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Rapid assemblage of diverse environmental fungal communities on public restroom floors

Abstract An increasing proportion of humanity lives in urban environments where they spend most of their lives indoors. Recent molecular studies have shown that bacterial assemblages in built environments (BEs) are extremely diverse, but BE fungal diversity remains poorly understood. We applied culture-independent methods based on next-generation sequencing (NGS) of the fungal internal transcribed spacer to investigate the diversity and temporal dynamics of fungi in restrooms. Swab samples were collected weekly from three different surfaces in two public restrooms (male and female) in San Diego, CA, USA, over an 8-week period. DNA amplification and culturing methods both found that the floor samples had significantly higher fungal loads than other surfaces. NGS sequencing of floor fungal assemblages identified a total of 2550 unique phylotypes (~800 per sample), less than half of which were identifiable. Of the known fungi, the majority came from environmental sources and we found little evidence of known human skin fungi. Fungal assemblages reformed rapidly in a highly consistent manner, and the variance in the species diversity among samples was low. Overall, our study contributes to a better understanding of public restroom floor fungal communities.

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Key words: Built environment; Indoor microbial ecology; Fungal diversity; ITS-1; Culture-independent methods. Next-generation sequencing.

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Practical Implications

Human exposure to fungal spores is associated with sick building syndrome, and fungi are known to trigger both allergies and asthma. Fungi have also increasingly been implicated in serious human diseases, such as aspergillosis and cryptococcal meningitis. Despite the clear importance of fungi in built environments, we know little about the true diversity and dynamics of indoor fungi because so many remain uncultured. In this study, we applied molecular culture-independent approaches to study fungal biodiversity in public restrooms at San Diego State University. Our results offer a better understanding of the diversity and sources of fungi in public restrooms, as well as insight into the speed at which such communities assemble. Together, these findings should assist efforts aimed at determining and monitoring true human indoor fungal exposure.

Introduction

Humans living in industrialized societies spend 90% of their lives indoors in artificial settings (Höppe and Martinac, 1998; Kelley and Gilbert, 2013). In the last decade, researchers have used increasingly sophisticated culture-independent molecular methods to show that humans share the built environment (BE) with an astonishing diversity of microorganisms (Kelley and Gilbert, 2013). Most of this research has focused on bacterial diversity, partly because of the general importance of bacteria to human health (Larsen et al., 2010) and the environment (Kendrick, 2011) but also because the molecular techniques for identifying microbial community diversity have worked best with bacteria (Tringe and Hugenholtz, 2008).

However, it has long been known that fungi are especially abundant and important in the BE (Solomon, 1975; Solomon et al., 1978). Fungal spores disperse readily and can travel over long distances, even thousands of kilometers (Kellogg and Griffin, 2006). Fungi grow well indoors on many types of building materials and, given sufficient moisture, can cause significant structural damage to buildings (Andersen

et al., 2011; Foto et al., 2005; Schmidt, 2007). In terms of human health, fungi are associated with sick building syndrome (Cooley et al., 1998; Crook and Burton, 2010; Shoemaker and House, 2005; Soeria-Atmadja et al., 2010) and are known to trigger both allergies and asthma (Allermann et al., 2006; Crook and Burton, 2010). Fungi are also increasingly implicated in some serious human diseases, such as aspergillosis and cryptococcal meningitis in immunocompromised patients (Low and Rotstein, 2011) and hospital-associated fungal infections such as candidemia, a leading cause of bloodstream infections in the United States (CDC, 2009). In the United States, several endemic fungi in soils and plants are also known to cause disease in healthy humans, including coccidioidomycosis (i.e., valley fever), blastomycosis, cryptococcosis, and histoplasmosis, with coccidiomycosis and cryptococcosis being the two primary endemic fungal diseases in the Southwestern United States where our study was conducted (CDC, 2009).

Given the importance of fungi in the BE and the large estimated fraction of unknown and uncultured environmental fungi, successful application of culture-independent molecular approaches should improve our understanding of diversity and dynamics of BE fungi. Fungal diversity, as is true of the diversity of other microbes, has been significantly underestimated by culturing (Pitkäranta et al., 2011; Tonge et al., 2014; Yamamoto et al., 2010). Development of broad-spectrum polymerase chain reaction (PCR) primers targeting the internal transcribed spacer region (ITS) in combination with multiplexing and NGS have allowed new insight into fungal diversity in many environments, including human skin (Findley et al., 2013), indoor air (Adams et al., 2013a,b), and others (Dollive et al., 2012; Ghannoum et al., 2010; Schoch et al., 2012). The ITS-1 and ITS-2 regions sit between the small subunit (SSU) ribosomal and large subunit (LSU) ribosomal RNA genes and flank the 5.8S ribosomal RNA gene. The ITS regions are not integrated into the final ribosomal subunits and evolve much more quickly than the SSU or LSU ribosomal RNA marker genes (Hillis and Dixon, 1991). This genetic variation allows for a much finer taxonomic resolution and a deeper understanding of the true fungal diversity in any given setting.

