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PAPER

Comparison of DNA extraction methodologies used for assessing fungal diversity *via* ITS sequencing

William R. Rittenour,*[a] Ju-Hyeong Park,[b] Jean M. Cox-Ganser,[b] Donald H. Beezhold[a] and Brett J. Green[a]

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Traditional methods of assessing fungal exposure have been confounded by a number of limiting variables. The recent utilization of molecular methods such as internal transcribed spacer (ITS) sequencing of ribosomal RNA genes has provided improved insight into the diversity of fungal bioaerosols in indoor, outdoor and occupational environments. However, ITS analyses may also be confounded by a number of methodological limitations. In this study, we have optimized this technology for use in occupational or environmental studies. Three commonly used DNA extraction methodologies (UltraClean Soil kit, High Pure PCR Template kit, and EluQuik/DNeasy kit) were compared in terms of sensitivity and susceptibility to PCR inhibitors in dust for three common fungal bioaerosols, *Aspergillus versicolor*, *Rhizopus microsporus* and *Wallemia sebi*. Environmental dust samples were then studied using each extraction methodology and results were compared to viable culture data. The extraction methods differed in terms of their ability to efficiently extract DNA from particular species of fungi (*e.g. Aspergillus versicolor*). In addition, the ability to remove PCR inhibitors from dust samples was most effective using the soil DNA extraction kit. The species composition varied greatly between ITS clone libraries generated with the different DNA extraction kits. However, compared to viable culture data, ITS clone libraries included additional fungal species that are incapable of growth on solid culture medium. Collectively, our data indicated that DNA extraction methodologies used in ITS sequencing studies of occupational or environmental dust samples can greatly influence the fungal species that are detected.

Introduction

Fungal spores, hyphae and fragments are ubiquitous bioaerosols in indoor, outdoor, and occupational environments.[1] Personal exposure to fungal bioaerosols may cause or exacerbate a wide variety of diseases including hypersensitivity pneumonitis, allergic rhinitis, and asthma as well as a variety of mycotic disorders.[2,3] Traditional methods of evaluating fungal bioaerosol exposure have included viable culture methods or non-viable spore counts. Both of these analytical methods rely on trained microbiologists to identify unique morphological phenotypes of fungal colonies or dispersive structures such as conidia. Although these methods have aided in the initial characterization of fungal diversity associated with indoor and outdoor environments,[4,5] there are several well addressed limitations associated with these methods of assessment. Viable culture methods favor fecund species that can outgrow slow-growing species on

[a]*Allergy and Clinical Immunology Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health, 1095 Willowdale Rd., Morgantown, West Virginia, USA. E-mail: ikx8@cdc.gov; Tel: +1 304-285-5721 ext 7*
[b]*Field Studies Branch, Division of Respiratory Disease Studies, National Institute for Occupational Safety and Health, Morgantown, West Virginia, USA*

Environmental impact

Fungi are ubiquitous microorganisms that can cause or exacerbate respiratory morbidity. Accurately assessing the species of fungi present in a given environment (*i.e.* indoor workspace) is of utmost importance in determining the effect that they may (or may not) be exerting on inhabitants. Molecular methods of fungal species identification (*e.g.* sequencing of the internal transcribed spacer [ITS] region of rRNA genes) are becoming more common in the assessment of fungal burdens in indoor and outdoor environments. In this report, we demonstrate that the method of DNA extraction can greatly influence the fungal species that are detected by ITS sequencing. This information is crucial to researchers interested in utilizing fungal ITS sequencing to assess the fungal burden in different environments.

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solid culture media. Varying nutritional and physiological requirements will hinder growth for some species but may be optimal for others, depending on the growth media chosen. To control for these limiting variables several different media and incubation temperatures may be required to capture the diversity of fungi in exposure assessment studies. Additionally, some species are incapable of growth on solid nutrient media and cannot be quantified using culture methods. Non-viable spore counts also have limitations such as the differentiation of amerospores, morphologically indiscernible dictyospores, and hyphal fragments.[6]

Given the limitations associated with traditional methods of assessment, recent developments in genomic sequencing technologies have enabled the differentiation of DNA sequence variation to characterize fungal diversity. Although several genomic loci have been used as targets for sequence comparison,[7–9] the most used locus is the Internal Transcribed Spacer (ITS) regions of the nuclear rRNA gene.[10] The ITS regions mutate rapidly, and are highly variable even between closely related species.[11] Given the sequence variability and the availability of thousands of annotated fungal sequences in public databases, the ITS regions have been recently utilized as targets for assessing fungal diversity. Universal fungal primers have been designed for the conserved regions that amplify the ITS through polymerase chain reaction (PCR).[12] These ITS amplicons can then be sequenced and compared to database sequences to identify the diversity of fungi present in the environmental sample. This technique has been used to study fungal diversity in soil,[13–15] frond tissues,[16] aerosol samples,[17] and dust samples from indoor environments.[18,19] These studies have identified a number of fungal taxa that have not been previously implicated in indoor environments, particularly those derived from the basidiomycetes.[17,19]

Despite the advantages of ribosomal DNA (*i.e.* the rRNA gene) sequencing, several limitations have been identified. Universal fungal primers may be biased toward certain fungal taxa[20] and the PCR amplification process may favor the amplification of shorter rDNA sequences.[21] These two factors may cause an overestimation of particular fungal taxa and misrepresent the fungal diversity of a particular sample. Correctly identifying a fungal taxon based on sequence similarity also requires well-curated reference sequences. Recent estimates place 10–20% of sequences to be incorrectly annotated.[22,23] To date, the influence of various DNA extraction methods on the identification of fungal diversity has, to our knowledge, remained relatively uncharacterized. Given the heterogeneous nature of environmental samples (surface dust, air particulate *etc*), different DNA extraction methods may have different efficacies. This aspect was recently observed by Pitkäranta *et al.*, who identified that prevalent indoor contaminants such as *Penicillium spp.* and *Aspergillus spp.* were not present in ITS sequence libraries. However, these genera were prominent in viable culture counts derived from the same samples.[19] Based on these observations, we hypothesize that certain fungal species may be more resistant to DNA extraction. Supporting this hypothesis, Haugland *et al.* demonstrated that smaller conidia yielded less DNA across several extraction methods.[24] To provide more reproducible and standardized methods for future exposure assessments utilizing this methodology, the objective of this study was to determine the effects of different DNA extraction techniques on sensitivity, PCR inhibition, and resultant fungal species detected in an rDNA amplicon clone library.

