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DNA analysis of outdoor air reveals a high degree of fungal diversity, temporal variability, and genera not seen by spore morphology

Catherine H. PASHLEY[a,*], Abbie FAIRS[a], Robert C. FREE[b], Andrew J. WARDLAW[a]

[a]Institute for Lung Health, Department of Infection, Immunity and Inflammation, University of Leicester, Leicester LE1 9HN, UK
[b]Department of Genetics, University of Leicester, Leicester LE1 7RH, UK

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ABSTRACT

Fungi are ubiquitous with many capable of causing disease by direct infection, toxicoses, or allergy. Fungal spores are present in outdoor air throughout the year, yet airborne diversity is poorly characterised. Airborne fungal spores are routinely counted by microscopy, enabling identification to genera at best. We generated traditional microscopic counts over a year, then used environmental sequencing techniques to assess and compare 3 d selected from the main fungal spore season. The days selected corresponded to one with a high quantity of spores unidentifiable by microscopy, and two representing dry and wet summer periods. Over 86 % of genera detected by sequencing were not routinely identifiable by microscopy. A high degree of temporal variability was detected, with the percentage of clones attributed to *Basidiomycota* or *Ascomycota*, and composition of genera within each phylum varying greatly between days. Throughout the year *Basidiomycota* spores were found at higher levels than *Ascomycota*, but levels fluctuated daily with *Ascomycota* comprising 11–84 % of total spores and *Basidiomycota* 7–81 %. No significant difference was found between the proportion of clones attributed to each morphological group detected by sequencing to that counted by microscopy ($P = 0.477$, 0.985, and 0.561). The majority of abundant genera detected by DNA analysis are not routinely identified by microscopy (>75 %). Of those, several are known human and plant pathogens, and may represent unrecognised aeroallergens.

Introduction

Aerobiology as a term was first used in the 1930s, and is defined as the study of biological particles present in the air, both outdoors (extramural) and indoors (intramural); and includes the study of airborne pollen grains and fungal spores. The pollen component is well characterised, with pollen monitoring networks established in many countries. In contrast, the airborne fungal load is poorly defined with few sites actively monitoring daily levels, due in part to limitations of traditional identification methodologies. Fungi are ubiquitous and fungal spores are present in outdoor air throughout the year, with many fungi exhibiting seasonal periodicity. The number of fungal spores per cubic metre of air can often exceed pollen concentrations by 100–1000 fold (Horner *et al.* 1995). Many airborne fungi are capable of causing disease by direct infection, toxicoses, or allergy. Incidences of allergy are rising, with fungal respiratory allergy affecting up to 30 % of atopic individuals. There is a clear association between life-threatening asthma and sensitisation to fungal allergens,

* *Corresponding author*. Tel.: +44 116 252 2936; fax: +44 116 252 5030.
E-mail address: chp5@le.ac.uk
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and studies have correlated outdoor spore concentrations with asthma symptoms (Black *et al.* 2000). The prevalence of fungal allergy in severe asthma ranges from 35 % to 70 % (Denning *et al.* 2006). With regard to agriculture, many important fungal plant pathogens are dispersed by wind or rain-splash, and are capable of causing severe losses in susceptible crops with significant economic consequences. Long-distance dispersal is an important survival strategy for many fungi, and has caused the spread of important diseases on a continental or global scale. The ability to detect these fungal pathogens from air samples can be used in an effort to devise or improve disease control methods (West *et al.* 2008).

Traditionally, airborne fungal biodiversity studies were based on culture-dependent methods which inevitably underestimate diversity, and grossly bias studies towards fungi that can be cultured on generic fungal growth media. The current standard device for most aerobiological studies is an automatic volumetric spore trap, with morphological identification of spores; limiting studies by the time and skill required to count them. Of the 40 or so fungal categories that can be recognised, some can be classified to genus, a few to species, but many have to be recorded in groups with similar characteristics (Lacey 1996).

PCR-based methods can detect and quantify biological material in air samples; and a number of total fungal and species-specific assays have been developed (Williams *et al.* 2001; Haugland *et al.* 2004). These assays, however, have primarily been designed to quantify fungi whose presence had previously been demonstrated from culture-based studies, and are therefore limited to the same biased view of fungal diversity. Before an assay to measure airborne spores can be developed, a comprehensive understanding of common airborne fungi is required without the bias of culture or limitations of morphological studies.

