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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

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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.
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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,

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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.

Following preliminary investigations (Supplementary Appendix 1) large subunit (LSU) rDNA was selected as the region to analyse, using primers described by Issakainen *et al.* (1999). Reactions were performed in 25 μL total volume containing 2–10 μL of template DNA; 0.25 pmol of each primer (Invitrogen, Paisley, UK); 0.25 mM each dNTP (Bioline, London, UK); 1× PCR buffer; and 1 U of HotStarTaq DNA Polymerase (Qiagen). The thermal profile (Biometra T3 thermocycler, Goettingen, Germany) was as follows: initial denaturing at 94 °C for 15 min, 40 cycles with denaturing at 94 °C for 20 s, annealing at 55 °C for 20 s, elongation at 72 °C for 90 s, and a final extension step at 72 °C for 7 min.

Cloning and restriction fragment length polymorphism (RFLP)

Amplification products were cloned using the pGEM® T-Easy cloning kit (Promega, Madison, WI, USA) following manufacturer's instructions. Colonies containing inserts were identified by blue–white selection, then screened for presence of a full length insert by reamplification using original primers and amplification conditions.

Colony PCR was followed by RFLP analysis to select optimal clones for sequencing. RFLP analysis was performed using 7.8 μL of the PCR-products with 1 U each of *Hae*III and *Hin*fI or *Hae*III and *Mse*I, in addition to 1× buffer (New England Biolabs, Ipswich, MA, USA) in a final volume of 10 μL. Restriction fragments from each double-digest were separated by gel electrophoresis in a 3 % metaphor® agarose (Lonza, Basel, Switzerland) gel stained with ethidium bromide. Using a specially designed algorithm, RFLP-patterns were scaled to a consistent size, and then imported into a custom-built program for visual comparison of gel patterns. Patterns were grouped according to similarity of RFLP-types.

DNA sequence analysis, taxonomic attribution, and statistical parameters

All clones representing unique or rare RFLP-types were sequenced. At least 25 % of pattern sharing clones were sequenced, including representatives from different days where relevant. When inconsistencies between RFLP-type and closest BLAST match were found, all clones representing that RFLP-type were sequenced. Clone sequences were determined using BigDye-Terminator v3.1 chemistry with either 3730XL sequencers (Applied Biosystems, Foster City, CA, USA) at The Genome Analysis Centre (was John Innes Genome Laboratory), Norwich; or 3730 sequencers (Applied Biosystems) at The Protein Nucleic Acid Chemistry Laboratory of the University of Leicester. Sequence data were manually inspected and trimmed, with closest taxonomic match determined by comparison with known sequences in GenBank (Benson *et al.* 2006) (March 2010) using the $BLAST_N$ (Altschul *et al.* 1990) database search method. Current fungal names were reported according to Index Fungorum (http://www.indexfungorum.org). If the closest match was to an uncultured clone, the first named hit was reported and details of the closest hit given in parentheses. GenBank sequences suspected of being incorrectly identified were compared to the sequence identities they were most similar to through a BLAST search, and to the sequences of other accessions for the same species. Accessions unrelated to their nearest BLAST hits were determined as being misidentified and consequently ignored.

Putative chimera (clones with <50 % overall coverage, matching one end of the sequence only) and clones with closest BLAST hits within the kingdom *Viridiplantae* were excluded from subsequent analyses. Sequences were aligned using ClustalW (Chenna *et al.* 2003), and distances created using PHYLIP v3.69 (Felsenstein 2005). Sequences were clustered into operational taxonomic units (OTUs) at 97 % homology using the furthest neighbour approach of mothur v.1.10 (Schloss *et al.* 2009). The get.oturep function of mothur was used to select representative sequences for RFLP-groups missing sequence data to get a conservative overview of the distribution of sequences within and between days. Venn diagrams, rarefaction curves, and abundance curves were determined using mothur.

Putative genera detected by sequence analysis were grouped into spore morphology groups as counted by traditional microscopy according to spore images available in the literature. Oomycetes were recorded as other fungal spores to conform with traditional aerobiology. A two-tailed Wilcoxon signed rank test was used to compare the percentage of clones per day assigned to each morphological counting group with the percentage of spores identified by traditional morphology, using GraphPad (Version 5; GraphPad Software Inc, CA, USA).

These sequence data have been submitted to the GenBank database under accession numbers: HQ691413–HQ691842.

Results

An environmental clone sequencing approach was developed to analyse airborne fungal diversity in greater detail than currently available using microscopic identification of airborne fungal spores. An overview of the methodology is shown in Fig 1. In brief, airborne particles were collected directly into a microcentrifuge tube sampling 16.5 L min^{-1} for 24 h, from which total genomic DNA was extracted. The fungal component was targeted by amplifying LSU rDNA with universal fungal primers. The fungal amplicons were separated by cloning, the clone libraries screened by RFLP analysis, and representatives of the different fungal entities sequenced then identified by comparison with sequences in GenBank.