Materials and methods

Fungal strains and culture

Fungal species were acquired from the American Type Culture Collection (ATCC) or the National Institute for Occupational Safety and Health (NIOSH) culture collection. The fungal species studied included the ascomycete, *Aspergillus versicolor* (ATCC 4448), the basidiomycete, *Wallemia sebi* (NIOSH strain 26-41-01), and the zygomycete, *Rhizopus microsporus* (NIOSH strain 17-59-14). These fungi were selected as representatives of three major fungal phyla and also because of their prevalence in indoor and occupational environments.[5] To compare differences in DNA extraction efficacy between different fungal species, the budding yeast *Geotrichum candidum* (UAMH 7863; a standard control for the quantitative PCR-based environmental relative moldiness index) was also included in qPCR analyses.[25]

To generate frozen stocks, spores were harvested from 14-day-old malt extract agar (MEA; 2% malt extract, 2% dextrose, 0.1% peptone, 1.5% agar) cultures in 0.05% Tween 20 (PBST). Spore concentrations were measured with a hematcytometer and adjusted to 10^7 particles/mL in 15% glycerol. Spores were aliquotted into 200 μl single-use volumes and transferred to −80 °C for storage.

Genomic DNA extraction procedures

Three DNA extraction methods were compared in this study: 1) a previously published method[26] that utilizes a combination of EluQuik (Whatman, Kent, UK) and DNeasy Blood and Tissue DNA extraction kits (Qiagen, Valencia, CA, USA); 2) the UltraClean Soil DNA isolation kit (MoBio Laboratories Inc., Carlsbad, CA, USA); and, 3) the High Pure PCR Template kit (Roche, Basel, Switzerland). For each kit, the cell lysis method employed involved a bead milling step, as this mechanical method of lysis has been shown optimal for DNA extraction.[24] The UltraClean Soil kit alternative method was utilized as described in the manufacturer's instructions. For the High Pure PCR Template kit, samples were lysed in 350 μL of the kit's lysis buffer using a 300 mg glass beads (Sigma-Aldrich, St. Louis, MO, USA) and a bead beater (BioSpec Products, Bartlesville, OK, USA) for 15 seconds at high speed. The samples were then centrifuged at 21,000 *x g* for 1 min, and the supernatant was mixed with 200 μL of the kit's binding buffer before application to the filter. The sample was then washed and eluted according to the manufacturer's instructions. In each extraction method, the DNA was eluted in 100 μL. The eluate was then reapplied to the filter for a final DNA volume of 100 μL. Genomic DNA extractions and rDNA amplification (see below) were performed at least in duplicate.

Sample preparation

Aliquotted frozen spore stocks were thawed and serially diluted to 10^6, 10^5, 10^4, 10^3, 10^2, and 10^1 spores per mL. Spores from the different species were combined at the ratio of 1:1:0.25

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(*A. versicolor*: *W. sebi*: *R. microsporus*). This ratio was utilized because *R. microsporus* spores typically contain four nuclei,[27] whereas *A. versicolor* and *W. sebi* are uninucleate. These desired spore concentrations were combined and adjusted to a final volume of 50 μL. The samples were then processed with each DNA extraction method as described above.

Different masses (0 mg, 5 mg, 10 mg, and 20 mg) of ultrafine Arizona test dust (Powder Technology Inc., Burnsville, MN, USA) were spiked with 10,000, 10,000, and 2500 *A. versicolor*, *W. sebi*, and *R. microsporus* spores, respectively. These samples were then processed with each DNA extraction method. The dust spiking experiments were performed at least twice. Two floordust samples collected from a water-damaged office building were analyzed for fungi by viable culture counts using methods described previously.[28] Genomic DNA was collected from 15 mg of the same office building dust samples using the three methods described above. Fungal rRNA genes were amplified, cloned, and sequenced from these dust samples as described below.

PCR, cloning, and sequencing

Several universal fungal primers were tested on various fungal DNA stocks, and two universal fungal primers, Fun18Sf[19] and ITS4[12] were selected for the study as PCR products were acquired for all fungal isolates tested in the preliminary experiments (data not shown). Fungal rDNA sequences were amplified using Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) according to the methods described previously.[19] Six 50 μL replicate PCR reactions were run for each sample using 5 μL of DNA template. These replicates were then combined and the rDNA amplicons were purified using a PCR purification kit (Axygen, Union City, CA, USA) according to the manufacturer's instructions. Five μL of this purified product was then run on a 1% agarose gel containing 0.4 μg/mL ethidium bromide and examined for amplicons using a ultraviolet gel doc (Alpha Innotech, Santa Clara, CA, USA). DNA extraction and PCR were performed three times to test the efficiency of each DNA extraction method at yielding amplifiable DNA. As an extraction-independent control, a large-scale method was used to extract DNA from mycelia or spores of the three standard fungal species.[29] The DNA concentrations of these controls was then measured with a NanoDrop spectrophotometer (Thermo Sci., Waltham, MA, USA) and mixed in equimolar concentrations. This control DNA was serially diluted and 300 pg (100 pg per species) was used in each of the six replicate PCR reactions described above.