In contrast to indoor air, which has been analysed from both residential and occupational environments, very few studies have used a molecular approach to study outdoor airborne fungal diversity. Those that have targeted various regions of the fungal nuclear ribosomal operon (rDNA) which is present in multiple copies, universally applicable, and has far greater representation in publicly available databases than any other region (Ward *et al.* 2004). Three studies have targeted 18s rDNA, analysing air samples from Phoenix Arizona, USA (Boreson *et al.* 2004), Boulder Colorado, USA (Fierer *et al.* 2008), and San Diego California, USA (Urbano *et al.* 2011). Three others have targeted internal transcribed spacer region (ITS) rDNA, two analysing air samples from Germany (Despres *et al.* 2007; Frohlich-Nowoisky *et al.* 2009), the third analysing air collected from Seoul, Korea (Lee *et al.* 2010). The time period during which individual air samples were collected varied from a few hours (Fierer *et al.* 2008; Urbano *et al.* 2011), to 24 h (Boreson *et al.* 2004; Lee *et al.* 2010) to several days (Despres *et al.* 2007; Frohlich-Nowoisky *et al.* 2009). Surprisingly, given the risk of bias being introduced through primer selection (Anderson *et al.* 2003), none of the previous airborne diversity studies have compared data generated using a molecular approach to data from more traditional microscopic analysis sampled simultaneously; although one compared a culture-dependant method to a DNA-based method (Urbano *et al.* 2011), unsurprisingly finding samples collected by culture were not similar to clones in the 18S rRNA gene clone library. This present study used environmental cloning and sequencing techniques to assess and compare airborne fungal diversity from a central UK location on 3 d during the main fungal spore season, and to compare the level of diversity detectable by the molecular approach to data generated using traditional microscopic analysis.

Materials and methods

Sample collection and microscopic analysis

Each sample was collected over a 24 h period from midnight, representing an individual day. Outdoor air samples were collected using two traps located 2 m apart on the roof of a building on the University of Leicester campus, 12 m above ground level in an urban area 60 m above sea level and approximately 1 km south of the city centre, recently shown to be sufficient for aeroallergen analysis for a 41 km area (Pashley *et al.* 2009). These traps sample air that has been thoroughly mixed by the turbulent boundary layer (Lacey & West 2006).

Samples for microscopic analysis were collected using a 7-d recording volumetric spore trap (Burkard Manufacturing Co. Rickmansworth, UK) with a flow rate of 10 L min^{-1}. Slides were stained with polyvinyl lactophenol cotton blue and analysed by microscopy at a magnification of 630×. A single longitudinal transverse of one field width was counted for each sample, as described previously (Corden & Millington 2001). Distinct spore morphology distinguished 17 fungi to the level of genus. Other fungi were categorised into closely related groups, such as *Aspergillus/Penicillium* (*Asp/Pen*)-type spores or into generalised groups including ascospores, hyaline, and coloured basidiospores (Supplementary Table 1).

Samples for molecular analysis were collected using a continuous volumetric cyclone sampler with wind orientation and an air throughput of 16.5 L min^{-1} (Burkard Manufacturing Co.). Airborne particles, including fungal spores and hyphae, were collected directly into a 1.5 mL microcentrifuge tube and stored at −80 °C prior to DNA extraction.

Temperature data were provided by the Leicester city council air quality group from a meteorological station 5 km from the trap site, and rainfall data were provided by the Met Office for Sutton Bonington meteorological station 24 km from the trap site.

DNA extraction and PCR amplification

Material trapped in the microcentrifuge tubes was eluted following vigorous agitation for 2 min with 100 μl of sterile 0.1 % Tween 80. Eluant was added to sterile 2 mL screw cap tubes containing 0.3 g ± 0.03 of 212–300 μm glass beads, 400 μl of buffer AP1, and 4 μl of 100 mg mL^{-1} RNase A (DNeasy plant kit, Qiagen, Crawley, UK), subjected to 2 min bead-bashing (BioSpec mini bead beater, Bartlesville, OK, USA) then incubated for 10 min at 65 °C. Total genomic DNA was extracted using the DNeasy plant mini kit (Qiagen) following manufacturer's instructions. Sterile water was used as a control.