Microscopic analysis

Airborne fungal spores were identified and counted *via* microscopy on 302 d during 2007. Spore counts were highly variable and positively skewed, ranging from 291 to 162 626 spores m^{-3} air d^{-1} (median = 6925, mean = 12 875; Supplementary Table 2). Total spore counts reached very high levels (>50 000 spores m^{-3} air d^{-1}) during the months of June, July, and August (Fig 2). Three days were selected for molecular analysis. The first day, hence-forth referred to as the mixed day, was selected based on fungal spore data. This day had a high total fungal spore count (ranked 24th in the year), a mixture of spores (representatives from 16 of the 24 recording categories, and multiple dominant spore types), and a high percentage of spores that could not be recognised to genera by microscopy (52.7 %). The second and third days represent dry and wet

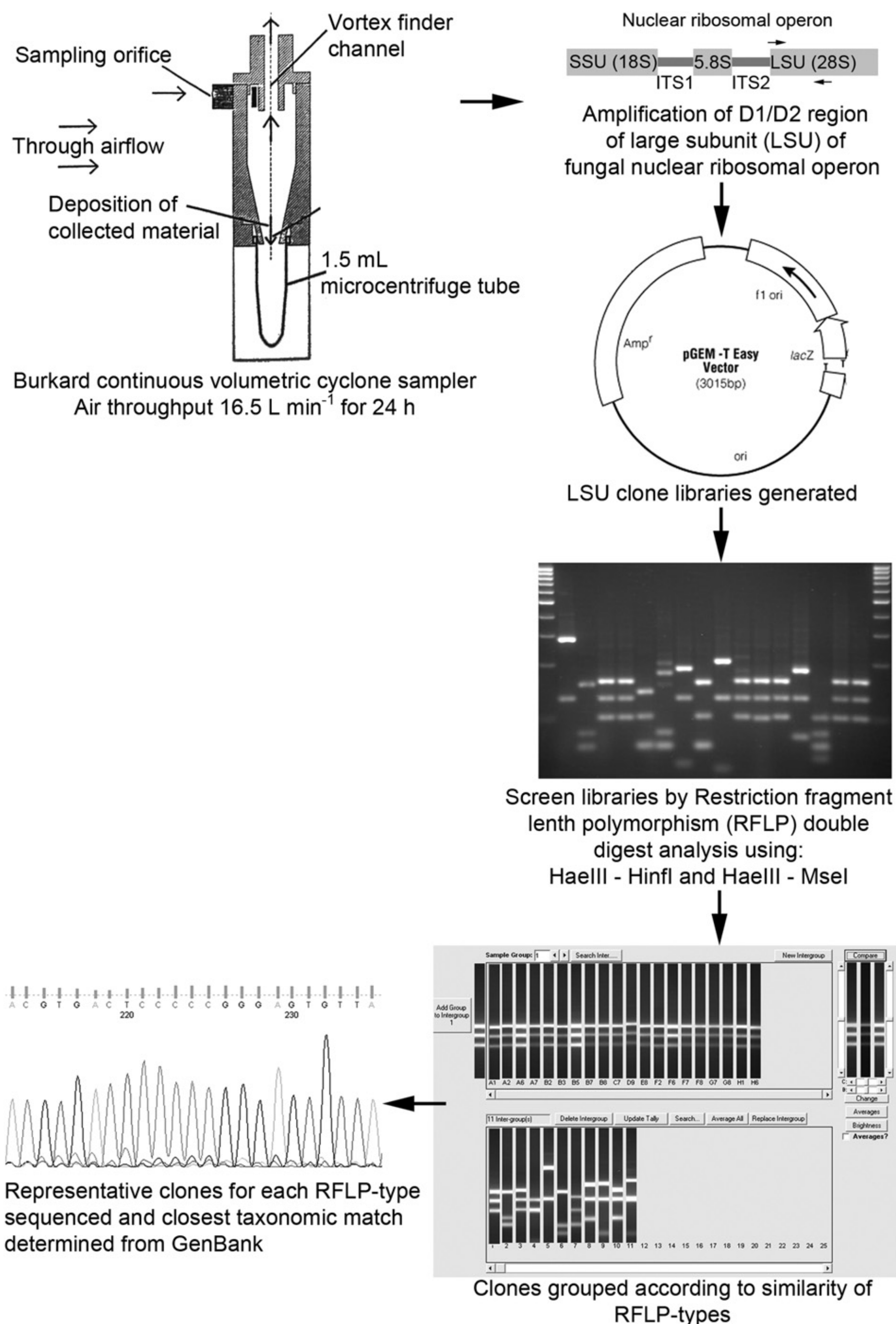

Fig 1 – Flow chart overview of the environmental clone sequencing approach used to analyse air samples.

periods during the fungal spore season. In contrast to the mixed day, both of these days had >50 % of total spores dominated by two fungal genera; *Sporobolomyces* and *Cladosporium* on the dry day and *Sporobolomyces* and *Tilletiopsis* on the wet day. The amount of pollen and hyphal fragments in the air varied between the 3 d; with the wet day having the lowest levels for both and the dry day the highest (Supplementary Table 1).

