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Research Article

A Method to Evaluate and Eliminate Fungal Contamination in Household Air Conditioners

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Background. Allergic fungal airway diseases, such as asthma and allergic bronchopulmonary mycosis (ABPM), are often difficult to manage with medical treatment alone; therefore, environmental fungal exposure should be accurately evaluated and minimized. In the present study, we established a method to evaluate and eliminate fungal contamination in household air conditioners (ACs). *Methods*. In the fall of 2020, an environmental survey of living rooms was conducted in 17 Japanese residences of patients with ABPM or related diseases. Household ductless minisplit AC units were disassembled to collect swab samples from the internal parts (filter, heat exchanger, blower fan, and air vent), followed by high-pressure washing. Fungal abundance and composition in swab samples and cleaning effluents of ACs as well as house dust and air samples were determined using quantitative PCR and next-generation sequencing of the internal transcribed spacer 1 region, respectively. A weighted UniFrac distance was calculated to analyze the similarity of the mycobiome among the samples. *Results*. All interior parts of ACs contained high levels of fungal DNA, with the blower fans being the most contaminated parts. *Cladosporium* and *Toxicocladosporium*, followed by *Aureobasidium*, *Aspergillus*, and *Rhodotorula*, were the most common fungi detected in the AC unit. High-pressure washing decreased fungal abundance by over 99% in all AC parts. Fungal abundance and composition in blower fans were strongly correlated with those in cleaning effluents. *Conclusion*. Interior parts downstream of heat exchangers in household ACs are the major sites of fungal contamination, possibly polluting the indoor air in the residences. High-pressure washing is highly effective for decontamination.

1. Introduction

Although there are millions of fungal species on the Earth, the majority of these rarely cause infections in immunocompetent people; however, certain fungi cause or worsen allergic fungal airway diseases, such as asthma, allergic fungal rhinosinusitis, and hypersensitivity pneumonia [1, 2]. A substantial proportion of patients with asthma, particularly those with severe disease and those using high-dose inhaled corticosteroids, are vulnerable to *de novo* sensitization [3] and are often sensitized to fungi [4]. Repeated exposure or colonization of fungi in the airways results in more refractory conditions, such as allergic bronchopulmonary aspergillosis/mycosis (ABPA/ABPM), requiring long-term treatment with systemic corticosteroids and leading to the destruction of airway structures, which occurs in 2.5% of adult patients with asthma [5]. Our nationwide survey of ABPA/ABPM revealed that patients of advanced age are more vulnerable to these diseases [6].

Substantial fungal burden exists in the living environment [7, 8]; therefore, indoor exposure to fungi is clinically important, as most people, particularly infants and the elderly, spend the majority of their time in their own residences [9]. Heating, ventilation, and air-conditioning (HVAC) systems effectively improve indoor air quality by reducing excess humidity and introducing fresh outdoor air [10]. Meanwhile, ductless minisplit air conditioners (ACs), commonly installed in Japanese residences, recirculate indoor air and do not ventilate with outdoor air. However, these systems can still reduce indoor airborne fungal load [11, 12] by controlling temperature and humidity [13–16].

Nonetheless, a caveat to using ACs is that these units can be contaminated with fungi and bacteria if not well managed [17–19], acting as a source of indoor pollution. Airborne fungal concentration increases from 40–920 to 5,500–100,000 colony-forming units·m^{-3} immediately after the operation of household ACs [17]. In our online survey, 37.1% of 1,006 Japanese participants reported a moldy odor from household ACs indicating fungal contamination [20]. ACs contaminated with fungi can impact the health outcomes of patients with allergic fungal airway diseases. In this context, we reported a case of ABPM in which repeated exacerbation was noted following exposure to blowout air from a contaminated AC, and the condition naturally resolved once the unit was discarded [21]. Therefore, the fungal burden in ACs must be accurately and appropriately evaluated to prevent the development and exacerbation of allergic fungal airway diseases.

Fungal burden in ACs has been assessed based on culture or next-generation sequencing (NGS) of samples obtained through swabbing the interior of ACs or collecting blowout air [8, 17]. However, little is known regarding (1) the most contaminated interior part of the AC unit that should be examined in the assessment of fungal load, (2) suitable methods to accurately determine the abundance and composition of fungi in the entire AC unit, and (3) appropriate methods to decontaminate ACs and their efficiency.

Indoor units of ductless minisplit ACs can be easily disassembled and cleaned via high-pressure washing using detergents and water at a relatively low cost and with less labor. In the present study, we performed fungal-specific quantitative PCR and mycobiome analysis of samples obtained from each interior part of disassembled ACs as well as from cleaning effluents before and after high-pressure washing to establish a method for evaluating fungal contamination in ACs and explore the efficiency of high-pressure washing in fungal decontamination.

2. Materials and Methods

2.1. Participants. We recruited patients living in Kanagawa, Japan, with allergic airway diseases, including ABPM, severe asthma, and related diseases with or without fungal sensitization. ABPM was diagnosed based on the criteria proposed by Asano et al. [22]. The present study was approved by the Institutional Review Board for Clinical Research of Tokai University (20R-129). The study was conducted in compliance with the principles of the Declaration of Helsinki of 1964 and its latest amendments. All participants provided written informed consent.

2.2. Survey of Residences. The survey was performed between September and November 2020, when the use of ACs during the summer ended and before the winter season started in Japan. We visited the participants' residences with a professional AC cleaning crew and collected indoor air, house dust, swab samples, and cleaning effluents from ACs in the living room. The samples were collected in the following order: (1) air, (2) house dust, (3) swabs from disassembled ACs before washing, (4) effluents of ACs at washing, and (5) swabs from cleaned ACs before reassembling.