In this study, we applied a multiplex PCR approach using an ITS-1 primer set conserved across the fungal kingdom and next-generation sequencing (NGS) to characterize the temporal dynamics of fungi in the BE using restroom surfaces as a model system. The previous study of Flores et al. (2011) on the bacterial diversity in restrooms found site-specific assemblages formed on various surfaces (e.g., the floor, toilet seat, and soap dispenser). More recently, Gibbons et al. (2015) found that restroom bacterial assemblages formed rapidly, were highly stable over time, and were primarily composed of bacteria from human skin and feces. Using DNA extractions from the same time series samples collected by Gibbons et al. (2015), we performed a molecular survey to determine the fungal diversity and dynamics on these surfaces. Analyzing the exact same samples collected by Gibbons et al. (2015) not only allowed us to assess the rate of accumulation of fungal diversity over time, but also allowed for direct comparisons between the identified fungal and bacterial assemblages. Finally, using the nearest relatives of the identified fungal sequences based on database comparisons, we determined the likely environmental sources of the restroom fungi and compared them with the sources of the bacteria from the same samples as reported by Gibbons et al. (2015).

Methods

Sample selection

Three surface types were sampled for this study: (i) the floor in front of the toilet seat, (ii) the toilet seat, and (iii) the soap dispenser pump (Gibbons et al., 2015). Samples were collected during an 8-week study between November 22, 2011 and January 31, 2012 from one male and one female restroom on the third floor of the North Life Sciences building at San Diego State University (SDSU), CA, USA. This occurred while school was in session, and the bathrooms were in constant use prior to sampling. Each of the three surfaces was sterilized with 10% bleach, allowed to sit for 20 min and rinsed with molecular biology-grade water. These surfaces were shown to be DNA- and RNA-free by epifluorescence microscopy using SYBR Gold (Invitrogen, Carlsbad, CA, USA) staining (Gibbons et al., 2015). The restrooms were opened for use 8 h after cleaning, and then, 1× phosphate-buffered saline (PBS) (Boston BioProducts, Ashland, MA, USA)-moistened sterile rayon-tipped swabs (MicroPur™ Swab P, Tonawanda, NY, USA) were used to collect samples from the three surfaces. Areas of 50.8 cm × 50.8 cm were swabbed for each sample. The entire surface was passed over once with the swab, which took approximately 45 s. The swab tips were broken off into 1.5-ml microtubes containing 500 μl of sterile 1× PBS solution and vortexed immediately for ten seconds. The samples were then stored at −20°C prior to further molecular analyses.

DNA extraction and PCR amplification

DNA extractions were performed on the samples using MO BIO PowerSoil® DNA Isolation Kits (MO BIO Laboratories, Carlsbad, CA, USA), and the extractions were stored at −20°C. To PCR amplify fungal ITS sequences from the samples, we used the ITS-1 forward

primer (ITS-1F ITS-1) sequence 5′-CTTGGTCATTTA GAGGAAGTAA-3′ (Gardes and Bruns, 1993), and the ITS-1 reverse (ITS-2 ITS-1) sequence used was 5′-GCTGCGTTCTTCATCGATGC-3′ (White et al., 1990). The positive PCR controls used were DNA extracted from *Saccharomyces cerevisiae* (i.e., baker's yeast) and a black mold that was growing in a home bathroom, while the negative control was molecular biology-grade water extracted by the same method as the positive controls. PCR conditions consisted of an optimized thermocycler protocol with an initial denaturing step of 95°C for 5 min, followed by 30 cycles of 98°C for 30 s, 55°C for 30 s, and 72°C for 60 s. The reaction ended with a final extension at 72°C for 10 min and was cooled and held at 10°C.

Fungal culture

Sabouraud dextrose agar (BD, Sparks, MD, USA) plates were inoculated with swab samples from the floor, toilet seat, and soap dispenser from two different female restrooms at SDSU. Samples used for culturing were collected on a different date from the original samples. Plates were incubated at 37°C for 24 h to encourage the growth of yeasts that thrive at human body temperature, and the same plates were then incubated at 25°C (room temperature) for 4 days to select for molds and room-temperature yeasts.

Multiplex NGS

Due to the lack of positive PCR amplification on soap dispenser and toilet seat surfaces, only the floor samples were used in multiplex (barcoded) PCR following the results of the fungal culturing and PCR. A unique identifier was added to the ITS-1 reverse primers to allow samples to be pooled together prior to sequencing. Specifically, a 12 base-pair Golay barcode was added to the 5′ terminus of the reverse primer (5′-*NNNNNNNNNNNN*CCGCTGCGTTCTTCATC GATGC-3′) (Fierer et al., 2008). The italicized Ns represents the different barcodes, the 'CC' is the linker, and the remaining nucleotides are a constant reverse primer used for all samples. PCR was performed on the 16 floor samples using the previously described reaction conditions but with 16 different barcoded reverse primers and a constant forward primer.

DNA amplicon sequencing

Samples were amplified using barcoded ITS-1 primers and submitted to the core facility at The Scripps Research Institute (La Jolla, CA, USA) where the samples were pooled at equimolar concentrations and sequenced on an Illumina MiSeq (Illumina, San Diego, CA, USA).