Delimitation of OTUs

Following PCR–RFLP screening of colonies containing full length inserts, 734 colonies were retrieved from the three libraries; 200, 232, and 302 representing the dry, wet, and mixed days respectively. Over 87 % of clones analysed were of fungal origin, 3 % represented *Chromista*, with the remaining either chimeric or *Viridiplantae* (Table 1, Supplementary Appendix 1). Sequences were grouped into 96 OTUs, of which 89 were fungal and seven *Chromista*. Over half (58.3 %) of the retrieved fungal and *Chromista* sequences had a closest match to a single species, 37 % matched to genus, whilst the remaining 4.7 % were represented by multiple genera (Supplementary Table 3). Only five of the 96 OTUs contained sequences with different closest matches; OTU036 and OTU067 contained two species from the same genera

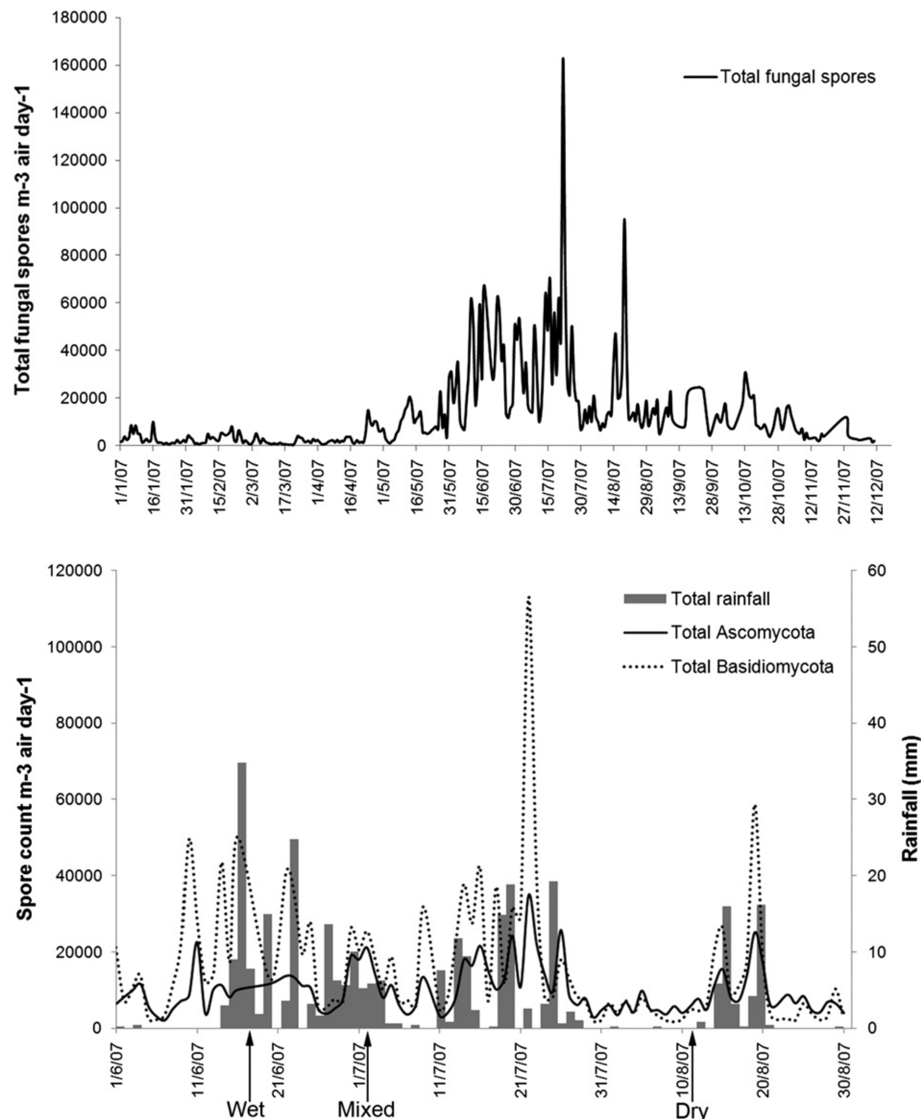

Fig 2 – Fungal spore counts recorded by microscopy during 2007. Top panel shows total fungal spore counts per cubic metre of air per day. Very high counts (>50 000 spore m^{-3} air d^{-1}, indicated on graph) were seen during the months of June, July, and August. Bottom panel shows the number of *Ascomycota* and *Basidiomycota* spores recorded, and the amount of rainfall each day during the months containing very high spore counts. The 3 d analysed in this study by molecular means are shown by arrows.

(*Entyloma* and *Aspergillus* respectively), OTU007 and OTU092 contained two species from closely related genera of *Cystofilobasidiaceae* and *Peronosporaceae* respectively. OTU026 contained two genera matching to class only. Within this OTU six clones were reliably identified as *Peniophora*, and another as *Kavinia* sp. (98.7 % homology), but this was 97.2 % homologous to *Peniophora* and may be a result of misidentification.