To collect airborne fungi, two sets of portable airborne bacterial samplers (IDC-500B; IDEC Inc., Japan) were used. Each device collected airborne fungi by impinging 500 L of air onto a membrane filter (JCWP09025, Omnipore Membrane Filters 10 μm pore/90 mm ø, Millipore, USA) soaked in phosphate-buffered saline (PBS) in a Petri dish. Using the two devices, 1,000 L of air was impacted onto membrane filters soaked in PBS, and the filters were processed with a stomacher containing 20 mL of PBS. House dust was collected with a vacuum cleaner (MC-PKL21A-W, Panasonic Corporation) in paper bags (SOP-210, Asahi Electric ELPA, Japan). Hair and sand were removed from the dust, and the samples were processed using a stomacher after adding 10 mL of PBS per 100 mg of house dust.

2.3. Survey and High-Pressure Washing of Household ACs. The filters, frontal area of heat exchangers, blower fans, and air vents of the ACs were scrubbed with a nylon Q-tip swab immersed in sterile PBS (ST-25, ELMEX). Two separate areas measuring 25 cm^2 were swabbed before washing the ACs, and one area measuring 25 cm^2 was swabbed after washing. The filter was removed from the outer frame of the AC, then vacuumed of dust, scrubbed with a brush using an alkaline detergent (pH 12), and rinsed with water. The interior parts of the ACs, such as heat exchangers, drain pans, blower fans, and air vents, were sprayed with alkaline detergent (pH 12), scrubbed with a brush, left for a few minutes, and then the components except for the drain pan were rinsed with a high-pressure washer (dirt on the drain pan was flushed without high-pressure washing). The AC cleaning effluent was collected in a 20 L polyethylene tank, and the volume of the effluent was determined.

2.4. Sample Homogenization. The membrane filter with 20 mL PBS, house dust in 10 mL PBS, and swabbed samples with 10 mL PBS were placed in a sterile stomacher bag with a filter (BagPage+, Interscience, France) and homogenized using a stomacher (MiniMix 100 P CC, Interscience) at the maximum paddle speed (paddle speed 4) for 10 min. Uniformly dispersed samples were then dispensed in 2 mL portions and stored at −80°C until DNA extraction.

2.5. DNA Extraction. The thawed samples were centrifuged at 20,000 × g for 20 min at 20°C. The pellets were suspended in 180 μL of buffer T1 and 25 μL of 22.4 mg·mL^{-1} proteinase

K (NucleoSpin Tissue Kit, Macherey-Nagel GmbH & Co. KG, Düren, Germany) and transferred to a tube filled with zirconia beads (ZircoPrep Mini, NIPPON Genetics, Japan) for bead disruption using Disruptor Genie (Scientific Industries, Inc., Bohemia, NY, USA) for 10 min, followed by incubation at 56°C overnight and at 70°C for 10 min in 200 μL of buffer B3 (NucleoSpin Tissue Kit). The disrupted/digested solution was placed on a Zymo-Spin III column (Zymo Research, Irvine, CA, USA) mounted on 2.0 mL collection tubes and centrifuged at 8,000 × g for 1 min to remove zirconia beads, followed by the addition of 210 μL of 99% ethanol. The samples were loaded onto a silica spin column (NucleoSpin Tissue Kit) to bind DNA and mounted on a vacuum manifold (Vac-Man® Laboratory Vacuum Manifold, Promega Corporation, Madison, WI, USA) connected to a vacuum pump (Wob-l Pump 2522C-05, Welch Vacuum–Gardner Denver Thomas, Inc., Mt. Prospect, IL, USA) with a fluid trap. Under -80 kPa vacuum pressure, the silica columns were washed with 500 μL of buffer BW and 600 μL of buffer B5 (NucleoSpin Tissue Kit). The columns were dried by centrifugation at 11,000 × g for 1 min, and the DNA was eluted two times with 50 μL of Tris–EDTA buffer (pH 8.0). Following alcohol precipitation, the samples were dissolved in 40 μL of nuclease-free water. DNA was quantified using a Qubit fluorometer with a dsDNA HS assay kit (Thermo Fisher Scientific, Waltham, MA, USA).

2.6. Semiquantitative PCR for Fungal Genome. Fungal abundance in the air, house dust, and samples obtained from the AC units was quantified using semiquantitative PCR according to the SYBR Green method. Briefly, a 10 μL PCR reaction mixture containing 1 μL of sample DNA, 5 μL of SYBR Green master mix (2x Fast SYBR, Thermo Fisher Scientific), a forward primer (ITS1: 5′-TCCGTAGGTGAACC TGCGG-3′), and a reverse primer (ITS2: 5′-GCTGCG TTCTTCATCGATGC-3′) [23] was loaded into the StepOnePlus Real-Time PCR System (Thermo Fisher Scientific) and amplified under the following conditions: initial denaturation at 95°C for 5 min, amplification for 40 cycles at 95°C for 20 s and 65°C for 30 s, and incubation at 4°C. Fluorescence intensity during PCR amplification was analyzed using the QuantStudio Design & Analysis Software (Thermo Fisher Scientific). The mycobiome genomic DNA mix (MSA-1010, American Type Culture Collection, Manassas, VA, USA) was used to construct a calibration curve, and the estimated genomic copy number was determined. Fungal abundance in each sample was described as the fungal genome copy number in a swabbed area equivalent of 25 cm^2 for AC units, equivalent to the total recovery volume of AC cleaning effluent, airborne fungi detected in 1,000 L of room air, and fungi detected in 1 g of house dust.