Fungal rDNA amplicons were cloned into the pDRIVE vector using a PCR cloning kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Ligated plasmids were then transformed into TOP10 chemically competent *Escherichia coli* cells (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. The transformants were spread onto Luria-Bertani (LB) agar plates containing 100 μg/mL ampicillin and a top layer of X-gal. The plates were then incubated overnight at 37 °C.[30] White colonies were selected and cultured in 2 mL LB media containing 100 μg/mL ampicillin overnight at 37 °C, and plasmid DNA was isolated with a miniprep kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. At first, the isolated plasmid DNA was screened for fungal rDNA inserts with primers Fun18Sf and ITS4. However, the percentage of positive transformants was typically high (~96–99%), so this pre-screening step was only used in preliminary experiments.

Plasmid DNA samples were loaded into 96-well plates and sent to the University of Georgia Genomics Facility for Sanger sequencing analysis. For testing standard spore stocks, only one sequencing primer was used for sequencing that amplified either the ITS1 or ITS2 region (depending on the orientation of the amplicons in the pDRIVE vector). This approach was sufficient for distinguishing between the three standard fungal species. The observed sum of clones across three independent DNA extraction experiments for each species was tested against an expected number (assuming no bias) with a χ^2 test in Microsoft Excel. For unknown dust samples, both a forward and reverse sequencing primer was used so that the full ITS1-5.8S-ITS2 region was obtained for optimal fungal speciation. Vector sequence data were trimmed, and forward and reverse sequences were assembled using Geneious Software (Biomatters Ltd, Auckland, New Zealand). Sequence data were then clustered into operational taxonomic units (OTUs) with MOTHUR software[31] using a 97% similarity cutoff. Sequences representative of each OTU were then used in a BLAST search against NCBI's database. The BLAST output file was processed with the MEGAN program[32] to determine the closest taxonomic relative to each sequence.

Quantitative PCR (qPCR)

Real-time PCR was used to quantify the amount of amplifiable DNA recovered following each extraction method. DNA was extracted from serial dilutions (10^5 to 10^2) of *A. versicolor* and *G. candidum* spores using each of the kits/protocols described above. Reactions used the Avers2-2 and Gcand2 primer/probe sets described previously (http://www.epa.gov/nerlcwww/moldtech.htm). The final volume of qPCR reactions was 25 μL with the following concentrations of PCR components: 1X TaqMan Fast Universal PCR Master Mix (Applied Biosystems), 1 μM forward primer (for Gcand2 assay), 5 μM forward primer (for Avers2-2 assay), 1 μM reverse primer, 0.2 μg/μL BSA, 100 nM probe, and 5 μL of template DNA. The cycling parameters were as follows: 50 °C for 1 minute, 94 °C for 20 seconds (initial denaturation), and then 40 cycles of 94 °C for 3 seconds and 60 °C for 30 seconds. Genomic DNA extraction and qPCR analysis of the standard spore/cell stocks was performed three times, with 3 replicates per qPCR reaction. The cycle threshold (Ct) values were then compared among genomic DNA extraction methods.

Results

Sensitivity of genomic DNA extraction methods

To test the efficacy of each DNA extraction method, different concentrations of spores were processed with each kit. Both homogenous and heterogeneous spore mixtures were processed in triplicate (Fig. 1). In general, the High Pure PCR Template kit was the most efficient extraction method and yielded amplifiable template from as little as 100 spores of each tested species (Fig. 1A). Conversely, the UltraClean soil kit was the least effective, requiring at least 10,000 *A. versicolor* and *W. sebi*

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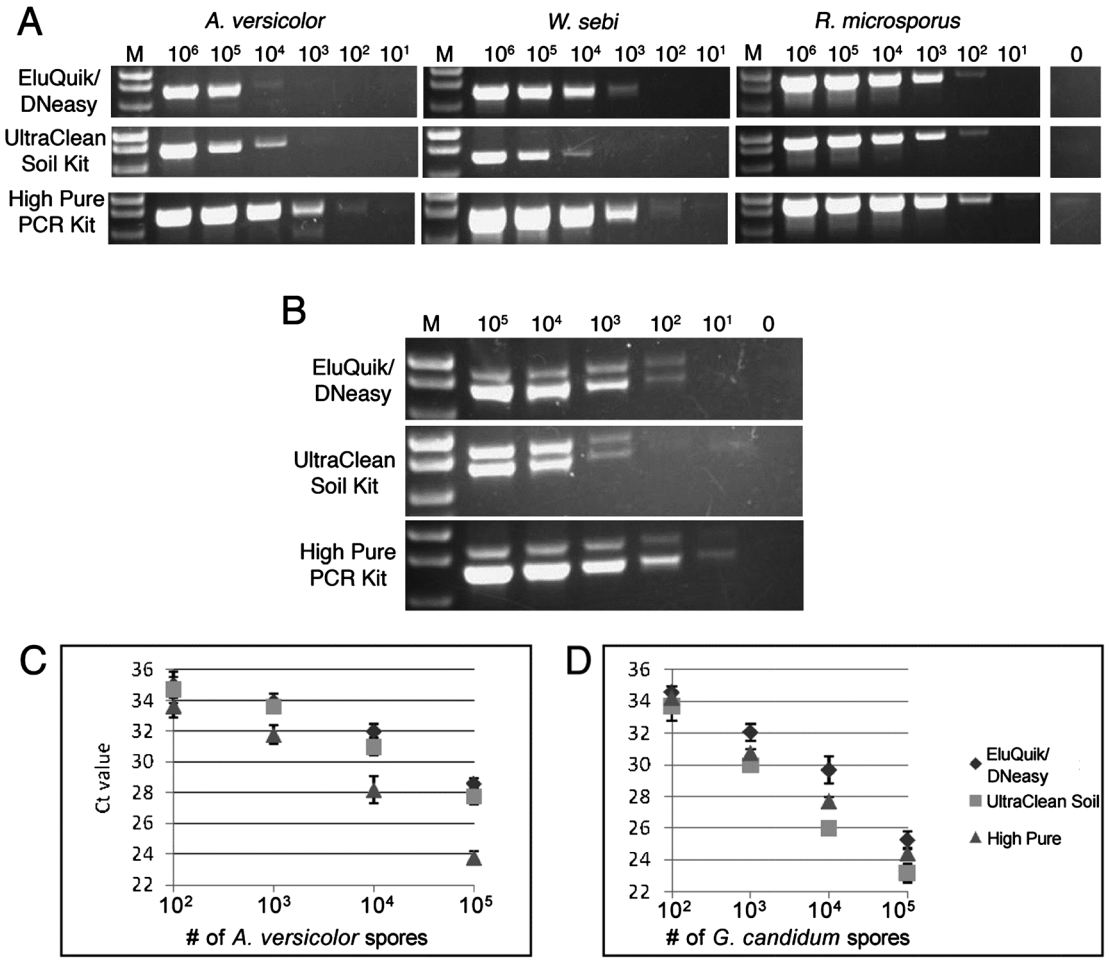