Over a third of clones could only be identified to genera. In most cases this was due to the inability of the LSU region to discriminate to species. Two notable examples were *Cladosporium* and *Ustilago*, representing 148 and 18 clones respectively (Supplementary Table 3), both of which contain multiple species that are 100 % identical in this region. Four OTUs could not be identified to genera. Of note, OTU061 represented 25 clones at >98 % similarity. These clones were equally close to three genera of *Dothideomycetes*; *Glonium*, *Didymosphaeria*, and *Didymella*; which are 100 % homologous in this region.

Two of the seven *Chromista* OTUs (OTU094 and OTU096, Supplementary Table 3) representing nearly 23 % of the total *Chromista* clones identified, had less than 95 % homology to their closest named match, *Phytophthora katsurae* and *Pythium monospermum* respectively. In contrast, less than 1 % of the fungal OTUs had <5 % similarity (Supplementary Table 3).

Abundance of fungal sequences

Rarefaction curves for fungal sequences detected in the air on each day do not reach a plateau (Fig 3). Relative abundance curves (Fig 4) showed the distribution of sequences across OTUs to follow the universal ‘hollow curve’, with most

Table 1 – Distribution of numbers of clones in the taxonomic kingdoms analysed on each day and on the 3 d combined, as absolute numbers of clones (*n*) and percentage of total clones analysed per day or 3 d combined (%). Within the kingdom *Fungi*, the number of clones attributable to the phyla *Ascomycota* and *Basidiomycota* is also shown.

	Mixed day		Dry day		Wet day		Combined	
	n	%	*n*	%	*n*	%	*n*	%
Total clones analysed	302	–	200	–	232	–	734	–
Putative chimera	1	0.3	36	18.0	0	0.0	37	5.0
Viridiplantae	19	6.3	7	3.5	7	3.0	33	4.5
Chromista	20	6.6	1	0.5	1	0.4	22	3.0
Fungi	262	86.8	156	78.0	224	96.6	642	87.5
Ascomycota	183	60.6	141	70.5	70	30.2	394	53.7
Basidiomycota	79	26.2	15	7.5	154	66.4	248	33.8

sequences being rarely found and relatively few abundant sequences. Each day had two OTUs representing >15 % of the total clones for that day. *Cladosporium* was in the top two on all 3 d, with *Botryotinia*, *Microdochium*, and *Tilletiopsis* being the other genera in the top two for the mixed, dry, and wet days respectively (Supplementary Table 4). Of the four, three are routinely counted by microscopy, however >75 % of the common genera detected each day were unidentifiable by microscopy (Supplementary Table 4). Including sequences that were not abundant, over 86 % of genera detected by sequencing were not identifiable by microscopy (Table 2). The least number of different genera and lowest percentage of unrecognisable genera were on the dry day. The percentage of unrecognisable ascomycetes was lower than basidiomycetes and oomycetes on all 3 d.

Distribution and higher-level classification of fungal sequences

The 3 d analysed were highly divergent, with only five OTUs being found on all 3 d (Fig 3 inset), and none being shared solely between the wet and dry days. Of the five shared OTUs one (OTU061) could not be identified to genus, two represented genera routinely counted by microscopy (OTU072 and OTU084; *Botrytis* and *Cladosporium* respectively), and two represented genera not routinely identified by microscopy (OTU004 and OTU056; *Cryptococcus* and *Microdochium* respectively) (Supplementary Table 3).

The higher-level phylogenetic classification of the *Fungi* (Hibbett *et al.* 2007) accepts seven phyla, 35 classes, and 129 orders, whilst the *Chromista* now contain ten phyla (Cavalier-Smith 2010). The sequences detected have been attributed to two phyla, 13 classes, and 32 orders from the kingdom *Fungi*; one phylum, one class, and two orders from the kingdom *Chromista* (Supplementary Table 5). The two fungal phyla, *Ascomycota* and *Basidiomycota*, each contain 15 accepted classes, of which six and seven were detected respectively. All *Chromista* sequences were from the phylum *Oomycota*, with >90 % of sequences from the family *Peronosporaceae*.

Comparison of fungal phyla and classes detected between days

In addition to the percentage of clones attributed to each phylum varying greatly between days, the composition of fungi within each phylum also varied (Fig 5, Supplementary Table 5). Only two classes of *Basidiomycota* were detected on all 3 d: *Tremellomycetes* and *Microbotryomycetes*, and these were the only *Basidiomycota* from the dry day; yet comprised only 20 % of the *Basidiomycota* on the wet day and <9 % on the mixed. *Exobasidiomycetes* represented nearly half the *Basidiomycota* clones on the wet day, <7 % on the mixed day and were nonexistent on the dry. The *Pucciniomycetes* and

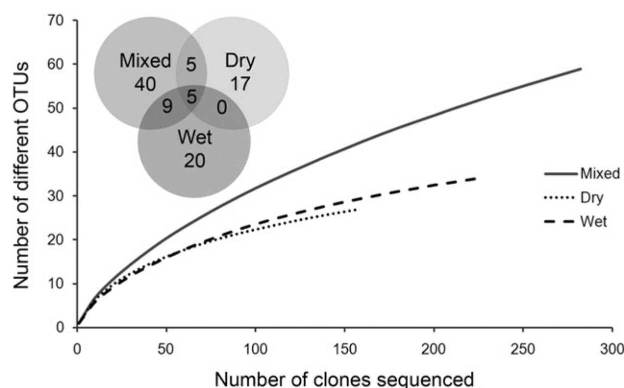

Fig 3 – Rarefaction curves depicting the effect of LSU sequence number on the number of OTUs at 97 % sequence similarity, identified from the 3 d. The overlapping OTUs between the 3 d are given as a Venn diagram in the inset.