Sequence analyses

Sequencing data were analyzed using QIIME (v. 1.8.0 Quantitative Insights into Microbial Ecology, www.qiime.org; Caporaso et al., 2010). Reads with a quality score less than 25 were filtered out (split_libraries.py; QIIME), and the remaining reads were assigned to samples according to the corresponding barcode. Operational taxonomic units (OTUs) were selected at a 97% similarity threshold using UCLUST (Edgar, 2010), denoting species-level similarity using closed-reference OTU picking using the UNITE ITS database (Kõljalg et al., 2005) as a reference (pick_otus.py; QIIME). One representative of each OTU was selected for downstream analyses, and taxonomy was assigned to each representative (pick_rep_set.py, assign_taxonomy.py; QIIME). Core fungal diversity of the public restroom was defined as fungi found in at least 25% (i.e., four of the 16) of the restroom floor samples. To determine beta-diversity, we used the weighted Bray–Curtis (Bray and Curtis, 1957) method, and the unweighted Binary-Jaccard (Jaccard, 1912) method to create pairwise distance matrices for principal coordinate analysis (PCoA), which were visualized using Emperor (Vázquez-Baeza et al., 2013) (beta_diversity.py, principle_coordinates.py, make_emperor.py; QIIME). Alpha-diversity analysis of each sample was calculated using the Shannon metric (alpha_diversity.py; QIIME), and box plots were created using the R Project (http://www.r-project.org/) package 'vegan' (Dixon 2003). Beta-diversity comparisons were completed using ANOSIM (compare_categories.py; QIIME). We tested whether particular OTU abundances differed significantly between gender and sampling date using false discovery rate (FDR) corrected ANOVA analyses (group_significance.py; QIIME). Fungal–fungal and fungal–bacterial family abundance correlation was determined using the R Project (http://www.r-project.org/) package 'corrgram' with the Pearson product moment correlation coefficient to produce a color-coded heat map.

Results and discussion

A total of 48 samples were collected from the floor, toilet seat, and soap dispenser surfaces from both male and female public restrooms at SDSU (Table 1; Gibbons et al., 2015). Of the 48 samples, only the 16 floor samples produced PCR amplicons using the ITS-1 fungal primers. The toilet seat and soap dispenser samples were all amplicon-negative despite multiple attempts to generate PCR products, indicating a low level of fungal biomass on these surfaces. Fungal culture efforts also found fungi easy to isolate from floor surfaces compared with the toilet seat and soap dispenser surfaces (Figure 1). Conversely, the previous study by Gibbons

Table 1 Restroom sample information (adapted from Gibbons et al., 2015)

Collection ID	Collection date	Gender	Surfaces[a]	Samples collected (*n*)
F1	11/22/2011	Female	Floor, toilet seat, soap dispenser	3
M1	11/22/2011	Male	Floor, toilet seat, soap dispenser	3
F2	11/29/2011	Female	Floor, toilet seat, soap dispenser	3
M2	11/29/2011	Male	Floor, toilet seat, soap dispenser	3
F3	12/06/2011	Female	Floor, toilet seat, soap dispenser	3
M3	12/06/2011	Male	Floor, toilet seat, soap dispenser	3
F4	12/13/2011	Female	Floor, toilet seat, soap dispenser	3
M4	12/13/2011	Male	Floor, toilet seat, soap dispenser	3
F5	1/10/2012	Female	Floor, toilet seat, soap dispenser	3
M5	1/10/2012	Male	Floor, toilet seat, soap dispenser	3
F6	1/17/2012	Female	Floor, toilet seat, soap dispenser	3
M6	1/17/2012	Male	Floor, toilet seat, soap dispenser	3
F7	1/31/2012	Female	Floor, toilet seat, soap dispenser	3
M7	1/31/2012	Male	Floor, toilet seat, soap dispenser	3
F8	1/24/2012	Female	Floor, toilet seat, soap dispenser	3
M8	1/24/2012	Male	Floor, toilet seat, soap dispenser	3
				n = 48 samples

[a]Only floor samples were sequenced (see Results and discussion).

et al. (2015) found that bacterial ribosomal RNA genes were readily amplifiable from all sample types.

Next-generation sequencing of the barcoded ITS-1 amplicons for the PCR-positive floor surfaces resulted in 3 334 181 reads prior to quality filtering, and 1 144 353 reads remained after quality filtering, which removed barcodes and primers and apportioned sequences into one of the 16 corresponding sample libraries. Reads per sample ranged from 44 939 to 112 358 sequences with a mean of 71 522. While only 34% of reads remained after splitting libraries, which is markedly lower than other studies using the same PCR primer set, the final sequencing depth was more than sufficient considering the small number of samples. UCLUST closed-reference OTU picking resulted in clustering of 76.8% of the 1 144 353 reads, while 23.2% did not to cluster with sequences in the UNITE database. A total of 2550 unique OTUs were identified in all 16 samples with a range between 579 and 1147 OTUs and an average of 806 OTUs per sample. The majority of OTUs (59%) were identified as fungi but were not classifiable to phylum or below with the UNITE ITS reference database, but are indeed unique OTUs within the UNITE database (Figure 2). Within the UNITE database, 30% of the OTUs are not identified below the phylum level. Figure 2 shows the top 25 most abundant genera within the 41% of the identified fungi. In total, the identifiable fungi in our samples comprised a total of 348 genera from 157 fungal families in five different phyla over all samples (Figure 2).

Analysis of the 'core fungal diversity', defined as taxa present in at least 25% of all samples, found that 114 of the genera were in at least 25% of the restroom floor samples (Tables S1 and S2). Thirty-six families of fungi were found in all restroom samples, including the Mycosphaerellaceae, a diverse fungal family containing many known plant pathogens which composed ~10% of the sequence reads, and Teratosphaeriaceae, which are common *Eucalyptus* pathogens (Pérez et al., 2013). *Eucalyptus* trees originating from Australia have been planted by the millions in San Diego since the early 1800s (Stanford, 1970). The family Pleosporaceae (3.4% of the reads identified in 100% of the restroom floor samples) is often found on wood, stems, and leaves of dead plants (Kodsueb et al., 2006). Sequences matching 29 different genera of fungi were found in all of the restroom samples, including *Cladosporium*, *Saccharomyces*, *Alternaria*, *Cryptococcus*, *Phoma*,