2.7. PCR for Amplicon Sequencing. Amplicon sequencing was performed using the Illumina MiSeq platform (Illumina Inc., San Diego, CA USA); all reactions were performed in a total reaction volume of 10 μL using TaKaRa Ex Taq® Hot Start Version Mix (Takara Bio Inc., Japan). Amplification was performed with a three-step PCR. The internal transcribed spacer (ITS) 1–5.8S rRNA–ITS2 region was amplified in the first PCR using the ITS1F KU DNA: (5′-CTYGGTCATTTAGAGGAASTAA-3′) [24] and ITS4: (5′-TCCTCCGCTTATTGATATGC-3′) primers. The reaction conditions were as follows: initial denaturation at 94°C for 2 min, followed by 30 cycles of amplification at 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min, and final extension at 72°C for 5 min. The second PCR was performed using 2 μL (maximum 1 ng·μL^{-1}) of each amplicon purified with Agencourt AMPure XP magnetic beads (Beckman Coulter Life Sciences, IN, USA) to amplify the ITS1 region using a primer set with the addition of Illumina Nextera-type adapter sequence: 1TS1 (5′-TCGTCGGCAGCGTC AGATGTGTATAAGAGACAGTCCGTAGGTGAACCTG CGG-3′) and ITS2 (5′-GTCTCGTGGGCTCGGAGATGT GTATAAGAGACAGGCTGCGTTCTTCATCGATGC-3′). The reaction conditions were as follows: initial denaturation at 94°C for 2 min, followed by 10 cycles of amplification at 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s, and final extension at 72°C for 5 min. The third barcoding PCR was performed using 2 μL of purified products (5 ng·μL^{-1}) with the addition of indices designed based on the Illumina Nextera PCR primers: 5′-AATGATACGGCGACCACCGAG ATCTACAC–[Index2]–TCGTCGGCAGCGTC-3′ (forward) and 5′-CAAGCAGAAGACGGCATACGAGAT–[Index1]–GTCTCGTGGGCTCGG-3′ (reverse). The reaction conditions were as follows: initial denaturation at 94°C for 2 min, followed by 10 cycles of amplification at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and final extension at 72°C for 5 min. The final products were pooled equimolars. The quality of the prepared library was assessed using the Fragment Analyzer System 5200 (Agilent, CA, USA) and the dsDNA 915 Reagent Kit (Advanced Analytical Technologies). Sequencing analysis was performed at 2 × 300 bp using the MiSeq System and MiSeq Reagent Kit v3.

2.8. Sequence Data Analysis. Taxonomic classification of the paired-end read sequencing data was performed using QIIME 2 [25] against the reference database UNITE (https://unite.ut.ee/). Taxonomic assignment was based on the best-matching genomes between the query sequence of NGS results and the reference sequence in the database. We selected the species with the highest alignment score, computed using BLASTN, as the species of interest in the sample. Visual reports were created using Krona Tools [26].

2.9. Statistical Analysis. The data are presented as median and interquartile range (IQR). Differences in the estimated fungal-specific ITS1 genome copy number in AC units, AC cleaning effluents, air samples, and house dust were analyzed using the Wilcoxon signed-rank test. Correlation was analyzed using Spearman's correlation test. A weighted UniFrac distance matrix [27–30] was calculated using QIIME 2 to compare the mycobiome in the cleaning effluent and in each part of the AC unit. The distance is zero if the mycobiome of the AC component is identical to that of the cleaning effluent and one if the mycobiome is entirely different. The weighted UniFrac distance from the mycobiome in the

Table 1: Demographic data and living environment.

	Total	Single-family house		Multiple-dwelling complex	
		Steel construction	Wooden construction	Reinforced concrete	Steel construction
Number, *n*	17	3	10	3	1
Male/female, *n*	8/9	1/2	7/3	0/3	0/1
Age, years, median (IQR)	71 (61–76)	72 (59–76)	71 (63–75)	61 (59–66)	80
Family members, median (IQR)	2 (2–2)	2 (2–2)	2 (2–8)	2 (2–2)	2
Building age, years, median (IQR)	26 (15–41)	23 (15–36)	22.5 (15–42)	26 (25–34)	30
Years of AC use, median (IQR)	9 (6–12)	6 (5–9)	8 (6.3–12)	9 (9–10)	5
Smokers, *n*	3	0	2	0	1
Pets, *n*	2	0	2	0	0
Carpets and rugs, *n*	9	3	4	2	0
Ornamental foliage plants, *n*	5	1	2	2	0
Aquarium, *n*	1	0	1	0	0
Drying laundry in the room, *n*	11	2	6	2	1
Moldy smell, *n*	2	0	0	2	0
Condensation, *n*	6	1	4	1	0
Automatic cleaning function of AC filter, *n*	12	3	5	3	1
Air purifier, *n*	6	2	3	1	0
Humidifier, *n*	4	1	3	0	0
Dehumidifier, *n*	2	0	2	0	0

AC: air conditioner; IQR: interquartile range.

cleaning effluent (distance zero) was analyzed using Kruskal–Wallis and Dunn's multiple comparison tests. *P*-values <0.05 were considered statistically significant. All analyses were performed using GraphPad Prism 9 version 9.5.0 (GraphPad Software, CA, USA).