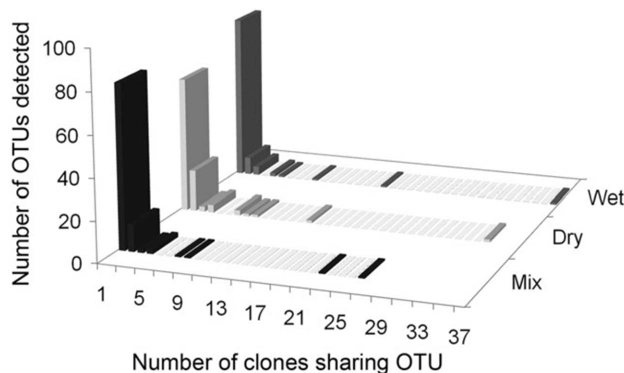

Fig 4 – Relative OTU abundance at 97 % sequence similarity for each of the 3 d sampled showing the universal 'hollow curve'.

Table 2 – Number of putative fungal and *Chromista* genera identified by sequencing (*n*), and the percentage of those genera (%) that cannot be identified to genera or group of closely related genera by routine microscopy. The number of different orders represented per phyla is also shown.

Kingdom	Phylum	Genera								Orders
		Mixed day		Dry day		Wet day		Combined		
		n	%	*n*	%	*n*	%	*n*	%	
Chromista	*Oomycota*	4	100.0	1	100.0	1	100.0	5	100.0	2
Fungi	*Ascomycota*	17	70.6	17	58.8	13	69.2	32	78.1	13
	Basidiomycota	25	88.0	3	66.7	18	88.9	35	91.4	19
Total		46	82.6	21	61.9	32	81.3	72	86.1	34

Ustilaginomycetes were only detected on the mixed day, and *Agaricostilbomycetes* only on the wet day. Of the 35 *Basidiomycota* genera detected, only three were found on the dry day of which one was unique to the dry day and the other two were shared by all 3 d. From the remaining 32 genera; 18 were unique to the mixed day, 11 unique to the wet day, and only seven shared between the mixed and wet days.

In contrast to the *Basidiomycota*, four of the six *Ascomycota* classes were detected on all 3 d. *Dothideomycetes* were the most commonly detected *Ascomycota* class, comprising almost half of fungal and *Chromista* clones analysed on the dry day, approximately a third on the mixed day and a fifth on the wet day. Of the 32 *Ascomycota* genera, eight, six, and seven were unique to the mixed, dry, and wet days respectively and only four shared between all days.

Airborne variation in Ascomycota and Basidiomycota levels

The traditional spore counts from this study show *Basidiomycota* to be frequently present in the atmosphere at higher levels than *Ascomycota* (Fig 2), although there were periods where levels were equal or reversed. On average 40 % of spores identified per day were *Ascomycota* and 42 % *Basidiomycota*, however, daily fluctuations were great (11–84 % *Ascomycota*, 7–81 % *Basidiomycota* per day). Over the year, *Basidiomycota* were abundant in higher quantities than *Ascomycota* on 57 % of days, rising to 65 % during the peak season. In absolute terms, over the course of the study period, >1.3 million *Ascomycota* spores were recorded compared with >1.9 million *Basidiomycota* (Supplementary Table 2). The 3 d analysed in detail had *Ascomycota:Basidiomycota* ratios of approximately 1:1.3, 1:0.9, and 1:5.0 according to microscopy (Supplementary Table 2) and 1:0.4, 1:0.1, and 1:2.2 according to molecular analysis (Table 1) for the mixed, dry, and wet days respectively.

Comparison between microscopic and molecular approach

Wilcoxon signed rank test showed no significant difference between the proportion of clones attributed to each spore morphology group detected by sequencing to the proportion of spores attributed to each group by microscopy ($P = 0.477$, 0.985, and 0.561 for the mixed, dry, and wet days respectively). The pairing was shown to be effective (Spearman coefficient $rs = 0.716$, 0.574, and 0.603; $P = <0.001$, 0.004, and 0.002 for