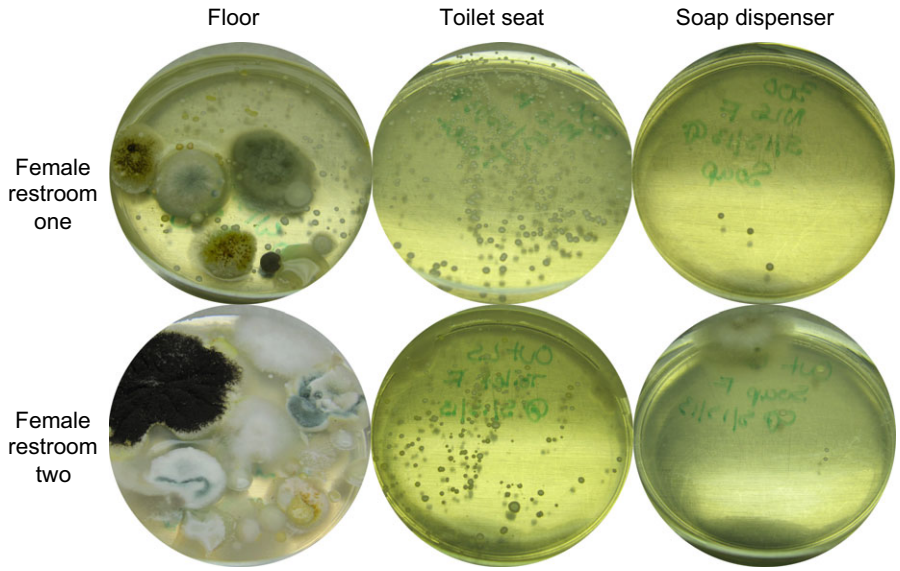

Fig. 1 Results using sabouraud dextrose agar to grow fungi from restroom surfaces. Fungal-specific agar plates were used to culture fungi from three different surfaces in the public restroom. Top row (left to right): female restroom 1 (F1), toilet seat, and soap dispenser. Bottom row (left to right): female restroom 2 (F2) floor, toilet seat, and soap dispenser.

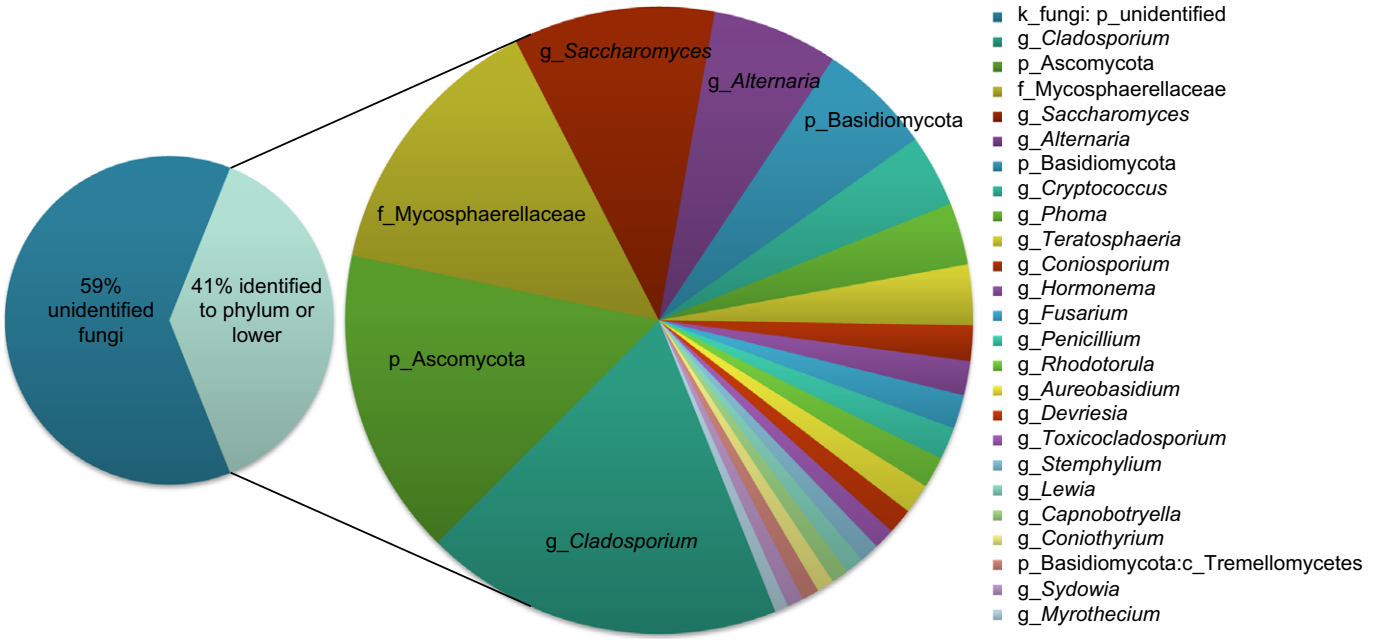

Fig. 2 Pie chart of the 25 most abundant identifiable fungal genera in the public restroom. This image shows a split pie chart highlighting the relative proportion of these 25 genera based on average relative abundance. Many of 41% of the community had a significant match to a known sequence while the rest were classified as fungi but were unidentifiable at the phylum level or below. The letters correspond to: k = kingdom, p = phyla, c = class, o = order, f = family, g = genus, and s = species. In the legend, genera are labeled clockwise in order of abundance, with *Cladosporium* being the most abundant and *Myrothecium* being the least abundant out of the top 25 fungal genera. The rightmost pie chart is drawn larger for improved readability.