3. Results

3.1. Demographic Data. Seventeen ACs in the living rooms of the residences of 13 patients with ABPM, 3 patients with severe asthma (one sensitized and two unsensitized to fungi), and 1 patient with bronchial mucus plugs were examined. The ACs under investigation were used for a median of 9 (IQR, 6–12) years. The other demographic data are presented in Table 1.

3.2. Fungal Abundance in the ACs. The copy number of the fungal ITS1 region in the interior of the AC filters is shown in Figure 1. Fungal abundance was highly variable, ranging from 10 to 10^7 genome copies per 25 cm^2. Blower fans were significantly more contaminated per unit area than filters ($P = 0.003$) and heat exchangers ($P = 0.007$). The number of years of AC use was significantly correlated with fungal abundance in AC effluent ($\rho = 0.51$; $P = 0.04$) (Supplemental Figure 1).

The estimated fungal-specific ITS1 genome copy numbers in the swabbed area of 25 cm^2 for each AC component are shown. The same AC components are connected by lines. The data were analyzed using the Wilcoxon signed-rank test.

The estimated fungal genome copy numbers on the heat exchanger, blower fan, and air vent were strongly correlated ($\rho = 0.66$–0.73) (Figure 2). In contrast, no correlation was noted between the estimated fungal genome copy numbers in these AC parts and those in the AC filter, house dust, and indoor air samples.

Correlations were analyzed based on Spearman's rank correlation coefficients for the estimated fungal-specific ITS1 genome copy numbers in the swabbed area of 25 cm^2 for each AC component, AC cleaning effluent per total recovery volume, 1,000 L of indoor air, and 1 g of house dust. Numbers in the matrix indicate correlation coefficients (ρ). In the heat map, red and blue indicate positive and negative correlations, respectively.

3.3. Efficacy of High-Pressure AC Washing and Correlation between Fungal Abundance in Samples from AC Components and Cleaning Effluent. High-pressure washing of ACs with detergent decreased fungal abundance by 99.6% (IQR, 98.1–99.9) in heat exchangers, 99.4% (IQR, 97.5–100) in blower fans, and 99.7% (IQR, 98.0–100) in air vents, indicating that this method is as efficient as hand washing of detached filters, which decreased fungal abundance by 98.3% (IQR, 92.5–99.9) (Figure 3). Therefore, fungal abundance in the cleaning effluent can be considered to represent the fungal burden in AC unit except for the filter. The estimated fungal genome copy number in the cleaning effluent of AC was significantly correlated with that in blower fans ($\rho = 0.54$; $P = 0.03$) but not with that in filters ($\rho = -0.06$; $P = 0.82$), heat exchangers ($\rho = 0.22$; $P = 0.40$),

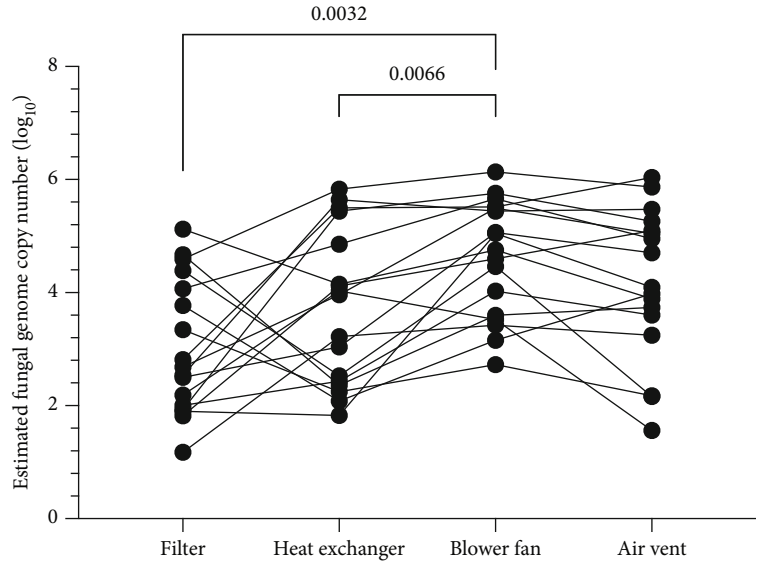

FIGURE 1: Fungal abundance in AC unit components.