Fig. 1 Efficiency of the different DNA extraction methods. **A**. Genomic DNA was isolated from different concentrations of spores using three different DNA extraction methods. **B**. Genomic DNA was isolated from different concentrations of a heterogeneous spore mixtures (containing a 1:1:0.25 ratio of *A. versicolor*: *W. sebi*: *R. microsporus* spores). 'M' = DNA marker, with the bands representing 1000 bp, 850 bp, and 650 bp from top to bottom, respectively. Lanes marked with '0' represent negative controls in which the input for DNA extraction was water containing no spores. Panels A and B are representative images of three independent experiments, where dilutions and DNA extractions were performed three separate times. **C.** qPCR standard curves of *Aspergillus versicolor* genomic DNA isolated with each of the three DNA extraction methods. **D.** qPCR standard curves of *Geotrichum candidum* genomic DNA isolated with each of the three DNA extraction methods. Legend displayed in panel **D** is applicable for panel **C**. Error bars show +/− standard error across three experiments, with three replicates per experiment.

spores for amplification (Fig. 1A). However, the UltraClean soil kit performed just as well as the other two kits regarding its ability to yield amplifiable DNA from as little as 100 *R. microsporus* spores (Fig. 1A). Notably, both the EluQuik/DNeasy and UltraClean kits failed to yield amplifiable DNA when ≤1000 *A. versicolor* spores were used as template (Fig. 1A). While there appeared to be species-specific variability in extraction efficiency, these variations were also applicable to the heterogenous spore mixture, with the High Pure PCR Template kit identified as the most sensitive, followed by the EluQuik/DNeasy kit, and lastly, the UltraClean soil kit (Fig. 1B).

To further test the efficacy of the different DNA extraction methods on different fungal species, real-time quantitative PCR (qPCR) was performed on DNA extracted from standard concentrations of *A. versicolor* and *G. candidum* spores. Across three separate experiments, DNA extracted from *A. versicolor* spores with the High Pure PCR Template kit consistently yielded lower cycle threshold (Ct) values (compared to the other extraction kits) for input spore counts from 10^3–10^5 spores (Fig. 1C). In contrast, this trend did not apply to *G. candidum* spores, where DNA extracted with the UltraClean Soil kit appeared to yield the lowest Ct values (Fig. 1D). These data suggest that the DNA extraction methods vary in their capacity for recovering DNA from various fungal species.

Species biases associated with genomic DNA extraction methods

Mixtures of *A. versicolor*, *W. sebi*, and *R. microsporus* spores containing a 1000:1000:1000 nuclear ratio were processed for DNA using the three extraction methods described previously. Ribosomal RNA genes were then amplified and clone libraries were constructed for sequence analysis (~95 clones). Overall, *A. versicolor* was significantly under-represented in clone libraries constructed from EluQuik/DNeasy and UltraClean amplicons (Table 1). The High Pure PCR Template kit provided the most reproducible clone library ratios that were not

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Table 1 Number of clones recovered with different DNA extraction kits

	No. of clones recovered[a]			
Extraction Method	A.v.	W.s.	R.m.	χ^b
EluQuik/DNeasy	6	51	38	<0.01[c]
UltraClean Soil	9	29	53	<0.01[c]
High Pure PCR	34	41	26	0.19
Standard DNA	20	43	31	0.01[c]

[a] number of clones with sequence identity to A. versicolor (A.v.), W. sebi (W.s.) or R. microsporus (R.m.). Values are sums across three independent experiments. [b] P-value are calculated from a chi-square goodness of fit test. [c] significance $p \leq 0.05$.

significantly different (Table 1). Interestingly, the control library constructed with rDNA amplicons from PCR reactions containing equimolar concentrations of standard DNA from each fungal species also had a bias against *A. versicolor* (Table 1). These data, coupled with the relatively low efficiencies of the EluQuik/DNeasy and UltraClean soil kits in yielding a suitable amount of *A. versicolor* DNA for amplification (Fig. 1A), suggest that DNA extraction, amplification, and cloning of rDNA amplicons may create a bias against certain fungal species.