Teratosphaeria, *Penicillium*, *Fusarium*, *Coniosporium*, *Hormonema*, *Fusarium*, *Penicillium*, and *Rhodotorula*. Although *Cladosporium* spp. were identified at a higher relative abundance than *Saccharomyces* spp., some *Cladosporium* were not identified to the species level. Thus, the most abundant identified species, per se, found in 100% of restrooms was *Saccharomyces cerevisiae*, which is a common fruit-associated fungus (Mortimer, 2000) and often used in fermentation and laboratory research (Karathia et al., 2011). In terms of potential pathogenic fungi, we did identify members of the genus *Cryptococcus* in all of the samples. However, none of the sequences were identified as *Cryptococcus gatii* or *Cryptococcus neoformans*, both of which can cause of cryptococcosis (Faganello et al., 2009). Moreover, none of the sequences were identified as the fungi known to cause coccidioidomycosis, blastomycosis, or histoplasmosis (although the latter is not endemic to the Southwestern United States). Incidentally, *Coccidiodes immitis* (i.e., the cause of 'valley fever') has been previously identified using this primer set (Dollive et al., 2012), and its sequence is also found in the UNITE ITS database.

Over the 2-month sampling period, the relative abundance of the five most commonly identified classes of fungi was remarkably stable in both male and female restrooms, although on day one phylum Ascomycota (class 'unidentified') and class Dothideomycetes fluctuated in their abundance compared to other days (Figure 3). The percent abundances of the 15 most abundant fungal classes showed similar stability over the course of the study (Figure 4). This is somewhat remarkable given the fact that the floor surface had only 8 h to accumulate each fungal assemblage. PCoA plots also suggested that fungal assemblages of samples collected on the same day tended to be more similar to one another than to other days based on Binary-Jaccard (ANOSIM; $r = 0.2299$, $P = 0.05$) and Bray–Curtis (ANOSIM; $r = 0.4576$, $P = 0.01$) beta-diversity metrics (Figure 5). We also found that the overall 'species' diversity of fungi, which were estimated by OTU counts, appeared to vary over time. Although not statistically significant, the Shannon diversity estimates found a lower mean diversity with a higher variance in the first 4 weeks of the study (November–December) compared to the last 4 weeks (December–January) (Figure S2). While 8 weeks do not appear to be a particularly long sample time, we note that December–January is typically the 'cold and rainy' season in San Diego, which could be expected to have a significant effect on fungal growth and dispersal. Additional long-term sampling follow-up studies are being performed to clarify the importance of seasonal effects on fungal diversity. The FDR-corrected analysis of variance (ANOVA) identified five OTUs with abundance levels that were significantly affected by the sampling date, including *Pleosporales* sp. ($P < 0.01$), *Serpula himantiodes* ($P < 0.01$), *Aspergillus fumigatus* ($P < 0.03$), and two uncultured unidentified fungal species (both $P < 0.05$). Considered together,

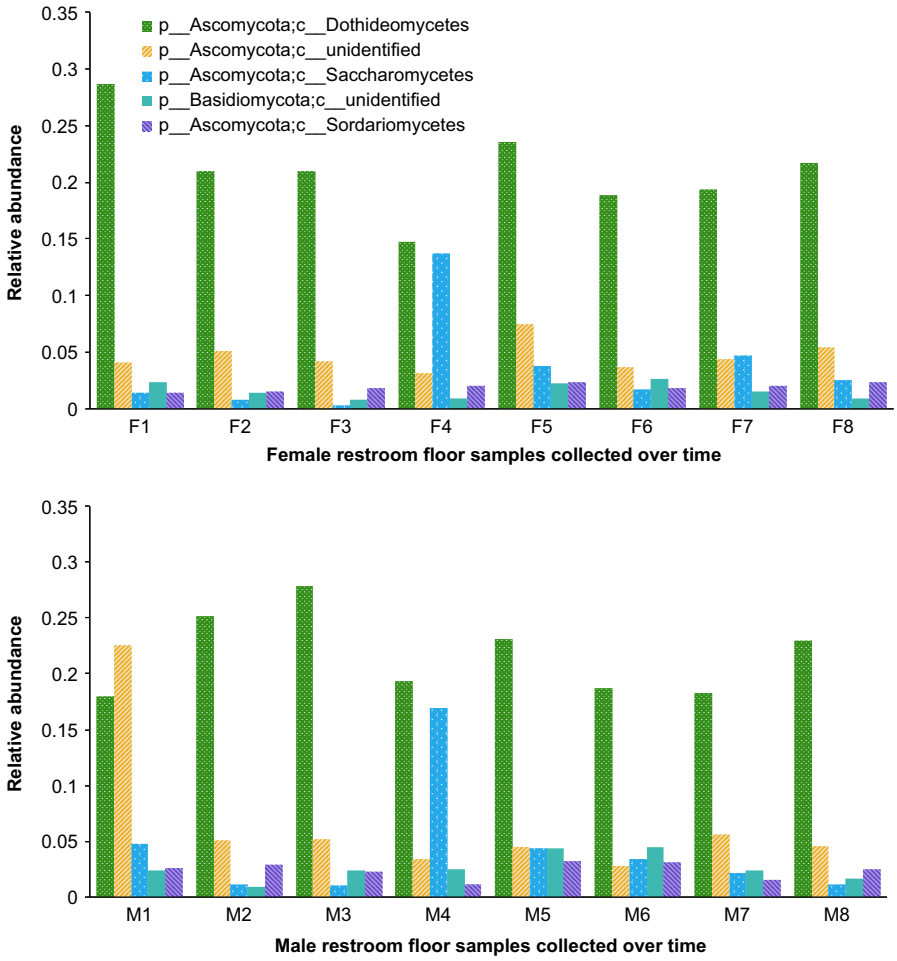

Fig. 3 Relative abundance of the top five most commonly determined classes of fungi arranged by collection time and grouped by gender. The *x*-axis indicates the restroom gender and time point, where F1 and M1 are female and male samples from week one. The colors correspond to each of the top identified fungal classes not including fungi that were unidentified at phylum level or lower (~59% of the OTUs). OTUs = operational taxonomic units.

these results indicated that external environmental factors and seasonal changes were likely the largest influence on the patterns of fungal diversity, although a much longer time course (i.e., many seasons in multiple locations) would be necessary to further examine this hypothesis.