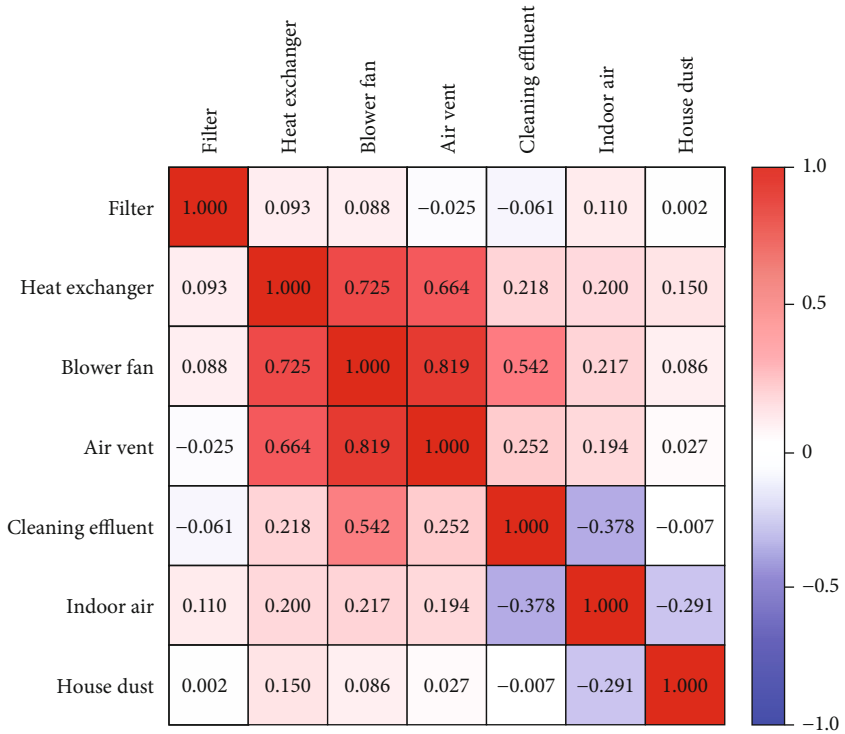

FIGURE 2: Heatmap of the correlation coefficient matrix of fungal abundance in AC components, cleaning effluent, indoor air, and house dust.

air vents ($\rho = 0.25$; $P = 0.33$), air ($\rho = -0.38$; $P = 0.14$), and house dust ($\rho = -0.01$; $P = 0.98$) (Figure 2).

The estimated fungal-specific ITS1 genome copy numbers in samples obtained from swabs of 25 cm^2 from each AC component before and after high-pressure cleaning with an alkaline detergent. Related parts are connected by lines. Parts are shown as closed circles before cleaning and open circles after cleaning. The data were analyzed using the Wilcoxon signed-rank test.

3.4. Mycobiome and Weighted-UniFrac Distance Analysis. Table 2 and Supplemental Tables 1–8 summarize the fungal genera frequently identified in the samples. *Cladosporium* and *Toxicocladosporium*, followed by *Aureobasidium*, *Aspergillus*, *Rhodotorula*, *Neoantrodiella*, *Alternaria*, and *Trametes*, were the most common fungi detected in samples from the AC unit, air, and house dust. Some fungi, such as *Aspergillus* and *Alternaria*, were identified both in filters and the interior parts of ACs, whereas other fungi, such as

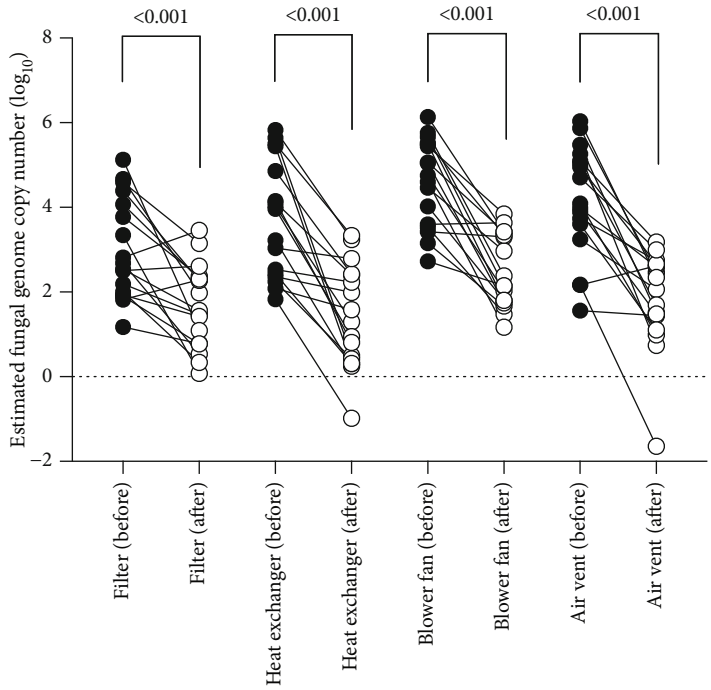

Figure 3: Fungal abundance in AC components before and after high-pressure washing.

Trametes and *Phlebia*, were mostly identified in filters but rarely in the inner parts of ACs.

The similarity of the mycobiome at the genus level between the AC cleaning effluents and AC components was examined using weighted UniFrac distance analysis. Relative distance from the mycobiome in the cleaning effluents was the shortest for the mycobiome in samples from blower fans (0.44, IQR, 0.19–0.55), followed by those in samples from air vents and heat exchangers (Figure 4). The distance for mycobiome in the cleaning effluent was significantly different from that in samples from all components ($P < 0.001$), except for blower fans ($P = 0.99$) and air vents ($P = 0.14$).

The weighted UniFrac distance from the mycobiome in the cleaning effluent is shown. The Kruskal–Wallis test was used to determine whether the distance of mycobiome at the genus level in samples from each component was significantly different from that in cleaning effluent (set as distance zero). Shaded boxes indicate groups without significant differences.

4. Discussion

In the present study, we investigated the magnitude of fungal contamination in the entire indoor unit of ductless minisplit ACs installed in Japanese residences, exploiting the unique structure of this type of AC, which can be easily disassembled and cleaned via high-pressure washing. Fungal-specific quantitative PCR demonstrated that blower fans and air vents were the most contaminated components of ACs. In addition, fungal abundance and composition in samples from blower fans, followed by air vents, were the most closely associated with those in AC cleaning effluent. The mycobiome in cleaning effluents is likely to reflect the contaminant fungi in the entire AC unit, as high-pressure washing could remove over 99% of the fungi from the indoor parts. Overall, the ACs were predominantly contaminated with fungi in the downstream parts of heat exchangers, such as blower fans and air vents.