Efficacy of genomic DNA extraction methods at removing PCR inhibitors associated with dust

When monitoring occupational environments for fungi, dust samples are often preferred over air samples as they provide a longer temporal interval of exposure.[33,34] However, dust samples may be replete with PCR inhibitors that can confound molecular analyses for fungi.[35] In order to test the ability of each DNA extraction method to remove these inhibitors, a heterogeneous mixture of spores was mixed with different masses of dust and then processed with the DNA extraction kits. PCR amplification of DNA isolated using the EluQuik/DNeasy method was completely inhibited by as little as 5 mg of the dust, while the High Pure PCR Template kit generated amplifiable DNA from samples spiked with 5 mg of dust but not from the 10 mg spiked samples (Fig. 2). In contrast, the UltraClean soil kit yielded amplifiable DNA from samples spiked with 10 mg of the dust,

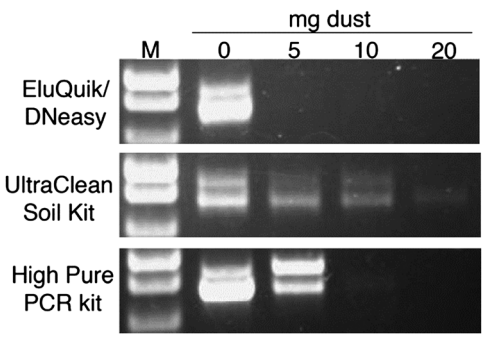


Fig. 2 Removal of PCR inhibitors by the different DNA extraction methods tested. DNA was isolated from 10,000 spores spiked with dust. Resultant DNA was used as template to amplify rDNA sequences. 'M' = DNA marker, with the bands representing 1000 bp, 850 bp, and 650 bp from top to bottom, respectively.

and although PCR inhibition was observable, faint bands were observed even when spiked with 20 mg of dust (Fig. 2).

Comparison of genomic DNA extraction methods on building dust samples

The observation of bias in standard spore stocks suggests that similar discrepancies may exist when environmental samples are processed. To test the effect that different DNA extraction methods may have on the results of fungal ITS sequencing studies, two separate floor dust samples (A and B) collected during a field study of occupational health problems in a water-damaged office building, were processed with each DNA extraction method. During the first screening, the EluQuik/DNeasy kit failed to yield amplifiable DNA from 15 mg of the two (A or B) dust samples (data not shown). In the second screening using the EluQuik/DNeasy kit, amplifiable DNA was obtained from 15 mg of dust from sample A, but not sample B. To overcome potential PCR inhibitors with the EluQuik/DNeasy kits, the dust sample (in 400 μl lysis/binding buffer mixture) was diluted 1:10 in the same volume of lysis/binding buffer before DNA extraction; this dilution enabled the amplification of DNA (data not shown).

Clone libraries of dust samples and comparison to viable culture methods

The ITS clone libraries generated from the two dust samples varied greatly among the three DNA extraction methods. The EluQuik/DNeasy method yielded a greater diversity of fungi than either the UltraClean Soil kit or the High Pure PCR Template kit (Fig. 3; Table 2). For sample A, there was very little agreement between the DNA extraction methods regarding the predominant fungal OTUs (Fig. 3A). Table 2 illustrates the EluQuik/DNeasy method yielded OTUs with ITS sequences very similar to an uncultured member of the Pleosporales (100% sequence identity), Cladosporium (99% identity), and *Fusarium* (99% identity) as the predominant genera, whereas the UltraClean soil kit yielded *Chaetomium*/*Thielavia* (96% identity) as the predominant OTU and the High Pure PCR Template kit yielded *Eurotium* (98% identity) as the predominant OTU (Table 2). However, the UltraClean Soil kit and the High Pure PCR Template kit both yielded *Eurotium halophilicum* (99% identity) as the predominant fungal OTU (~77%) in the dust sample B (Fig. 3B, Table 2). The EluQuik/DNeasy kit yielded only 15.4% *E. halophilicum* clones in the sample B, and also yielded a relatively high number of clones (compared to the other extraction methods) of the genus *Pichia* (92% identity; Fig. 3B; Table 2). These data demonstrated that the method of DNA extraction can greatly influence the outcome of ITS sequencing/cloning studies of fungal diversity.

Discussion

The decreasing costs of molecular analyses, coupled with the well-documented limitations of viable and non-viable analyses of fungi in environmental samples, has spurred the use of DNA analyses for assessing fungal loads in soil, dust, and air samples.[14,17–19] Although the sensitivities of different methods have been empirically tested, the effects of varying DNA

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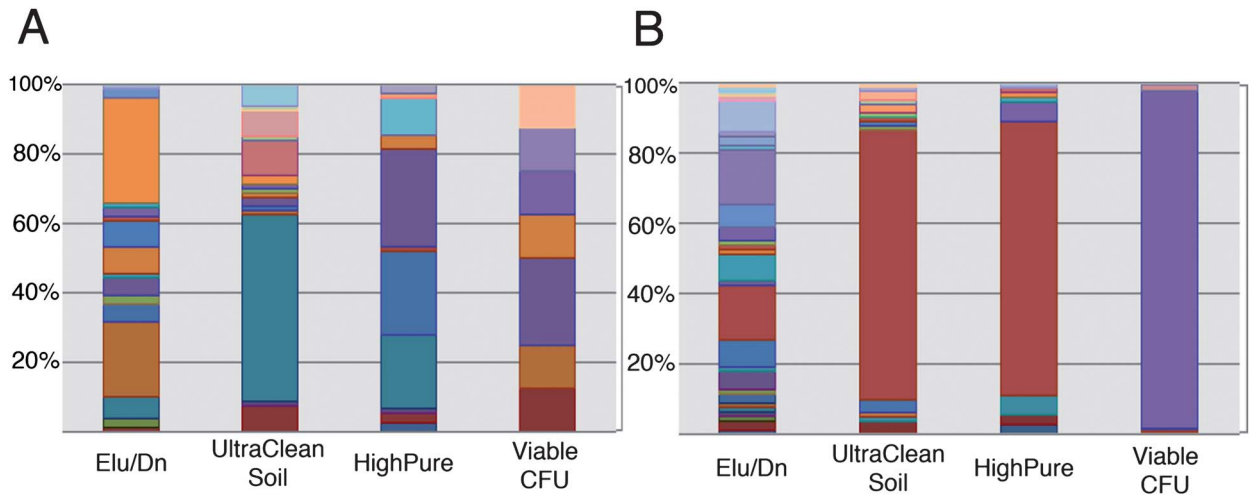


