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OCCUPATIONAL ALLERGIES (JA POOLE, SECTION EDITOR)

Emerging Insights into the Occupational Mycobiome

Brett J. Green[1]

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Abstract

Purpose of Review The evolution of molecular-based methods over the last two decades has provided new approaches to identify and characterize fungal communities or "mycobiomes" at resolutions previously not possible using traditional hazard identification methods. The recent focus on fungal community assemblages within indoor environments has provided renewed insight into overlooked sources of fungal exposure. In occupational studies, internal transcribed spacer (ITS) region sequencing has recently been utilized in a variety of environments ranging from indoor office buildings to agricultural commodity and harvesting operations.

Recent Findings Fungal communities identified in occupational environments have been primarily placed in the phylum Ascomycota and included classes typically identified using traditional fungal exposure methods such as the Eurotiomycetes, Dothideomycetes, Sordariomycetes, and Saccharomycetes. The phylum Basidiomycota has also been reported to be more prevalent than previously estimated and ITS region sequences have been primarily derived from the classes Agaricomycetes and Ustilaginomycetes. These studies have also resolved sequences placed in the Basidiomycota classes Tremellomycetes and Exobasidiomycetes that include environmental and endogenous yeast species.

Summary These collective datasets have shown that occupational fungal exposures include a much broader diversity of fungi than once thought. Although the clinical implications for occupational allergy are an emerging field of research, establishing the mycobiome in occupational environments will be critical for future studies to determine the complete spectrum of worker exposures to fungal bioaerosols and their impact on worker health.

Keywords Allergy · Fungi · Sequencing · Occupational · Mycobiome

Introduction

Workers can be exposed to a broad diversity of bioaerosol sources in their work environment. Bioaerosols include airborne reproductive propagules and particles derived from acellular viruses, prokaryotes such as bacteria and archaea, and eukaryotes including plants (pollen, fern/moss spores, and algae), fungi, microscopic animals (arthropods, crustaceans), and even insect debris and excreta [1•]. Compared to other bioaerosol sources, personal exposure to fungi continues to be a public health burden and community concern in the USA.

The proliferation of fungi can become problematic within indoor environments with water damage following infiltration/leaks or natural disasters such as hurricanes or flooding. Damp building materials can lead to the growth and establishment of fungal contaminants, and when disturbed, conidia, spores, or fragments of hyphae can become aerosolized into the breathing zone of the worker [2]. The adverse health effects of these fungal exposure scenarios have been the subject of two international consensus reports published by the National Academy of Sciences, Committee on Damp Indoor Spaces and Health [3], and the World Health Organization (WHO) [4]. Subsequent meta-analyses conducted in the USA and abroad have identified additional associations between visible fungi, odor, and respiratory morbidity in damp indoor environments highlighting the potential breadth of adverse health effects that follow personal fungal exposure [5–7]. Recent

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✉ Brett J. Green
dox6@cdc.gov

[1] Allergy and Clinical Immunology Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention, 1095 Willowdale Road, Morgantown, WV 26505, USA

estimates have placed the annual economic cost of illnesses resulting from exposure to dampness and fungi in the USA to be $3.7 billion for allergic rhinitis, $15.1 billion for asthma morbidity, and $1.7 billion for asthma mortality [8].

Fungi can also contaminate organic materials found in occupational environments [2, 9]. Disturbance (abiotic or biotic) can lead to occupational exposures that can exceed 1×10^7 colony forming units (CFU)/m^3 [9]. These occupational exposure scenarios can result in respiratory morbidity [9]. Although occupational health studies have identified the relevance of fungal exposures in the workplace, the spectrum of fungi that contributes to worker exposure has been restricted due to limitations with existing hazard identification methods. For example, culture methods select viable spores or hyphal fragments that are capable of germination and colony growth on the selected nutrient medium and generally select species placed in the genera *Aspergillus*, *Cladosporium*, and *Penicillium* in addition to 20 other commonly detected genera that are described elsewhere [10]. However, cultured air or dust samples may consist of mycelia sterilia (hyphae) or non-culturable fungi that cannot be identified. Microscopic methods also provide the enumeration of morphologically discernible viable and non-viable fungal spores collected on an optically clear adhesive or membrane. Taxonomic placement of certain spore morphologies such as unicellular amerospores that contain similar morphological attributes can be microbiologically challenging to identify and can confound taxonomic placement. These existing knowledge gaps have been reviewed elsewhere [11•] and have hindered the allergy community's understanding of the complete spectrum of fungal bioaerosols that contribute to worker exposures and clinical symptoms.