Fungal abundance and composition were examined in the ACs of living rooms in the present study because Japanese residences are predominantly equipped with ACs in the living room (90.2%), followed by the bedroom (50.1%) [20]. Furthermore, a Canadian study reported that airborne spore concentrations were the highest in living rooms [31]. Fungal abundance and composition in samples from various parts of ACs were examined with fungal-specific quantitative PCR and NGS because unviable or difficult-to-grow fungi cannot be detected using culture-based methods but are in fact clinically important for allergic sensitization [32, 33].

The present study demonstrated that fungal abundance and composition in samples from blower fans and air vents—the downstream parts of the heat exchangers—most closely reflected the mycobiome in the cleaning effluent, which represents the entire mycobiome contaminating the indoor units of household ACs. Previous studies have demonstrated that condensation occurs mostly on heat exchange coils, leading to fungal growth [34–36]. However, obtaining swab samples from the rear side of heat exchangers is difficult, where condensation occurs and wet dust containing fungi accumulates. Therefore, sampling at the downstream blower fans rather than the frontal edge of heat exchangers is appropriate for the assessment of fungal contamination of ACs.

Mycobiome analysis identified *Cladosporium* spp., *Toxicladosporium* spp., *Aspergillus* spp., *Penicillium* spp., and *Simplicillium* spp., which have been previously reported as

Table 2: Top 50 frequently detected fungal genera.

	Genus	Total	Air conditioners					Air	House dust
			Filter	Heat exchanger	Blower fan	Air vent	Cleaning effluent		
1	*Cladosporium*	88	14	14	11	12	12	10	15
2	*Toxicocladosporium*	79	10	14	12	14	15	5	9
3	*Aureobasidium*	35	2	9	5	4	5	3	7
4	*Aspergillus*	31	5	3	3	2	4	5	9
4	*Rhodotorula*	31	3	3	6	4	6	2	7
6	*Neoantrodiella*	26	11	5	2	2	1	1	4
7	*Alternaria*	23	3	2	4	4		2	8
8	*Trametes*	21	11	2	1	1	1	4	1
9	*Acrodontium*	19	1	2	4	4	6	1	1
10	*Phlebia*	17	11	1	2		1		2
11	*Hyphodontia*	15	8		1	2	1	1	2
11	*Penicillium*	15	1		3	1	5	3	2
13	*Pseudopithomyces*	16	5	3		1		1	6
14	*Candida*	15	1			3	3		8
15	*Exophiala*	14	1	2	3	1	3	3	1
15	*Sterigmatomyces*	14	2		1	2	2	1	6
15	*Curvularia*	14	4	1		1			8
18	*Zasmidium*	13	3	1	3	3	2	1	
18	*Ganoderma*	13	7	3		1		2	
20	*Talaromyces*	12	1	1	2	3	3	1	1
20	*Peniophorella*	12	8			1		1	2
20	*Ceriporia*	12	9	1	1				1
20	*Schizophyllum*	12	7	1		2			2
24	*Simplicillium*	11			3	1	4	2	1
24	*Sporidiobolus*	11			3	4	3	1	
24	*Peniophora*	11	6	1		2		2	
27	*Meyerozyma*	10	1		2	2	1	1	3
27	*Knufia*	10	2	1	1	3	2	1	
27	*Lachnum*	10		1	4		3	1	1
30	*Naganishia*	9		1	2	1	1		4
30	*Phoma*	9	1		1			1	6
32	*Phellinus*	8	5	1		1	1		
32	*Resinicium*	8	4	2	1				1
32	*Microporus*	8	7		1				
32	*Bjerkandera*	8	7	1					
36	*Meripilus*	7	1	1		1	1	1	2
36	*Rigidoporus*	7	6			1			
36	*Ophiosphaerella*	7	3	1		1	1		1
36	*Perenniporia*	7	3	2	2				
40	*Xeromphalina*	6	4			1		1	
40	*Wallemia*	6	3	1				1	1
40	*Didymella*	6	1	2					3
40	*Phialocephala*	6	1	1	2		1	1	
40	*Stereum*	6	3		1	1		1	
45	*Catenulomyces*	5	1		1	1	1		1
45	*Coprinellus*	5	1			2	1	1	
45	*Tricholosporum*	5	2	1		1	1		
45	*Xylodon*	5	3		1	1			

Table 2: Continued.

	Genus	Total	Air conditioners					Air	House dust
			Filter	Heat exchanger	Blower fan	Air vent	Cleaning effluent		
45	*Debaryomyces*	5	1						4
50	*Neodevriesia*	4			1	1	1	1	
50	*Ramularia*	4	1		1	1		1	

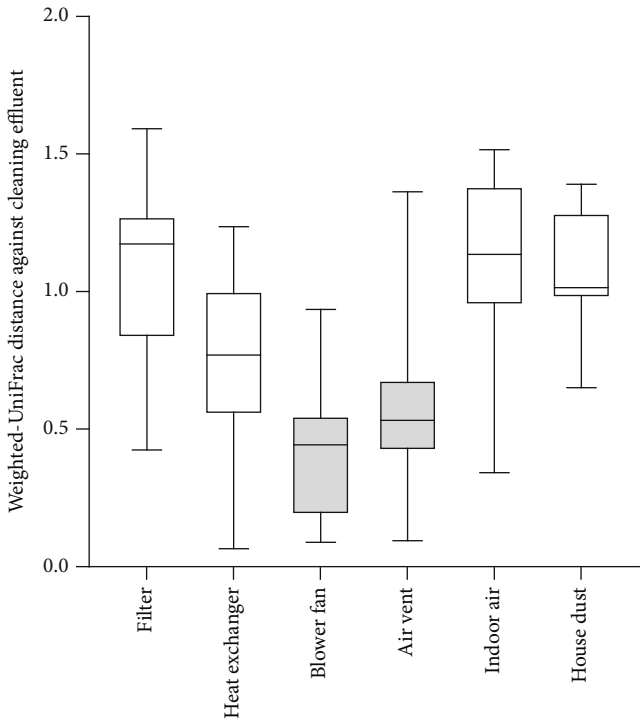

Figure 4: Comparison of mycobiome at the genus level in samples from AC components and cleaning effluent.