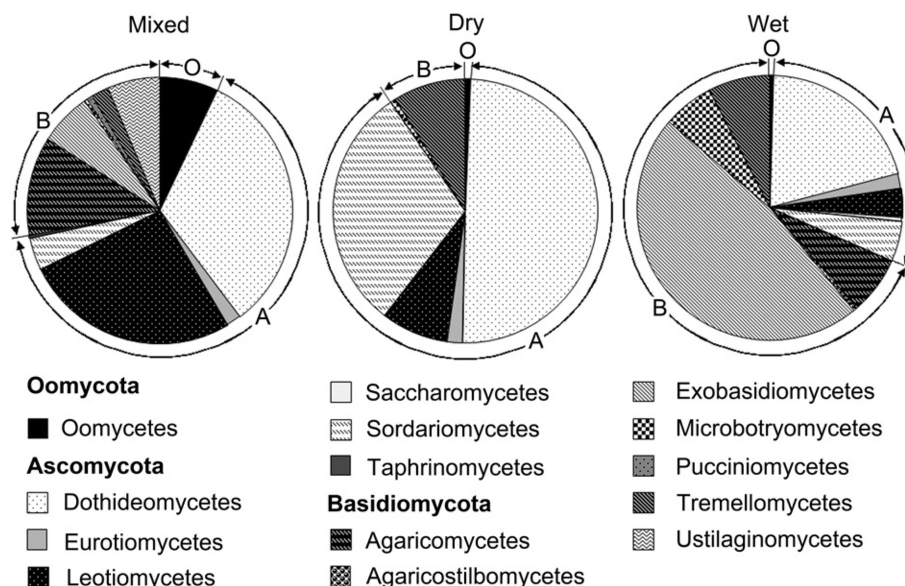

Fig 5 – Taxonomic distribution, at the level of phyla and class, of fungal and *Chromista* clone sequences. Percentages of sequences associated with the classes detected on each day are shown as pie charts.

the mixed, dry, and wet days respectively). Most genera identified through sequencing were not identifiable by routine microscopy; but of those that were detected by both approaches, abundance levels were comparable for some genera. For example, *Cladosporium* on the dry day and *Tilletiopsis* on the wet day represented just over 30 % of the clones sequenced, and just under 30 % of spores recorded by microscopy. In contrast on the mixed day *Botryotinia* represented 25 % of the clones sequenced but only 0.2 % of the spores identified by microscopy (Supplementary Table 4).

Discussion

This is the first study that we are aware of that has used a molecular approach to determine airborne fungal biodiversity and compared the data generated to results from direct microscopic identification of airborne fungal spores. This is also the first study to apply a molecular approach to identify air sampled within the UK. Traditional aerobiological studies identify some spores to genera, but many are recorded under broader morphological classifications. In this study nearly half the fungal spores recorded in 2007 could not be identified morphologically to the level of genus or closely related genera. In contrast, by sequencing the LSU region, over 95 % of clones could be identified to genus or putative species; a clear improvement. We used 97 % sequence homology to delimitate OTUs. At 97 % similarity, within ITS sequences, the different OTUs are often regarded as different fungal species (Frohlich-Nowoisky *et al.* 2009); and ITS is generally accepted to be more diverse than LSU. We found that the vast majority of OTUs contained sequences matching the same closest species or genus, demonstrating that 97 % sequence homology is a good indicator of taxonomic relatedness using LSU rDNA.

Over half of the clones sequenced were identifiable to putative species, with the majority of the remainder only identifiable to genus. In most cases this was due to the inability of the LSU region to discriminate to species. This region was selected following preliminary experiments comparing 18S rDNA, ITS spacer region 1, and LSU, detailed in Supplementary Appendix 1. Primers were screened based on their ability to amplify ten fungal species representing different fungal genera of common airborne fungi, the degree of variation between the amplified fragments for each taxon, and their success at amplifying the various components in mixed samples of known constitution. Efforts continue to reach a formal decision on the DNA barcoding region for fungi, with many mycologists informally adopting ITS for most fungi and LSU for yeasts (Seifert 2009; Eberhardt 2010). Whilst ITS gives superior resolution in groups with longer amplicons, for common airborne ascomycete genera such as *Cladosporium*, *Penicillium*, and *Fusarium* ITS, like LSU, has insufficient variation to unequivocally identify species (Seifert 2009). Alternative regions exist with greater species level discrimination for particular genera, for example translation elongation factor 1-α (EF1-α) for *Fusarium* (Chandra *et al.* 2011) and β-tubulin for *Aspergillus* (Balajee *et al.* 2009). Unfortunately preliminary experiments found neither EF1-α (James *et al.* 2006) nor β-tubulin (Einax & Voigt 2003) was able to readily amplify our panel of common airborne fungi.

Environmental sequencing projects are identifying a vast quantity of sequences from undescribed putative fungal species with <95 % homology to named fungi (Hibbett *et al.* 2009), and these can be used to highlight areas from the fungal kingdom that may benefit from targeted molecular studies. In this study whilst nearly a quarter of *Chromista* clones had <95 % homology to their closest named match, less than 1 % of the fungal clones were that divergent. All fungal sequences detected in this study were attributable to either *Ascomycota* or *Basidiomycota* from the subkingdom *Dikarya* and representing about 98 % of described fungal species (James *et al.* 2006), although DNA from the *Glomeromycota* and *Chytridiomycota* phyla were present within the putative chimeric sequences (Supplementary Appendix 1). Many of the fungal environmental sequencing projects have focused on soil or mycorrhizal fungi. The lower proportion of sequences from putative undescribed fungal species in this study may reflect a historical bias towards naming and studying *Dikarya* that readily spread through the air compared to, for example, *Glomeromycota*, the root colonising fungi that are not able to be identified to species without the application of molecular methods (Öpik *et al.* 2009). The other studies that have applied a molecular approach to identify airborne fungi were also dominated (Boreson *et al.* 2004; Frohlich-Nowoisky *et al.* 2009; Lee *et al.* 2010; Urbano *et al.* 2011), or solely represented (Despres *et al.* 2007; Fierer *et al.* 2008), by fungi from the subkingdom *Dikarya*.