Amplification of the internal transcribed spacer (ITS) region has recently improved the identification of fungal populations within the built environment. The results derived from these studies have established new associations between overlooked fungal yeasts and asthma development and severity [12, 13••]. Further, the significance of these datasets has been highlighted in a recent consensus report titled "Microbes of the Built Environment" published in 2017 by the National Academies of Sciences, Engineering, and Medicine [14••]. In contrast, ITS region sequencing has only recently been employed in a limited number of occupational environments that have included indoor office buildings [15–17], biomethanization plants [18], and biowaste sorting facilities [19, 20••], as well as agricultural commodity and harvesting operations [21–23]. Although these initial studies have provided preliminary insights into occupational fungal exposures, the concordance between fungal communities across different occupational sectors currently remains unknown. This review aims to provide the allergist an overview of the emerging fungal communities or "mycobiomes" that have been resolved in occupationally focused studies. Further, the clinical relevance of these datasets and implications for allergic sensitization will be briefly discussed.

ITS Region Sequencing

The recent emergence of molecular techniques to detect fungal bioaerosols overcomes several limitations of traditional methods and has enabled the spatial and temporal examination of fungal community distributions in indoor and outdoor, as well as soil horizon samples. In the indoor air quality (IAQ) field, Haughland and colleagues were among the first researchers to develop species-specific primers for hydrophilic fungal contaminants such as *Stachybotrys chartarum* [24]. Subsequent optimization of DNA extraction procedures and refinement of mold-specific quantitative polymerase chain reaction (MS-qPCR) enabled the detection and quantification of over 100 fungal species within the built environment [11•, 25–27]. Laboratories in academic, government, and commercial sectors have used this methodological approach to assess fungal contamination and personal exposure. In health surveys, MS-qPCR analyses have shown the increased prevalence of *Chaetomium globosum*, *Aspergillus fumigatus*, *A. niger*, *A. unguis*, and *Eurotium* species in the homes of asthmatic children [28–30]. Statistically significant associations between childhood asthma and the combination of *A. ochraceus*, *A. unguis*, and *Penicillium variabile* have also been identified using MS-qPCR approaches [31]. Although MS-qPCR has provided much needed quantitative data in IAQ-focused studies, the MS-qPCR panel primarily consists of selected culturable fungi placed in the phylum Ascomycota whereas many other environmentally ubiquitous fungi are not included.

Contemporary ITS region sequencing methods have increasingly been utilized over the last two decades to identify fungal communities (Fig. 1). Studies employing this methodological approach have provided the allergy research community renewed insight into the diversity of the kingdom fungi. The development and application of clone library sequencing technologies, and more recently high-throughput methodological approaches such as Roche 454, and Illumina MiSeq as outlined in Fig. 1, has provided the elucidation of fungal phyla including the prominent aeroallergen phyla: the Ascomycota, Basidiomycota, and Zygomycota [32]. These methodological approaches have been utilized in recent IAQ studies, and the resultant datasets have provided improved resolution of fungal communities, the impact of spatial, and temporal and environmental variables on richness and diversity, as well as providing unique epidemiological insight into associations between fungal diversity and adverse health effects.