contaminants [10, 17, 36]. In addition, *Alternaria* spp. and *Trametes* spp., which are common fungi in the outdoor environment, were identified. *Alternaria* spp. were identified both in filters and interior parts of ACs, whereas *Trametes* spp. and *Phlebia* spp. were mostly identified in filters but not in interior parts, suggesting that the former cannot readily grow inside ACs.

Several approaches have been employed to decontaminate household ACs. Approximately 80% of AC users perform some measures for AC maintenance [20]; among these, manual or automatic cleaning of filters is the easiest approach. Patients with asthma are more conscious regarding the maintenance of ACs and use biocidal detergent sprays more frequently than those without asthma [20]. Notably, spraying the cleaner from the frontal side of heat exchangers may be ineffective, as fungal contamination in ductless minisplit AC units predominantly occurs in the downstream area of heat exchangers, as evidenced in the present study. This is because components downstream of the heat exchanger are typically very moist, and fungi and bacteria are more likely to inhabit the moist surfaces of these components [37]. The present study demonstrated that disassembling and cleaning the entire indoor unit of ACs through high-pressure washing with detergent and water is highly efficient in reducing fungal abundance.

Urbanization of residences and lifestyles has changed indoor fungal composition and the location of contamination. In addition to the conventional strategies, such as vacuum or passive dust sampling and active airborne dust sampling for the survey of indoor fungi [37], the present study has demonstrated the importance and strategy to examine fungi in the ACs. However, the present study has several limitations. First, our study was not designed of examining the relationship between environmental fungi and the occurrence of ABPM. Previous studies have demonstrated the link between indoor bacteria or fungi and allergic disease states such as asthma [37, 38]. However, it was not practical for us to collect a large number of participants during the coronavirus disease (COVID-19) pandemic. We are now conducting a study examining the effects of integrated environmental intervention, including high-pressure cleaning of ACs, on the outcomes of patients with ABPM (UMIN000048857) based on the results of the present study. Second, we did not clarify the factors associated with the magnitude of fungal contamination of ACs, as the observed fungal abundance and composition in ACs were the result of repeated exposure to the indoor environment over 9 (IQR, 6–12) years. We noted a nonlinear correlation between the period of AC use and the degree of its contamination, which is consistent with our previous observation that moldy odor develops more frequently in ACs used for longer periods [20]. However, because of the long-term usage of ACs, we could not evaluate the contribution of each specific indoor environmental factor to fungus contamination of ACs. Third, although we demonstrated that high-pressure washing is effective in cleaning ACs, there is no data on how long the effects persist. To answer these questions and improve the management of ACs, a future study reexamining ACs cleaned in the previous season is warranted.

5. Conclusions

Ductless minisplit ACs in Japanese residences are highly contaminated by fungi and may spread the contaminants into the indoor air and environment. Examining fungal abundance and composition in blower fans and air vents downstream of heat exchangers is the most suitable approach for evaluating fungal contamination in the entire AC unit. High-pressure washing is highly efficient for the fungal decontamination of disassembled ductless minisplit ACs, and the cleaning effluent may act as another useful sample for the evaluation of fungal contamination.

Data Availability

The dataset used in this study is available from the corresponding author upon request.

Conflicts of Interest

Kazuhiro Harada, Chikao Maeda, and Fumitoshi Ogino are employees of Duskin Co., Ltd., which provides professional AC cleaning services.

Authors' Contributions

Yoshiki Shiraishi did the conceptualization, methodology, investigation, data curation, validation, formal analysis, writing—original draft, writing—review and editing, visualization, and funding acquisition. Kazuhiro Harada, Chikao Maeda, and Fumitoshi Ogino were worked on the resources and investigation. Yu Suzuki and Tadashi Imanishi performed the software and formal analysis. Naoki Okada, Katsuyoshi Tomomatsu, and Tsuyoshi Oguma supervise the resource and investigation. Yoshika Sekine executed the investigation and resources. U Yanagi did the conceptualization, methodology, resources, and investigation. Koichiro Asano carried out the conceptualization, resources, writing—review and editing, supervision, project administration, and funding acquisition.

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Supplementary Materials

Table S1: the top 30 fungal genera in indoor air, house dust, swab samples, and cleaning effluents of ACs in the living room. Table S2: the top 30 fungal genera in filters of ACs. Table S3: the top 30 fungal genera in heat exchangers of ACs. Table S4: the top 30 fungal genera in blower fans of ACs. Table S5: the top 30 fungal genera in air vents of ACs. Table S6: the top 30 fungal genera in the cleaning effluent of ACs. Table S7: the top 30 fungal genera in indoor air. Table S8: the top 30 fungal genera in house dust. Figure S1: the correlation between the years of AC use and fungal accumulation. *(Supplementary Materials)*

References

[1] S. Ueki, Y. Fukutomi, Y. Miyabe, T. Yamada, T. Oguma, and K. Asano, "Allergic fungal diseases in the upper and lower airways," *Eosinophilic Lung Diseases*, vol. 95, pp. 119–140, 2022.

