Aerodynamic Particle Sizer	Madelin and Johnson (1992)	1.3 × 2.9	Madelin and Johnson (1992)
			<i>Cladosporium cladosporioides</i>	1.75–1.87	1.14–1.17	Aerodynamic Particle Sizer	Reponen <i>et al.</i> (1996)	3–11 × 2–5	Cole and Samson (1984)
Aaltr	>10.4	n.d. ^d	<i>Alternaria alternata</i>	19	n.a.	Impaction and microscopy	McCartney <i>et al.</i> (1993)	18–83 × 7–18	Cole and Samson (1984)
Enigr	>10.5	n.d. ^d	<i>Epicoccum nigrum</i>	n.a.	n.a.	n.a.	n.a.	15–25	Cole and Samson (1984)

Abbreviation: n.a., not available in the literatures.

^aSpecies detectable by qPCR assay.

^bGeometric mean aerodynamic diameter.

^cGeometric s.d. aerodynamic diameter.

^dNot determined owing to the skewed particle-size distributions.

allergenic and pathogenic fungi. Geometric means (d_g) and geometric s.d. (σ_g) of aerodynamic diameters were calculated according to Equations 1 and 2, respectively. Afumi, Cclad2, PenGrp3 and PenAsp1mgb showed aerodynamic diameter peaks of $d_g = 3\text{--}6\ \mu\text{m}$ whereas Aaltr and Enigr were significantly larger, with $d_g > 10\ \mu\text{m}$ (Figure 4). The observed tendencies were consistent with results characterized by 454 pyrosequencing (Figure 2)

where *Aspergillus*, *Cladosporium* and *Penicillium* were enriched in the $< 9\ \mu\text{m}$, and *Alternaria* and *Epicoccum* were enriched in the $> 9\ \mu\text{m}$ stage. *Aspergillus* and *Penicillium* produce unicellular asexual spores $< 5\ \mu\text{m}$, whereas *Alternaria* and *Epicoccum* produce multicellular dictyospores $> 10\ \mu\text{m}$ (Cole and Samson, 1984). The aerodynamic diameters of the unicellular fungi characterized in the atmosphere by qPCR were larger than those determined

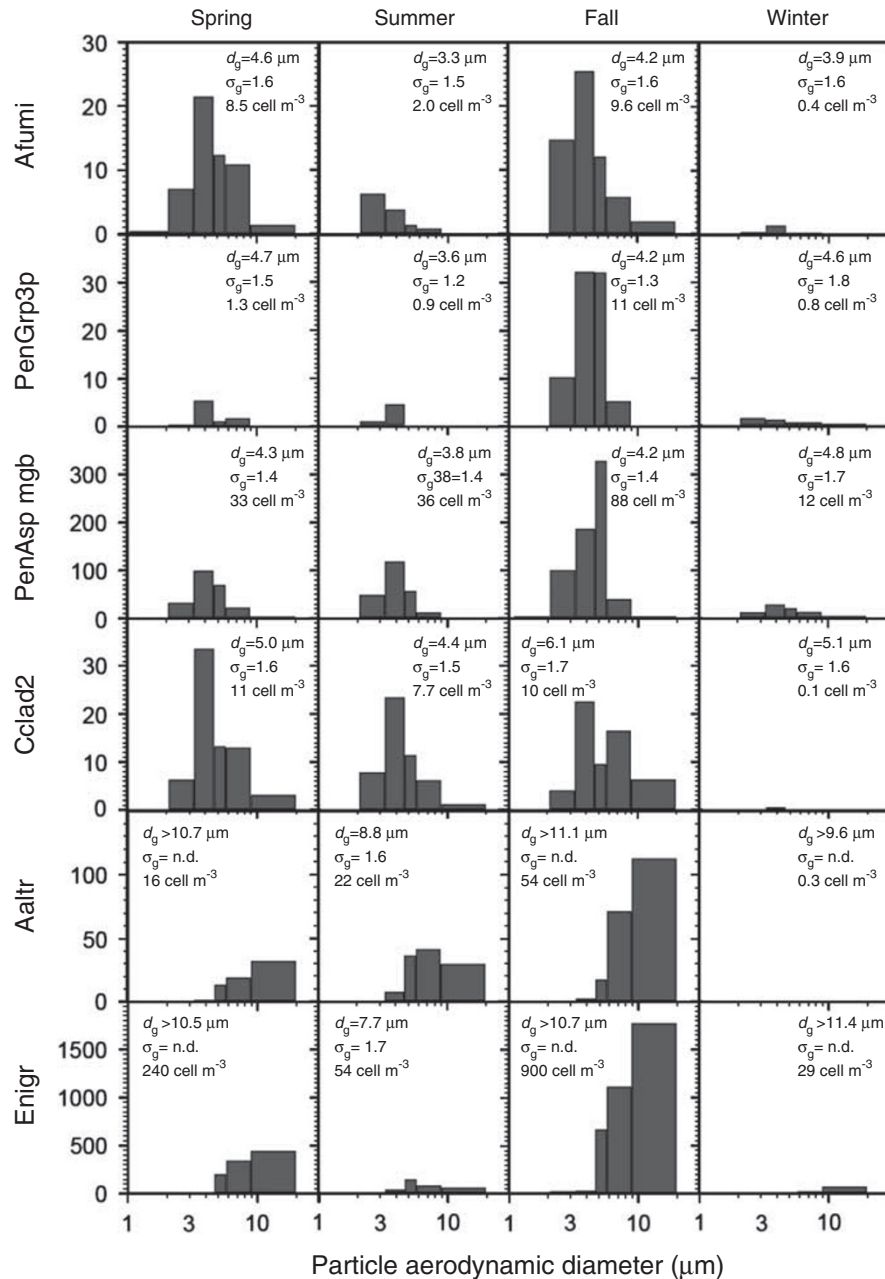


Figure 4 The seasonal particle-size distributions of airborne fungi determined by qPCR (y axis, $\Delta N/\Delta \log d_a$ (cell m^{-3})). An upper limit of particle size for the stage of $> 9.0 \mu\text{m}$ is set at $20 \mu\text{m}$ to calculate geometric means of aerodynamic diameter (d_g) and geometric s.d. (σ_g) of particle-size distributions of airborne fungi. The abbreviation n.d. means 'not determined' owing to the skewed particle-size distributions.