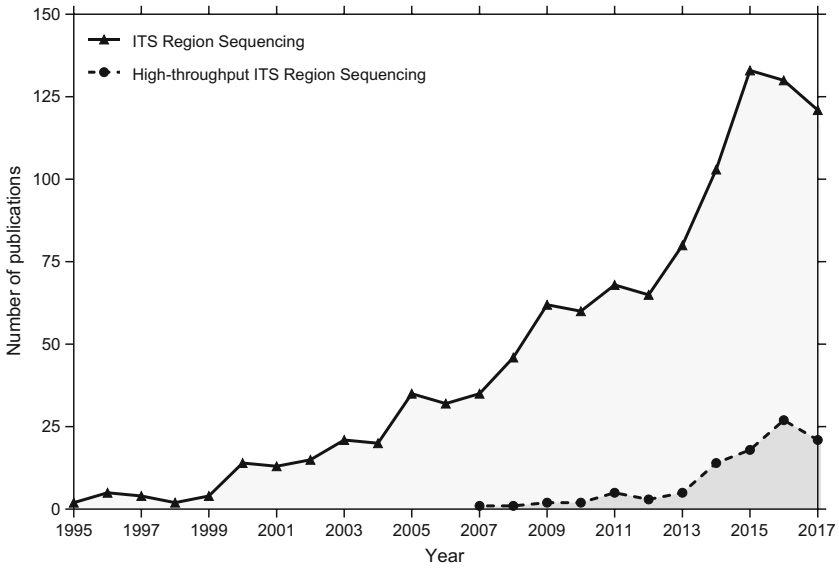

Fig. 1 The annual number of peer-reviewed journal articles focused on "ITS Region Sequencing" that have been published between 1995 and 2017. Data were acquired from PubMed searches utilizing the search terms "ITS Region Sequencing" or "High-throughput ITS Region Sequencing" to identify manuscripts that used this methodological approach with the aim of discriminating fungi in environmental studies

Amplified ITS region sequences are the principle genetic markers used to identify fungal communities. Often referred to as the "fungal barcode," the ITS region consists of two highly variable spacers, ITS 1 and ITS 2, that are flanked by conserved ribosomal RNAs (18s, 5.8S, and 28S) as shown in Fig. 2 [33]. The homology of ribosome encoding genes (18s, 5.8S, or 28S) allows the design of universal fungal ITS primers to amplify fragments containing either the ITS 1, ITS 2, or ITS 1 and 2 regions that can be differentiated to genus and even species taxonomic ranks [33–38]. As the genetic marker is a nuclear ribosomal repeat unit, there are multiple copies that provide up to 100-fold more templates compared to single-copy genes [33]. In addition to ITS regions, other gene encoding sequencing regions have also been utilized to identify fungal species including β-tubulin, calmodulin, and the mitochondrial gene CO1 [37, 39]. Targeting the ITS region in surveys of indoor and occupational environments has improved the identification of fungi compared to culture-dependent approaches where morphological examination can result in misidentifications [40, 41]. However, several sequence amplification considerations within the analysis matrix could introduce selection biases and should be considered before utilizing ITS region sequencing methods. Examples of these limitations have been reviewed or discussed elsewhere and include primer design, sample extraction and purification, eukaryotic contamination, and homology of ITS region sequences within specific fungal orders, as well as various bioinformatic analyses and workflow considerations [33, 34, 38, 42–50].

Occupational Fungal Community Analysis

Surveys of indoor environments utilizing contemporary ITS region sequencing methods have provided improved resolution of fungal communities [33]. Initial reports of fungal community composition have shown air and dust samples to be primarily composed of the phylum Ascomycota and to include sequences placed in the classes Dothideomycetes, Sordariomycetes, Leotiomycetes, and Eurotiomycetes [15, 16, 51, 52]. Sequences derived from overlooked sources of Basidiomycota have additionally been resolved [17]. The class Agaricomycetes placed in the phylum Basidiomycota is one of the most frequently detected fungi in ITS region sequence surveys of indoor and outdoor environments. This class accounts for approximately 20% of all fungi [53] and produces fruiting structures termed basidiocarps (mushrooms) and is characterized as wood-decaying species [54]. Other Basidiomycota sequences resolved in studies of the indoor environment have been placed in the classes Tremellomycetes, Exobasidiomycetes, and Ustilaginomycetes [12, 13••, 15–17, 41]. Representative sequences that have been commonly identified included previously overlooked environmental yeast species placed in the genus *Cryptococcus*, as well as the lipolytic endogenous yeast species, *Malassezia restricta*, that causes superficial fungal mycoses. Utilization of high-throughput DNA sequencing in epidemiological studies has shown that increased asthma risk in children was associated with yeasts such as *Cryptococcus* species [12, 13••] and the genus *Volutella* has been associated with increased asthma severity in a cohort of asthmatic children in Connecticut and Massachusetts [12]. Both of these fungal genera have been previously overlooked as sources of personal

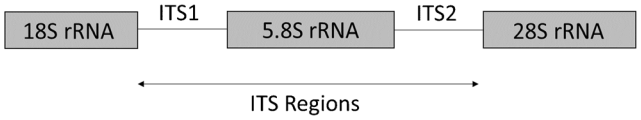

Fig. 2 The internal transcribed spacer region (ITS). The ITS region includes three coding regions and two internal transcribed spacer regions (ITS 1 and ITS 2) of the nuclear ribosomal repeat unit

exposure in health surveys. In addition to effects on health endpoints, ITS region sequencing has also shown high fungal richness to be related to having pets, water leaks, and urban homes [55]. It is important to note that each of these overlooked fungal sources are difficult to culture and identify using traditional methods due to shared morphologies.