Majority of genera sequenced are unidentifiable by microscopy

The most common pollen and fungal aeroallergens are abundant taxa that are present in the air for periods long enough for individuals to become sensitised. Of the five OTUs shared between all 3 d, two represented genera not routinely identified by microscopy; *Cryptococcus* and *Microdochium*. The two most common genera detected on the mixed and dry days, and the most common on the wet day, were genera normally counted using microscopy; *Cladosporium*, *Botrytis* (anamorphic *Botryotinia*), and *Tilletiopsis*. The second most common genus on the wet day was *Microdochium*, a genus containing known plant pathogens that would not have been identified by microscopy, demonstrating the advantage of using a molecular approach to study fungal aerobiology. In fact >75 % of the common genera detected each day, and 86 % of all genera sequenced, were unidentifiable by microscopy, although some spores may have been counted within morphological similar genera, for example some species of *Chalastospora* would be counted morphologically as *Cladosporium*.

The percentage of ascomycetes unrecognisable by microscopy was lower than basidiomycetes and oomycetes on all 3 d. This may reflect a historical bias towards studying ascoymycetes due to their ease of culture, but is also likely due to the high number of hyaline basidiospores within the airspora that could not be identified to fungal genera even by a highly trained specialist mycologist. More extensive fungal aerobiology may enable representatives from 126 fungal genera to be distinguished (Magyar *et al.* 2009); however, comparing the expanded list of identifiable genera to the genera detected by molecular means in this study still results in >80 % being unidentifiable.

Airborne levels of Basidiomycota often higher than Ascomycota, but with high temporal variability

The reported proportion of *Ascomycota* to *Basidiomycota* within air samples can vary greatly depending on the study and method of analysis. A recent molecular study from Germany observed 64 % of the fungal airspora to be *Basidiomycota* (Frohlich-Nowoisky *et al.* 2009), higher than previous culture-based studies, and proposed two hypotheses to explain this: (1) They may be enriched in the atmosphere relative to the biosphere; and/or (2) the ratio of *Basidiomycota* to *Ascomycota* may have been underestimated in earlier studies. The importance of ascospores and ballistospores in the atmosphere has been known about since the introduction of automatic volumetric spore traps in the 1950s. These spore types had either been trapped inefficiently by earlier sedimentation methods, or produced no fruiting bodies or alternative anamorphic forms in culture (Lacey 1996). The *Basidiomycota* contains more than 31 000 species representing about a third of all described fungal species (Kirk *et al.* 2008) and ranges from large fruiting body producing organisms such as mushrooms to microfungi and yeasts. Most members reproduce mainly by basidiospores, although a number also produce asexual conidia (Ingold & Hudson 1993). Many are difficult or impossible to culture on standard agar media which tend to favour fast-growing heavily sporulating species (Frankland *et al.* 1990) such as the anamorphic *Ascomycota*.

In agreement with Frohlich-Nowoisky *et al.* (2009), over the course of the year *Basidiomycota* were found at higher levels than *Ascomycota*, but this was dramatically variable on a daily basis. This study found a high degree of temporal variability. The days analysed were selected to maximise this discovery, specifically targeting days from a very dry and very wet period. Precipitation is required for the release of spores from many actively wet spore discharging *Ascomycota*, with concentrations known to increase during and after rainstorms. The release and resultant airborne concentrations of actively wet spore discharging *Basidiomycota* are correlated with relative humidity rather than precipitation; whilst dry discharged spores from fungi such as *Aspergillus*, *Penicillium*, and *Cladosporium* are mostly emitted when dry, warm, and windy conditions prevail (Elbert *et al.* 2007). It is therefore probable that meteorological conditions explain some of the differences in fungal diversity detected from earlier molecular studies. Boreson *et al.* (2004), Fierer *et al.* (2008), and Lee *et al.* (2010) only sampled during dry periods and found ratios comparable with the dry day in this study. In contrast Frohlich-Nowoisky *et al.* (2009) detected a ratio of 1:1.8 over a period of 1 y; comparable with the mixed and wet days in this study, and the microscopic data when averaged over a year. This emphasises the necessity of taking into consideration meteorological data, time of year, and length of sampling when comparing studies of seasonal fungi.

Relative proportions of fungal genera identified by microscopy and DNA correlate

Traditional aerobiology recognises about 40 fungal categories, only some of which can be classified to genus (Lacey 1996). The rarefaction curve generated from the sequence data in this study did not reach a plateau, indicating that more diversity would have been detected had more clones been analysed. Nonetheless, 96 OTUs were detected representing 72 genera, the vast majority of which were not recognisable by microscopy.