Fig. 3 Graphical representation of the diversity of fungi in two floor dust samples (A and B) collected from a water-damaged office building, each processed with the different DNA extraction kits and also sampled for viable cultures. Different colored blocks represent different operational taxonomic units (OTUs). See Table 2 for detailed percentages of each OTU.

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extraction protocols remains a caveat in the field of fungal ITS sequencing. In this study, the DNA extraction methods tested showed differences in (1) the ability to purify DNA from low quantities of template spores for PCR amplification, (2) the efficacy of extraction among species (*e.g. A. versicolor*, *W. sebi, R. microsporus, and G. candidum*), and (3) the susceptibility to PCR inhibitors. When processed with different DNA extraction kits, dust samples collected from a water-damaged office building yielded variable ITS clone libraries. Particularly, the species composition of the ITS clone library generated with the EluQuik/DNeasy method differed greatly from the composition of the libraries generated with the other two kits. These data demonstrate the importance of empirically testing DNA extraction methodologies prior to exposure assessment studies. Additionally, the variability observed in clone numbers generated with different DNA extraction techniques further demonstrates the care that must be taken when interpreting results of the prevalence of various fungal species.

The High Pure PCR Template kit was found to be the most sensitive DNA extraction method for *A. versicolor* and *W. sebi* spores (Fig. 1A). Interestingly, extraction efficiency was not only dependent on the methodology, but also on the fungal species from which DNA was being extracted. Quantitative PCR data showed that the High Pure PCR Template kit was most efficient for *A. versicolor* spores, but the UltraClean Soil kit was the most efficient for *G. candidum* spores (Fig. 1C). Also, DNA derived from *R. microsporus* spores was extracted in greater concentrations than *W. sebi* or *A. versicolor* across all three extraction kits (Fig. 1A). This observation may be partially explained by *R. microsporus* containing 4 nuclei per spore (*i.e.* more DNA to be extracted per spore), compared to *W. sebi* and *A. versicolor* that are uninucleate.[27] However, this difference only partially explains this observation as the EluQuik/DNeasy extraction kit required a 100-fold greater number of *A. versicolor* spores (10,000) for ITS amplification, compared to *R. microsporus* (100 spores; Fig. 1A). Another contributing factor may be associated with the spore wall composition of these fungi, which are likely to differ substantially. Although the spore wall of *A. versicolor* has not been studied in detail, the spore walls of other *Aspergillus* species are covered with a rodlet layer composed of cystein-rich hydrophobin proteins.[36–38] In addition to their small size, the rodlet layer confers elasticity that may functionally protect the spores during the bead beating steps.[39] The High Pure PCR Template kit may be more efficient at extracting *A. versicolor* DNA because it uses a lysis buffer that contains 4 M urea, a strong protein denaturant that may assist in degrading the rodlet layer of *A. versicolor* spores. In contrast, the EluQuik lysis buffer contains guanidine thiocyanate that is structurally similar to urea, but denatures proteins by a different mechanism.[40] The EluQuik lysis buffer is also diluted in binding buffer before the lysis step, which may reduce its efficacy. In contrast, the UltraClean Soil kit lysis buffer contains sodium dodecyl sulfate (SDS) as the main denaturant, which is not efficient at solubilizing hydrophobic proteins.[41] Other variables, such as bead size, type, and speed/length of bead beating are also likely to contribute to DNA extraction efficiency. Currently, we are testing the different lysis methods for their efficacy and aim to determine the role of the *Aspergillus* rodlet in protecting spores during DNA extraction.

When heterogeneous spore mixtures were tested, a similar pattern of sensitivity was observed between each DNA extraction kit. The High Pure PCR Template kit was the most sensitive, followed by the EluQuik/DNeasy kit, and the UltraClean Soil kit (Fig. 1B). Amplicons from the 10^3 treatment were cloned into an *E. coli* library and sequenced to test the biases associated with each kit. The EluQuik/DNeasy and UltraClean kits exhibited a bias against *A. versicolor*, likely caused by the inefficiency of these kits at generating amplifiable DNA from *A. versicolor* (Table 1; Fig. 1A). In contrast, an equal distribution of clones among the three species was observed with the High Pure PCR Template kit (Table 1). The practical implication of these data is clear; environmental samples containing equal concentrations of *A. versicolor* that are analyzed with either the UltraClean Soil kit or the EluQuik/DNeasy kit would detect fewer *A. versicolor* than the other two fungi, even though they are present in equal concentrations. Interestingly, an equimolar DNA mixture that

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Table 2 Percentage of fungal OTUs recovered in two building dust samples (A and B)