In terms of the origins of the identified fungal assemblages, based on the matches to known fungal taxa the vast majority of the restroom fungi appear to come from environmental sources [e.g., soils, plants (Dean et al., 2012), and rock surfaces (Gueidan et al., 2011)] rather than human sources. A culture-independent study of residential surfaces in a university-housing complex also found that outdoor fungi were the most important contributor to passive fungal assemblages on surfaces (Adams et al., 2013b). This stands in sharp contrast to the bacterial assemblages from the same samples. Although it does not appear that the fungi themselves come from human sources, Hospodsky et al. (2014) demonstrated that humans contribute to the dispersion of fungi in air and may bring fungi indoors with them; however, this does not necessarily mean that the identified fungi are human-associated, which was not tested in this study.

Two previous studies by Flores et al. (2011) and Gibbons et al. (2015) determined that the primary source of bacteria found on restroom surfaces originated from human skin followed by the human gut with a small percent from the environment (Flores et al., 2011; Gibbons et al., 2015; Knights et al., 2011). Similar studies of homes, offices, and hospital environments have also found a predominance of skin-associated bacteria on surfaces (Bokulich et al., 2013; Brooks et al., 2014; Dunn et al., 2013; Hewitt et al., 2012, 2013). If fungi found in the restroom were primarily skin-associated, we would have expected to find more fungi in the toilet seat and soap dispenser surface samples, which come into direct contact with human skin and were dominated by skin bacteria. As these surfaces did not yield evidence of many fungi either via culturing or PCR, we analyzed the sequence abundance in the floor samples of the most common