The impact that these contemporary studies have had on the IAQ field has recently been outlined in a report entitled "Microbiomes of the Built Environment" that was published by the National Academies of Sciences, Engineering, and Medicine [14••]. ITS region sequencing surveys of the indoor environment highlight a much broader spectrum of fungi that could impact personal exposure and respiratory health. Understanding the diversity of microorganisms present in the environment and how personal exposure modulates downstream immune responses are critical steps that will help improve our future knowledge of the role of microbial populations in the development of allergic disease.

To date, there has been a paucity of studies that have utilized ITS region sequencing studies to survey occupational environments. Table 1 lists a collection of occupational studies that have employed ITS region sequencing and the fungal taxa most frequently identified. Working environments assessed using this methodological approach have included indoor office buildings [15–17], biomethanization plants [18], and biowaste sorting facilities [19, 20••], as well as agricultural commodity and harvesting operations [21–23] (Table 1). These studies have utilized either ITS clone library approaches or more recent high-throughput sequencing platforms such as Illumina MiSeq. Like studies conducted in the built environment, the mycobiome of occupational environments primarily consists of sequences placed in the phyla Ascomycota and Basidiomycota. Compared to culture-dependent datasets, a much higher number of operational taxonomic units (OTUs) have been identified in studies utilizing this molecular platform ranging from as low as 25 OTUs to as high as 5255 OTUs (Table 1). Sequences placed in the phylum Basidiomycota have additionally been resolved and included the classes Agaricomycetes, Ustilaginomycetes to environmental Tremellomycetes fungal yeasts placed in the genus, *Cryptococcus*.

Several studies have characterized fungal communities within indoor office buildings [15–17]. Fungal communities identified in office environments have been primarily placed in the phylum Ascomycota and included the orders Capnodiales, Dothideales, Eurotiales, Pleosporales, and Saccharomycetales (Table 1). Sequences derived from *Aspergillus* spp., *Cladosporium* spp., *Penicillium* spp., and *Aureobasidium microstictum* were especially prominent [15–17] (Table 1). Fungal species placed within these genera are commonly detected in culture-dependent studies and are characterized sources of allergen within the built environment. The phylum Basidiomycota has also been identified in indoor office environments and OTUs derived from this phylum were 46-fold more prevalent compared to the number of fungal species resolved using traditional methods of fungal exposure assessment [17]. The diversity of Basidiomycota included non-pathogenic *Cryptococcus* species [15–17], plant pathogenic smuts such as *Ustilago syntherismae* [17], heteroecious rust species, *Thekopsora areolate* [15, 16], and endogenous *Malassezia* yeasts [15, 16], as well as sequences derived from plant pathogen genus *Rhizoctonia* and brown rot fungi *Antrodia* that are taxonomically placed within the class, Agaricomycetes [15]. Although the clinical significance of these newly identified occupational sources of fungal exposure requires further clinical characterization, several species placed in the class, Agaricomycetes, have been identified in cases of occupational allergy and respiratory morbidity in mushroom-processing facilities. Allergens derived from these species (*Coprinus comatus*, *Psilocybe cubensis*, and *Schizophyllum commune*) have been characterized and listed in the WHO, International Union of Immunological Societies allergen nomenclature (http://www.allergen.org/). Epidemiological studies have also identified associations between *Cryptococcus* species and the development [13••] and severity of asthma [12], whereas smuts placed in the genus *Ustilago* have been identified in cases of occupational hypersensitivity pneumonitis [56]. To date, the clinical significance of many of these overlooked sources of fungal exposure in office environments remains unknown and requires further clinical evaluation.

ITS region sequencing of samples derived from agricultural commodity processing operations and biowaste sorting facilities has provided further insight into workforce exposures [18, 19, 20••, 21, 22]. Sequences placed in the phylum Ascomycota have predominantly been identified in these workplace settings and included genera placed in the order Eurotiales such as *Aspergillus*, *Penicillium*, and *Talaromyces* species [18, 19, 20••, 21, 22]. In an evaluation of organic dust toxic syndrome (ODTS) among workers employed in a grass seed-manufacturing facility in Denmark, *A. fumigatus* was a prominent source of worker exposure [21, 57]. Dust samples were additionally composed of sequences placed in the phylum, Zygomycota, and included *Rhizopus microspores* that were identified in dust implicated in ODTS cases [21]. Additional sources of worker exposure to the Ascomycota in waste sorting environments have been resolved and included *Tricothecium* [21] and *Davidiella* [18], as well as yeasts such as *Candida* and *Blastobotrys* species [20••]. Clinically relevant fungal taxa placed in the order Pleosporales that expresses homologous Alt a 1 allergen [58] were identified in a wheat grain production facility and included *Epicoccum nigrum*, *Alternaria ethzedia*, and *Didymella exitialis* (Table 1) [22]. Sequences placed in the Basidiomycota were also resolved in these occupational environments and included the Agaricomycetes in biomethanization facilities [18], as well