previously by aerosolized pure culture fungi and also larger than microscopically determined fungal dimensions (Table 1). Previous culture-based methods for environmental airborne fungi and time-of-flight based methods applied to pure culture (Madelin and Johnson, 1992; McCartney *et al.*, 1993; Reponen *et al.*, 1996) may underestimate fungal quantities in larger particles as they are incapable of quantifying multiple cells in an aggregate (Heikkilä *et al.*, 1988).

Knowledge about the aerodynamic diameter in which fungi are present in the atmosphere is critical for determining the respiratory tract penetration and deposition and thus understanding environmental aspects of allergic airway diseases. Aerodynamic diameter also has great influence on particle behavior in indoor and outdoor air (Riley *et al.*, 2002; Nazaroff, 2004). With the exception of water-damaged buildings where fungal growth may be

encouraged, indoor fungal aerosols are generally considered to be of outdoor origins (Burge, 2002). Unicellular allergenic and pathogenic fungi have geometric mean aerodynamic diameters in the 4–5 μm range. Particles in this size range have limited efficiency for penetrating building envelopes, are efficiently removed by the ventilation system filters in many buildings, deposit slowly onto upward surfaces, and can be resuspended with moderate efficiency from flooring during human occupancy (Thatcher and Layton, 1995; Riley *et al.*, 2002; Nazaroff, 2004). In contrast, the multicellular spores are typically $>10 \mu\text{m}$. Particles of this size would not penetrate building envelopes, would settle rapidly onto upward surfaces, and would be strongly resuspended during human occupancy. Respiratory deposition patterns are markedly different for 4–5 μm particles as compared with $>10 \mu\text{m}$ particles. The smaller particle sizes, as presented for *Aspergillus*, *Cladosporium* and *Penicillium* show significant penetration to and deposition in the pulmonary regions of the human lung (Yeh *et al.*, 1996; Cho *et al.*, 2005). The larger particles would deposit mainly in the naso-oro-pharyngolaryngeal region of the respiratory tract (Yeh *et al.*, 1996). Indeed, it has been documented that *A. fumigatus* causes allergic bronchopulmonary aspergillosis (Greenberger, 2002) whereas *Alternaria* and *Epicoccum* cause allergic fungal sinusitis and are found in nasal sinuses of hypersensitive patients (Noble *et al.*, 1997).

Seasonal variations

Important seasonal trends with respect to fungal concentration, ecology and size were observed. Consistent with seasonal trends previously observed by culture analysis of airborne fungi in the north-eastern United States (Shelton *et al.*, 2002), the concentrations were smallest in winter for all species analyzed whereas the qPCR analyses Aaltr, Afumi, Enigr, PenGrp3 and PenAsp1mgb showed the highest concentrations in fall (Figure 4). The wintertime levels of airborne fungi, while lower than during other seasons, are nevertheless still substantial. Figure 4 shows high relative prevalence of PenAsp mgb and Enigr in winter as compared with the other species reported. These species are also prominent in the outdoor winter sampling data by Shelton *et al.* (2002). These findings indicate the potential wintertime sources for both background/natural fungal materials as well as contributions from anthropogenic disturbances in the urban setting (e.g., resuspension by motor vehicles). The relative abundance of allergenic species also peaked in the fall, whereas the relative abundance of pathogens was highest in the spring. Although the concentrations of fungi showed clear seasonal trends (Figure 4), with few exceptions (*Schizophyllum* and *Penicillium*), the relative abundances of fungal materials did not vary considerably

(Figure 2). Interestingly, the geometric means of aerodynamic diameters were smallest in summer for all the species analyzed (Figure 4).

Conclusions

Aerodynamic diameter is a crucial parameter for understanding fungal fate and transport in the environment. This study provides the most in-depth characterization of size-resolved taxonomic compositions of atmospheric fungi to date. Results demonstrate that fungal diversity in the atmosphere is higher than previously reported and may rival that of the terrestrial environment. Taxonomic composition of airborne fungi vary strongly with aerodynamic diameter and by season, with allergens clustering in the largest size ranges and the fall season, whereas fungal pathogens were typically observed with aerodynamic diameters $<4.7 \mu\text{m}$ and were most abundant during the spring. *In situ* measurements of fungal aerodynamic diameter in the atmosphere are different (and typically larger) than diameters determined from pure culture studies. Owing to their ability to identify a broad spectrum of allergenic and pathogenic fungal species, molecular-based methods are an important technology for human exposure studies that seek to better understand the elusive nature of fungal allergy and invasive fungal infections.

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Author contributions

NY and JP designed the research; NY, JQ, DH and HR-Y performed the research; NY, KB, WWN and JP analyzed the data; NY, KB and JP wrote the paper. The order in which the contributors are listed was agreed amongst the investigators. The first listed (NY) made the greatest contributions to the paper.

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