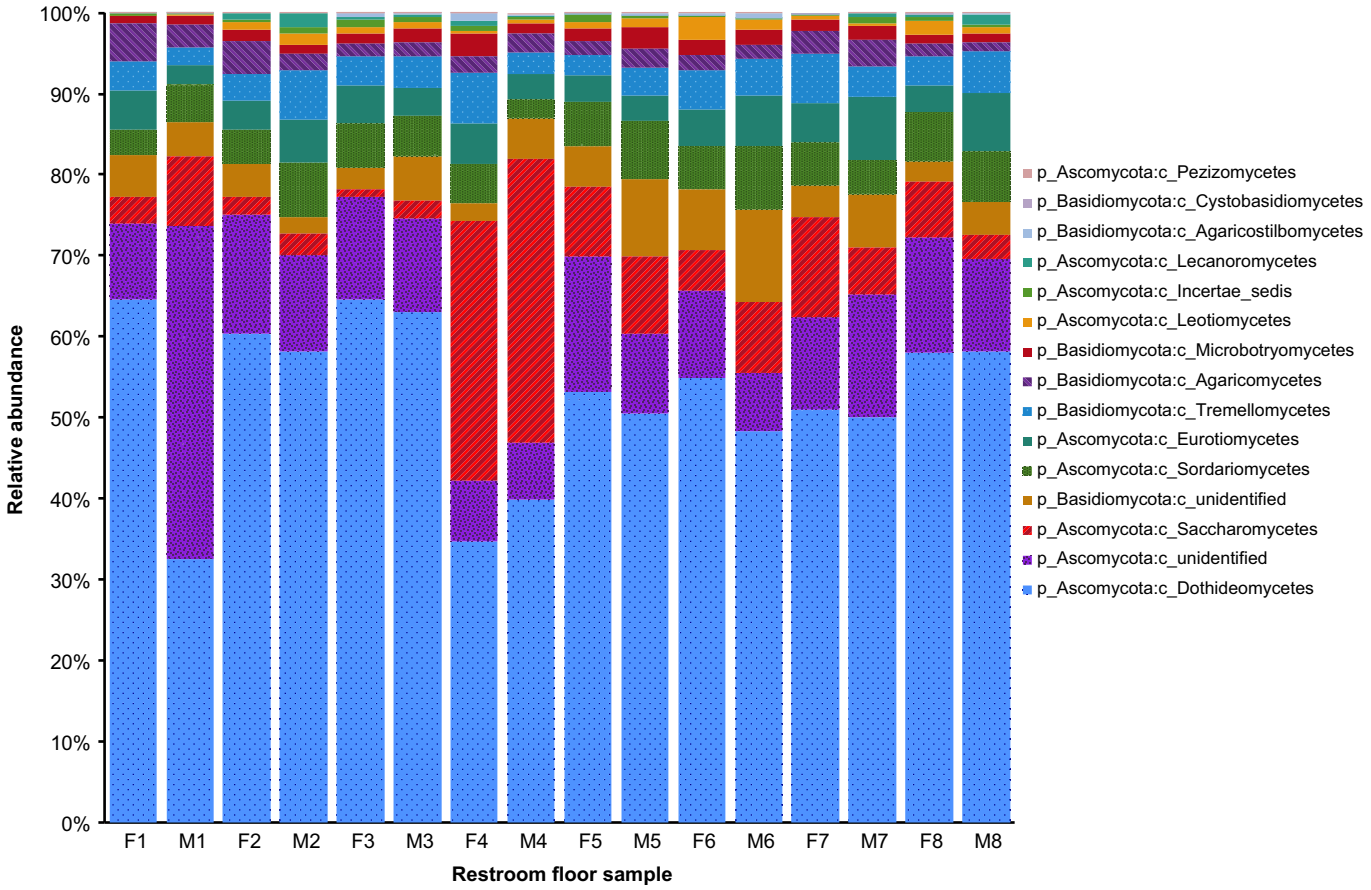

Fig. 4 Relative abundance of the 15 most abundant fungal classes found on the public restroom floor displayed as relative abundance. (In legend and figure, fungal classes are listed top to bottom, with least abundant on top and most abundant on bottom.) Along the *x*-axis are the identification numbers of the restroom floor samples over 2 months, where F1 and M1 are from the first week and F8 and M8 are from the last week (F = female restrooms, M = male restrooms). The colors correspond to each of the top identified fungal classes, not including the ~59% of the fungal OTUs that matched sequences which were unidentified at phylum or lower. OTUs, operational taxonomic units.

skin-associated fungi, which are *Malassezia* spp. (Findley et al., 2013). This genus has previously been shown to be identifiable by the primer set we used (Adams et al., 2013b; Dupuy et al., 2014), and 91 different reference sequences from this genus are also present in the UNITE ITS database (Kõljalg et al., 2005). However, a thorough search of our dataset found trace evidence of this genus (~0.01% of reads) on just two of the restroom floor samples despite deep sequencing. This result was surprising because skin-associated fungi would be expected to be relatively common in the public restroom as skin cells and their associated bacteria slough off readily from human bodies (Hospodsky et al., 2012). However, we note that most of our data come from just one surface (floor) as the other two surfaces we tested did not yield many fungi. A more thorough sampling of all the surfaces in restrooms might have revealed more skin-associated fungi, although it is also possible that the public restroom floor is a very distinct type of indoor environment that is not readily colonized by skin fungi. As more fungal ITS-1 sequence datasets become available, the hypothesis that fungi assembling in the public restroom are of environmental origin could be more directly tested by comparing BE 'sink' environments to a variety of 'source' environments, including humans and soils (Knights et al., 2011).

Given the samples were taken from floor surfaces, shoes appear to be the most likely source of the environmental fungi rather than passive deposition from the air particularly because these diverse assemblages formed within a few hours. This inference is also supported by the fact that we did not find diverse fungal communities on the toilet seat or soap dispensers (not normally touched by shoes). Certainly, passive accumulation of fungi in the air plays a generally important role in establishing BE fungal communities (Adams et al., 2013a,b). However, our data indicate that walking also may play a significant role and that future studies should aim to quantify the respective roles of air and shoe dispersal on indoor fungal assemblages.

When Gibbons et al. (2015) characterized the beta-diversity of bacterial communities using 16S sequencing, they found significant differences between