Table 1 Collection of occupational studies that have employed ITS region sequencing. The listed fungal taxa include sequences that accounted for the highest relative abundance of sequences identified in each study

Occupational environment	Sample	Source	Occupational task	Location	ITS region	Sequencing methodology	OTUs	Fungal taxa			Reference
								Phylum	Order	Species	
Nursing home building	Dust sample	Moisture-damaged building, control building	Healthcare, nursing	Finland	ITS1 and ITS2	ITS clone library—Sanger sequencing	394	Ascomycota	Capnodiales	*Cladosporium magnusianum*	[16]
										Cladosporium tenuissimum	
								Basidiomycota	Tremellales	*Cryptococcus victoriae*	
									Malasseziales	*Malassezia restricta*	
									Pucciniales	*Thekopsora areolata*	
Office building	Dust sample	Moisture-damaged buildings, pre and post remediation	Office work	Finland	ITS1 and ITS2	ITS clone library—Sanger sequencing	305	Ascomycota	Eurotiales	*Penicillium* spp. (*P. chrysogenum* group and *P. commune* group)	[15]
									Capnodiales	*Cladosporium* spp. (*C. sphaerospermum* group, *C. cladosporioides* group, and *C. herbarum* group)	
									Dothideales	*Aureobasidium* spp. (*A. pullulans*), *Hormonema* (*H. dematioides* and *Hormonema* spp.)	
									Pleosporales	*Phoma* spp. (*P. herbarum* and *P. macrostoma*)	
										Leptosphaerulina chartarum	
									Saccharomycetales	*Saccharomyces cerevisiae*	
								Basidiomycota	Tremellales	*Cryptococcus* spp.	
`									Malasseziales	*Malassezia* spp.	
									Pucciniales	*Thekopsora areolata*	
									Cantharellales	*Rhizoctonia* spp.	
									Polyporales	*Antrodia* spp.	
Office building	Dust sample	State office building	Office work	Vermont	ITS1 and ITS2	ITS clone library—Sanger sequencing	216	Ascomycota	Dothideales	*Aureobasidium microstictum*	[17]
									Eurotiales		

Table 1 (continued)

Occupational environment	Sample	Source	Occupational task	Location	ITS region	Sequencing methodology	OTUs	Fungal taxa			Reference
								Phylum	Order	Species	
										Aspergillus penicillioides, *Penicillium* spp.	
									Pleosporales	*Pithomyces chartarum*	
								Basidiomycota	Ustilaginales	*Ustilago syntherismae*	
									Tremellales	*Cryptococcus* spp.	
Biomethanization facilities	Static air sample	Organic waste processing	Sorting wastes and compostin-g activities	Canada	ITS1	Illumina MiSeq—two-step dual--indexed PCR	5132	Ascomycota	Eurotiales	*Penicillium* spp.	[18]
										Talaromyces spp.	
									Capnodiales	*Davidiella* spp.	
									Pleosporales	*Epicoccum* spp.	
								Basidiomycota	Hymenochaetales	*Hyphodontia* spp.	
									Polyporales	*Ganoderma* spp.	
									Agaricales	*Cortinarius* spp.	
Waste sorting plant	Static air sample	Paper, cardboard, food packaging, and other waste sorting	Sorting wastes	France	V1 vari-able regio-n, 18S rDN-A	GS-FLX pyrosequen-cer 454 Life Sciences	38–42	Ascomycota	Eurotiales	*Aspergillus* spp. *Penicillium* spp.	[19]
								Basidiomycota	Wallemiales	*Wallemia* spp.	
Composting plant	Static air sample	Domestic waste, pig carcasses	Sorting, screening, and filling compost	Canada	ITS1	Illumina MiSeq—two-step dual--indexed PCR	5255	Ascomycota	Eurotiales	*Penicillium* spp.	[20••]
									Saccharomycetales	*Candida* spp.	
										Blastobotrys spp.	
Wheat grain production	Soil samples, static air sample	Wheat grain farming	Harvester operation	Switzerland	ITS1	GS-FLX pyrosequen-cer 454 Life Sciences	197	Ascomycota	Pleosporales	*Epicoccum nigrum* *Alternaria ethzedia* *Didymella exitialis*	[22]
									Capnodiales	*Passalora robiniae*	