	Sample A					Sample B			
Species[a]	Elu/Dn[b]	Soil[b]	HighPure[b]	Viable CFU %[c]	Species[a]	Elu/Dn[b]	Soil[b]	HighPure[b]	Viable CFU %[c]
Coniosporium sp.	0	0	2.7	0	*Alternaria sp.*	1.3	0	2.8	0
Aspergillus sp.	1.3	7.5	2.7	12.5	*Aspergillus sp.*	2.6	3.7	2.8	0.5
Cephalosporium sp.	2.5	0	0	0	*Botryobasidium*	1.3	0	0	0
Chaetomium globsum	0	1.3	1.3	0	*Candida sp.*	1.3	0	0	0
Chaetomium/thielavia	6.3	53.8	21.3	0	*Capnobotryella sp*	1.3	0	0	0
Cladosporium sp.	21.5	1.3	0	12.5	*Chaetomium/Thielavia*	1.3	0	0	0
Uncultured Sordariomycetes sp. (FR682418)	5.1	1.3	24.0	0	Chaetothyriales sp. FGPMC2	2.6	0	0	0
Corynascus verrucosus	0	0	1.3	0	*Cladosporium sp.*	0	0	0	1
Epicoccum nigrum	2.5	0	0	0	*Coniozyma leucospermi*	1.3	0	0	0
Eurotium spp	5.1	2.5	28.0	25.2	*Cryptococcus hungaricus*	5.1	0	0	0
Exophiala nigra	1.3	0	0	0	*Davidiella sp.*	1.3	1.2	5.6	0
Fusarium oxysporum	7.6	1.3	4.0	12.5	*Dothioraceae sp.*	0	1.2	0	0
Fusarium sporotrichioides	7.6	0	0	0	*Epicoccum nigrum*	7.7	3.7	0	0.05
Hypocrea lixii	1.3	0	0	0	*Eurotium halophilicum*	15.4	76.5	77.8	0
Kabatiella microsticta	0	1.3	0	0	*Fusarium graminearum*	0	1.2	0	0
Penicillium chrysogenum	2.5	1.3	0	12.5	*Fusarium oxysporum*	1.3	0	0	0
Penicillium janthinellum	1.3	0.0	0	0	*Galactomyces geotrichum*	7.7	0	0	0
Uncultured Pleosporales (AM901892)	30.4	2.5	0.0	0	*Ganoderma sp.*	1.3	0	0	0
Pyrenochaeta lycopersici	2.5	0	0	0	*Leptosphaeria sp.*	0	1.2	0	0
Uncultured Saccharomycetales (AM902050)	0	10.0	0	0	*Leptosphaerulina sp.*	1.3	1.2	0	0
Trichocladium asperum	0	1.3	0	0	*Mucor racemosus*	1.3	0	0	0
Trichoderma harzianum	0	0	0	12.5	*Penicillium chrysogenum*	3.8	0	5.6	95.5
Chaetothyriales sp.	0	0	10.7	0	*Penicillium decumbens*	0	0	1.4	0
Ustilago syntherismae	0	0	1.3	0	*Phaeococcomyces nigricans*	0	0	1.4	0
Verticillium nigrescens	1.3	0	0	0	*Phaeosphaeria* sp. I147	6.4	0	0	0
Wallemia sebi	0	7.5	0	0	*Phlebia uda*	0	0	1.4	0
Chaetothryales sp.	0	1.3	0	0	*Phoma sp.*	0	1.2	0	0
Rhizophlyctis rosea	0	0	2.7	0	*Pichia sp.*	15.4	0	0	0
Rhizopus sp.	0	6.3	0	0	*Pleosporales sp.*	1.3	0	0	0
Yeast - other	0	0	0	12.5	*Rhizopus microsporus*	0	2.5	0	0
Total no. of clones[d]/CFUs[e]	**79**	**80**	**75**	**34,500**	*Rhodotorula sp.*	2.6	0	0	0
Total no. of OTUs[f]	**15**	**7**	**11**	**7**	*Stachybotrys chartarum*	0	0	0	1.9
					Trapeliopsis glaucolepidea	0	1.2	0	0
					Trichosporonales sp.	1.3	0	0	0
					Ulocladium chartarum	0	0	0	0.5
					Umbilicaria kappeni	0	2.5	0	0
					Uncultured Ascomycete	9.0	0	1.4	0
					Uncultured Basidiomycete	1.3	0	0	0
					Uncultured Lasiosphaeriaceae	1.3	0	0	0
					Ustilago	0	1.2	0	0
					Volutella colletotrichoides	1.3	0	0	0
					Wallemia sebi	1.3	1.2	0	0
					Total no. of clones [d]/CFUs[e]	**78**	**81**	**72**	**513,160**
					Total no. of OTUs[f]	**17**	**9**	**6**	**6**

[a] closest hit in BLAST analysis of sequenced ITS region. [b] percentage of clones in the library that contained a particular ITS sequence. [c] percentage of colonies recovered for a particular species during viable culture analysis. [d] total number of clones analyzed for a particular DNA extraction method. [e] total number of colony forming units calculated for a given dust sample based on recovery of viable cultures. [f] total number of different operational taxonomic units (OTUs) observed in a clone library or viable culture collect.

was included as a control in this experiment exhibited a clone distribution that differed significantly from what was expected assuming no bias (Table 1). Although we eliminated the possible bias introduced by the different DNA extraction techniques with this control, we could not control for possible PCR amplification and cloning biases that may occur during this process. Biases are well-documented in bacterial rRNA gene amplification studies,[42,43] and different universal fungal primers may exhibit amplification biases to specific fungal taxa.[20] Currently, we are testing PCR amplification and cloning biases that may be introduced during ITS amplification and cloning.