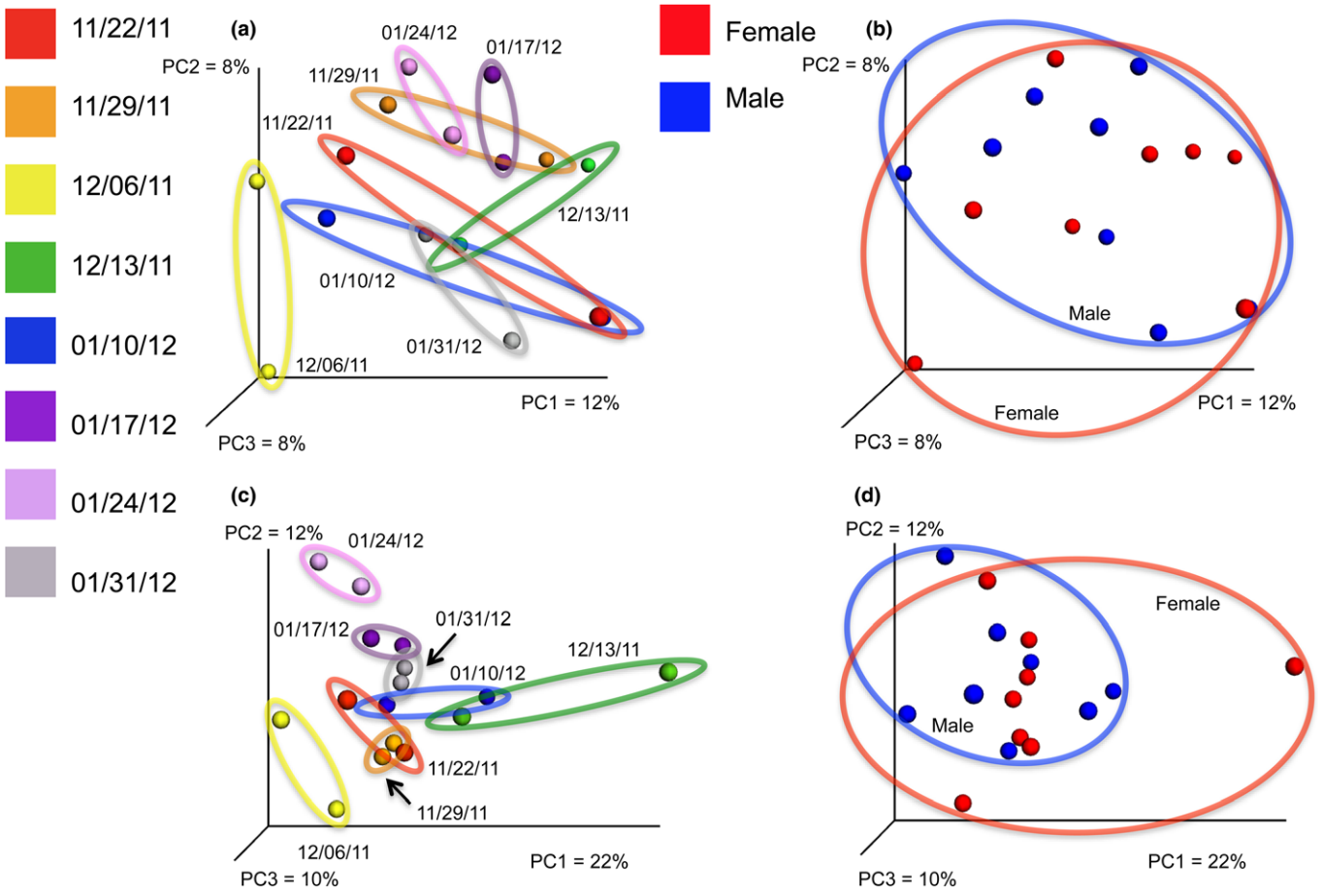

Fig. 5 Beta-diversity PCoA plots for date and gender on restroom floor samples. Beta-diversity analysis performed with the unweighted Binary-Jaccard metric for (a) date and (b) gender. Beta-diversity analysis performed with the weighted Bray–Curtis metric for (c) date and (d) gender. PCoA = principal coordinates analysis.

the bacterial diversity of male and female toilet seats but no significant difference in the bacterial diversity between the floor samples in male and female restrooms. For the floor fungal communities, we also did not find significant differences in beta-diversity between male- and female-associated communities with either Binary-Jaccard (ANOSIM; $P = 0.62$) or Bray–Curtis metrics (ANOSIM; $P = 0.71$; Figure 5). ANOVA tests corrected for multiple comparisons did not identify any OTUs that were significantly affected by restroom gender.

When viewing the rank abundance according to the Whittaker plot (Figure S3), the top 25 highest-ranked OTUs, which make up only 1% of the 2550 OTUs, accounted for 55% of the OTU abundance over all reads, meaning most organisms found in this environment are rare. Whittaker plots are used to visualize relative species abundances of ecological communities, as well as species richness and evenness. The slopes of the Whittaker curves have very steep gradients, indicating very uneven fungal assemblages in these samples, all of which are dominated by a small number of highly abundant OTUs.

Finally, we looked at the relationship between the fungal and bacterial diversity. Specifically, we did a pairwise correlation between the bacterial and fungal family abundances. After correcting for multiple comparisons, we only found two significant correlations, one of which was actually between plant chloroplasts and fungi (Figure S1). Abundances of OTUs matching to plant chloroplasts (Plantae: Streptophyta), often amplified by bacterial primers because of the chloroplast's evolutionary relationship to Cyanobacteria, were positively correlated with some fungal plant pathogens, including Pleosporaceae ($r = 0.78$, $P < 0.001$) and Teratosphaeriaceae ($r = -0.52$, $P < 0.05$). The soil- and drinking water-associated bacterial family Sphingomonadaceae (Hageskal et al., 2009; Narciso-da-Rocha et al., 2014; Vaz-Moreira et al., 2011) was found to be moderately positively correlated ($r = 0.65$, $P < 0.01$) with the fungal family Filobasidiaceae, which contains the soil genera *Cryptococcus* and *Filobasidium* (Karpouzas et al., 2009). Given that the putative sources of the fungi and bacteria are so different, it is perhaps not surprising to observe so few significant correlations.

Conclusion

Our analysis of fungal assemblages on restroom floor surfaces showed that fungi in public restrooms can be extremely diverse, yet can also form rapidly and in a highly predictive manner. These results suggest it should be possible to develop a baseline of expected environmental fungal diversity for a particular geographic locale. Development of a comprehensive seasonal fungal database will be important for fungal surveillance efforts and for detecting significant departures from typical conditions. Such departures could then be linked to incidences of human exposure and potentially to novel fungal pathogens.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Fungal-bacterial and fungal-fungal correlations, determined by Pearson product moment correlation coefficient using the R Project package 'corrgram'. Bacterial names are colored in blue and fungal names in red. The letters correspond to: k = kingdom; p = phyla; c = class; o = order; f = family. Each taxon was identified to the family level when possible, or the lowest level of taxonomy identification available. Positive correlations are indicted in blue and negative correlations in red. Significance levels are indicated by asterisks: $***P \leq 0.001$; $**P \leq 0.01$; and $*P \leq 0.05$.

Figure S2. Alpha diversity box-plots using Shannon diversity for public restroom floor samples. Values were averaged combining both male and female restrooms over 2 week blocks (four blocks total, where each block represents two male and two female restroom floor samples.). The height of the boxes indicates Shannon diversity variance and the central line is the average Shannon diversity within the sampling blocks.

Figure S3. Whittaker plot showing the OTU rank abundance for each sample.

Table S1. Genera identified in at least 25% of restroom floor samples constituting the core diversity of this environment. The letters correspond to: k = kingdom, p = phyla, c = class, o = order, f = family, and g = genus.

Table S2. Species identified in at least 25% of restroom floor samples constituting the core diversity of this environment. The letters correspond to: k = kingdom, p = phyla, c = class, o = order, f = family, g = genus, and s = species.

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