Table 1 (continued)

Occupational environment	Sample	Source	Occupational task	Location	ITS region	Sequencing methodology	OTUs	Fungal taxa			Reference
								Phylum	Order	Species	
										Cladosporium cladosporioides	
								Basidiomycota	Tremellales	*Cryptococcus victoriae*	
Grass seed production	Personal air sample, dust sample (reference seed dust), dust sample (ODTS seed dust)	Grass seed dust	Seed preparation, forklift operation	Denmark	ITS2	Illumina MiSeq	25	Ascomycota	Eurotiales	*Aspergillus fumigatus*	[21]
									Hypocreales	*Tricothecium* spp.	
								Zygomycota	Mucorales	*Rhizopus microsporus*	
Outdoor *Cannabis sativa* farm	Personal air sample, static air sample	Harvested and processed cannabis plants	Harvesting and processing	Washington	ITS1 and ITS2	ITS clone library—Sanger sequencing	216	Ascomycota	Leotiomycetes	*Botrytis cinerea*	[23]

as *Wallemia* species and *Cryptococcus victoriae* in a waste sorting plant [19] and wheat production facility [22], respectively. Studies of outdoor biowaste and agricultural commodity processing demonstrate the ubiquity of pre-established fungal contaminants such as *A. fumigatus* but additionally highlight overlooked fungal sources placed in the Ascomycota and Basidiomycota to be more prevalent than previously estimated and contribute to worker exposures.

Worker exposure to fungal bioaerosols in the emerging US cannabis industry was recently evaluated by the National Institute for Occupational Safety and Health (NIOSH) [23]. Fungal communities were determined using ITS region sequencing of outdoor area and personal air samples collected during a Health Hazard Evaluation of an outdoor organic production facility located in Washington State. Outdoor area samples were primarily composed of the Basidiomycota and included sequences placed in the class, Agaricomycetes. In contrast, personal air samples were composed of sequences derived from the Ascomycota plant pathogen, *Botrytis cinerea*, the causal source of gray mold disease that affects cannabis stems and buds [59–61]. Previous occupational health studies have shown *B. cinerea* to be prevalent in greenhouse environments [62, 63] and worker exposure has been implicated in cases of allergy and hypersensitivity pneumonitis [64–69]. Other assessments of fungal communities associated with cannabis plants have been recently conducted within the cannabis industry. These studies have amplified ITS 2 sequences from cannabis flower samples and demonstrated fungal communities placed in the order Eurotiales that included species such as *Aspergillus versicolor*, *A. terreus*, *A. ostianus*, *A. sydowii*, *Penicillium citrinum*, *P. steckii*, and *P. paxilli* [70, 71]. Compared to other occupational environments, workers in the cannabis production industry may be exposed to fungal plant pathogens, and disturbance activities could result in worker exposure. These preliminary studies also show that fungal communities may vary according to the season and geographical location.

Conclusions

Occupational studies that have utilized contemporary ITS region sequencing methods have provided renewed insight into the fungal communities present in occupational environments. In addition to traditionally identified fungal aeroallergen sources placed in the Ascomycota, these studies have resolved a broad diversity of sequences placed in the classes Eurotiomycetes, Dothideomycetes, Sordariomycetes, and Saccharomycetes. ITS region sequencing studies have also shown an increased prevalence of Basidiomycota sequences compared to datasets captured using culture-dependent approaches. The class Agaricomycetes and yeast species such as *C. victoriae* placed in the order Tremellales have been more prominently detected in surveys of occupational environments. Fungal sequences derived from the classes Agaricomycetes, Tremellomycetes, and Ustilaginomycetes have been consistently identified across diverse occupational environments. Currently, the clinical significance of many of these overlooked sources of occupational exposure remains unknown and requires further clinical assessment. Utilization of contemporary sequencing methods in indoor and occupational exposure surveys will help identify the mycobiome that contributes to worker exposures.

Compliance with Ethical Standards

Conflict of Interest The authors declare no conflicts of interest relevant to this manuscript. The findings and conclusions in this report are those of the author(s) and do not necessarily represent the official position of the National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention. Mention of any company or product does not constitute endorsement by the National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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