Sampling efficiency of cyclone samplers is approximately 100 % for particles down to 4 μm. Cyclone samplers trap fungal spores and hyphal fragments, and can collect the full size range of airborne fungal spores (generally 1–50 μm (Gregory 1973)), unlike filter traps that only collect particulate matter (PM) $<$10 μm (PM2.5 or PM10). DNA-based analyses cannot distinguish between DNA from spores or hyphae. Concentrations of hyphal fragments in the air are generally low, although peaks can occur in the summer months with considerable daily variations (Harvey 1970). Hyphal fragments have the potential to impact on human, animal, and plant health as pathogen inoculum and source of allergens (Green *et al.* 2005; Green *et al.* 2006), and have been implicated in contributing towards asthma severity (Delfino *et al.* 1997). The amount of recorded hyphae varied between the 3 d, however, there was no significant difference between the proportion of clones attributed to each spore morphology group by sequencing or microscopy. This suggests that the effect of hyphae was minimal and that a DNA-based approach could be used as a guide to spore levels. Free DNA from decayed spores and tissue fragments could be a confounding factor; however, DNA in aerosols is most likely to originate from captured spores which can resist environmental stress and survive atmospheric transport, whereas free DNA or DNA in fungal tissue may be rapidly degraded by atmospheric photooxidants (Despres *et al.* 2007; Frohlich-Nowoisky *et al.* 2009).

Abundance of some genera detected by both approaches was comparable, for example *Cladosporium* on the dry day and *Tilletiopsis* on the wet. For others there was discrepancy between the two techniques, most notable being *Botrytis* which was found to be highly abundant by molecular means and not by microscopy. Interestingly, *Botrytis* is believed from microscopic studies to be one of the less prevalent fungi, and yet allergy to it in Europe is believed to be prevalent (Jurgensen & Madsen 2009). By microscopy only the distinctive anamorphic conidia can be identified by microscopy, and some discrepancy may arise due to DNA from the teleomorph or hyphal fragments. However, the molecular and microscopic abundance data pairing was shown to be effective, implying that overall the genera abundance data were comparable.

Abundant genera detected by DNA analysis which may represent unrecognised aeroallergens

One of the main impacts of fungal bioaerosols is as causative agents of disease, with large economic and ecological consequences. Fungal bioaerosols are also associated with noninfectious diseases such as allergies and asthma (Horner *et al.* 1995), and several studies have correlated outdoor spore concentrations with asthma symptoms (Black *et al.* 2000). Over 80 genera of fungi have been associated with respiratory tract allergy, and the majority of these were originally identified through microscopy or culture-based assays. Most early research on fungal allergens focused on anamorphic fungi,

however, later work suggested that prevalence of hypersensitivity to basidiospore and conidial allergens may be comparable and that intensity of exposure may determine clinical relevance (Horner *et al.* 1995). The most commonly studied allergenic fungi are anamorphs of ascomycetes such as *Alternaria*, *Aspergillus*, *Cladosporium*, and *Penicillium*; genera that are often prevalent in the air and can be readily identified using traditional approaches. Over three quarters of the genera found to be abundant by molecular analysis are not routinely identified by microscopy. Of those, several contain species that are known plant pathogens (for example, *Phytophthora*, *Entyloma*, *Peniophora*, *Microdochium*, *Phaeosphaeria*, *Mycosphaerella*, *Gnomonia*, *Itersonilia*, and *Ramularia*), human and animal pathogens (*Cryptococcus* and *Acremonium*), and insect pathogens (*Lecanicillium*), and may represent currently unrecognised aeroallergens.

This study was not intended to be a complete guide to airborne fungal spore diversity but was designed to show the potential of using a molecular approach to study air samples collected on a single day, the time scale used in traditional microscopy based studies. A recognised limitation of this study is that the cyclone sampler used draws air into one microcentrifuge tube at a time preventing analysis of parallel samples without acquiring further samplers. Nonetheless, this study suggests a DNA-based approach targeting LSU has great potential for detecting much higher diversity of airborne fungal load than traditional microscopy, particularly at the level of genus. Whilst absolute levels of the different fungal types within a single day cannot be elucidated without validation experiments to correlate quantities of DNA to spore numbers, this study has shown levels of spores and DNA to be correlated. DNA analysis has potential for comparing day-to-day variation for each fungal type, and it is these fluctuations that are important for establishing links between fungal load and respiratory symptoms in humans. Adopting a DNA-based approach to analysis could revolutionise fungal aerobiology, particularly utilising high throughput sequencing approaches to initially characterise diversity, followed by a microarray-type approach to monitor daily fluctuations of fungi. Fungal aerobiology is currently much less studied than pollen; due in part to the labour-intensive nature of the work due to the far higher levels, smaller physical size, and more diverse nature of airborne spores compared to airborne pollen grains. A molecular approach has potential for a single laboratory to simultaneously analyse samples from a network of samplers from multiple sites. It would also be less prone to bias inherent with interpersonal variation in identifying spores by microscopy. The experimental approach used allows the creation of a databank on fungal biodiversity in the air, which can be expanded upon. This databank could constitute a tool to identify the fungal biodiversity in routine samples by methods such as high throughput sequencing or through the development of a microarray.

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Supplementary material

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