Several limitations associated with fungal ITS sequencing of environmental samples have been identified. Variation between viable culture data and ITS sequencing data associated with *Penicillium* and *Aspergillus* genera have been recently reported.[17,19] In prior studies,[14,19] and in the dust sample B of the present study, *Penicillium* and/or *Aspergillus* species dominated the viable culture counts but were not observed in ITS clone libraries. The molecular basis for these observed discrepancies were not explored in the previous studies, but data presented in the current study suggest that DNA extraction methodologies, and possibly PCR amplification or cloning, may introduce biases

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that preclude the detection of *Aspergillus* and possibly other amerospore producing fungal genera. Other studies also have demonstrated different DNA extraction techniques to be effective at isolating DNA from some fungal taxa but ineffective at isolating DNA from *Aspergillus* species. For example, Fredricks *et al.*[44] demonstrated that some DNA extraction methodologies were reproducible for *Candida albicans*, but were ineffective for detecting *A. fumigatus*. To overcome the limitations associated with DNA extraction methods, recent studies have shown that several PCR targets within the rDNA and ITS regions reduce biases associated with any particular primer pair.[18,19] Furthermore, a DNA extraction step is often replicated and combined prior to PCR to reduce sample-to-sample variation.[18,19] However, these strategies would not overcome the partially recalcitrant nature of amerospores.

The methods used to process environmental samples for molecular analyses may greatly influence the interpretation of the diversity of fungal bioaerosols in indoor and other occupational environments. In the present study, office building dust samples that were processed with different DNA extraction techniques resulted in variable clone libraries (Table 2; Fig. 3). As in the preliminary spiking experiments, analysis of environmental samples further demonstrated the biases associated with various DNA extraction methodologies. In the sample B clone library generated with the EluQuik/DNeasy kit, *Pichia spp.* was identified as a predominant fungal genus (Table 2). However, this genus was not present in the other clone libraries generated using the UltraClean Soil or High Pure PCR Template kits; the clones in those libraries were predominantly *Eurotium halophilicum* (Table 2). Importantly, the viable culture data for sample B were dominated by *Penicillium chrysogenum*, and neither *Pichia spp.* or *Eurotium halophilicum* were identified. These data demonstrate the advantages offered by ITS sequencing in fungal exposure assessment: the ability to identify fungal species that would often be overlooked using traditional exposure assessment methods or even more recently published molecular methods such as the Environmental Mold Relativity Index.[45] *E. halophilicum* is a rarely identified xerophilic species that grows slowly, and only on specific culture media with low water potential.[46–48] Compared to viable culture data associated with these samples, a number of other fungal species including *Alternaria*, *Rhodoturula*, *Rhizophlyctis*, *Wallemia*, and *Umbilicaria* were identified in the clone libraries but were not detected in viable culture. On the other hand, there were several species of fungi identified in the viable cultures that were not observed in the ITS clone libraries, namely *Trichoderma harzianum* (sample A), *Stachybotrys chartarum* (sample B), and *Ulocladium chartarum* (sample B). These species are readily culturable and were present in low quantities in the viable counts, which are likely reasons why they were observed in culture but not in the ITS clone libraries. Many of the species identified in the ITS clone libraries have specific nutrient and temperature requirements for growth on solid culture medium (*e.g. E. halophilicum*), and as a result, may not be detected when viable culture methods are used in exposure assessment studies. Many of these species are also not included in the ERMI primer/probe panel (http://www.epa.gov/nerlcwww/moldtech.htm). Fungal ITS sequencing does not allow quantification of detected fungal species, and ITS sequence similarities of 97% do not necessarily correlate OTUs to the species level. However, such sequencing studies do provide a basis for further investigation into the role that under-detected species may play in the health effects of residents and workers.

Environmental and occupational dust samples contain PCR inhibitors that may interact with DNA or DNA polymerase and hinder molecular analyses.[35] In the present study, the ability to remove PCR inhibitors was evaluated and the UltraClean Soil kit was most effective at yielding amplifiable DNA, even from samples spiked with 10 mg of Arizona test dust (Fig. 2). The High Pure PCR Template kit was also able to amplify DNA from samples spiked with up to 5 mg dust. These results are not surprising, since these two kits contain specific steps that aim to eliminate PCR inhibitors. In contrast, the EluQuik/DNeasy method does not have a PCR inhibitor removal step and as a result, this kit failed to yield amplifiable DNA from samples spiked with the standard dust (Fig. 2). While Arizona test dust does not necessarily represent the dust samples of indoor environments, it is a standardized, commercially available dust product that allowed for testing the efficacy of the different DNA extraction methods in removing PCR inhibitors. Consistent with the spiked Arizona road dust experiment, the EluQuik/DNeasy inconsistently yielded amplifiable DNA from the two building dust samples tested in this study. Although the EluQuik/DNeasy method has been demonstrated as effective at removing PCR inhibitors from dust compared to more crude DNA extraction methods,[26] our data indicated that this method is not consistently effective at removing PCR inhibitors from environmental dust samples. The level of PCR inhibitors in template samples are likely to differ among various indoor and occupational environments, and several DNA extraction methods should be tested for optimum DNA recovery. In such cases, detection efficiency of such genera as *Aspergillus* and *Penicillium* may have to be traded for the ability to amplify fungal ITS sequences in the presence of strong PCR inhibitors. An alternative strategy would be to test and employ *Taq* polymerase mutants that function better in the presence of PCR inhibitors.[49]

Conclusions

Although there are methodological limitations that need to be explored further, the results of this study demonstrated that the High Pure PCR Template kit is a sensitive and reproducible method that can be utilized in fungal diversity studies of indoor and occupational dust. Although the other tested methods (UltraClean soil and EluQuik/DNeasy kits) have been previously utilized in studies, biases against *Aspergillus* and potentially other amerospore genera must be taken into consideration. However, dust samples that contain high levels of PCR inhibitors may require soil DNA extraction kits (such as UltraClean) to overcome inhibition at the expense of DNA extraction efficiency. Future studies will utilize the efficacy of the High Pure PCR Template kit to identify previously overlooked fungal species and study the possible health effects associated with occupational exposure to these fungi.

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