[2] A. J. Wardlaw, E. M. Rick, L. Pur Ozyigit, A. Scadding, E. A. Gaillard, and C. H. Pashley, "New perspectives in the diagnosis and management of allergic fungal airway disease," *Journal of Asthma and Allergy*, vol. 14, pp. 557–573, 2021.

[3] K. Watai, Y. Fukutomi, H. Hayashi et al., "De novo sensitization to aspergillus fumigatus in adult asthma over a 10-year observation period," *Allergy*, vol. 73, no. 12, pp. 2385–2388, 2018.

[4] K. Masaki, K. Fukunaga, M. Matsusaka et al., "Characteristics of severe asthma with fungal sensitization," *Annals of Allergy, Asthma & Immunology*, vol. 119, no. 3, pp. 253–257, 2017.

[5] D. W. Denning, A. Pleuvry, and D. C. Cole, "Global burden of allergic bronchopulmonary aspergillosis with asthma and its complication chronic pulmonary aspergillosis in adults," *Medical Mycology*, vol. 51, no. 4, pp. 361–370, 2013.

[6] T. Oguma, M. Taniguchi, T. Shimoda et al., "Allergic bronchopulmonary aspergillosis in Japan: a nationwide survey," *Allergology International*, vol. 67, no. 1, pp. 79–84, 2018.

[7] A. Barberán, J. Ladau, J. W. Leff et al., "Continental-scale distributions of dust-associated bacteria and fungi," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 112, no. 18, pp. 5756–5761, 2015.

[8] K. Izawa, A. Kubosaki, N. Kobayashi et al., "Comprehensive fungal community analysis of house dust using next-generation sequencing," *International Journal of Environmental Research and Public Health*, vol. 17, no. 16, p. 5842, 2020.

[9] J. Vitte, M. Michel, A. Malinovschi et al., "Fungal exposome, human health, and unmet needs: a 2022 update with special focus on allergy," *Allergy*, vol. 77, no. 11, pp. 3199–3216, 2022.

[10] J. S. Nascimento, L. M. Volcão, K. C. Costa, M. G. S. Cavalcanti, and B. H. A. Galvão, "Assessment of the abundance and diversity of airborne fungi in two different air conditioning systems in Paraíba, Brazil," *International Journal of Advanced Engineering Research and Science*, vol. 7, no. 10, pp. 342–347, 2020.

[11] A. Kalwasinska, A. Burkowska, and I. Wilk, "Microbial air contamination in indoor environment of a university library," *Annals of Agricultural and Environmental Medicine*, vol. 19, no. 1, pp. 25–29, 2012.

[12] A. Niesler, R. L. Górny, A. Wlazło et al., "Microbial contamination of storerooms at the Auschwitz-Birkenau museum," *Aerobiologia*, vol. 26, no. 2, pp. 125–133, 2010.

[13] M. Golofit-Szymczak and R. L. Gorny, "Microbiological air quality in office buildings equipped with dventilation systems," *Indoor Air*, vol. 28, no. 6, pp. 792–805, 2018.

[14] H. Wu and J. W. C. Wong, "Current challenges for shaping the sustainable and mold-free hygienic indoor environment in humid regions," *Letters in Applied Microbiology*, vol. 70, no. 6, pp. 396–406, 2020.

[15] D. Wu, Y. Zhang, C. Zhao et al., "Temporal variation of airborne fungi in university library rooms and its relation to environmental parameters and potential confounders," *Environmental Science and Pollution Research International*, vol. 28, no. 11, pp. 14068–14079, 2021.

[16] X. Zhang, J. Liang, B. Wang, Y. Lv, and J. Xie, "Indoor air design parameters of air conditioners for mold-prevention

and antibacterial in island residential buildings," *International Journal of Environmental Research and Public Health*, vol. 17, no. 19, p. 7316, 2020.

[17] K. Hashimoto, H. Oda, Y. Saito, M. Akimoto, T. Nojiri, and Y. Kawakami, "Isolation of Simplicillium sympodiophorum and Toxicocladosporium irritans from the blowout air of household air conditioners," *Biocontrol Science*, vol. 26, no. 2, pp. 105–111, 2021.

[18] K. Hatayama, Y. Oikawa, and H. Ito, "Bacterial community structures in air conditioners installed in Japanese residential buildings," *Antonie Van Leeuwenhoek*, vol. 111, no. 1, pp. 45–53, 2018.

[19] K. Watanabe, U. Yanagi, Y. Shiraishi, K. Harada, F. Ogino, and K. Asano, "Bacterial communities in various parts of air-conditioning units in 17 Japanese houses," *Microorganisms*, vol. 10, no. 11, p. 2246, 2022.

[20] N. Okada, Y. Shiraishi, K. Tomomatsu, T. Oguma, and K. Asano, "Moldy odor from air conditioners in the residences of Japanese participants with and without asthma," *Indoor Air*, vol. 32, no. 11, Article ID e13156, 2022.

[21] N. Okada, T. Oguma, Y. Shiraishi et al., "Repeated exacerbation by environmental exposure and spontaneous resolution of allergic bronchopulmonary mycosis," *The Journal of Allergy and Clinical Immunology. In Practice*, vol. 9, no. 1, pp. 558–560.e1, 2021.

[22] K. Asano, A. Hebisawa, T. Ishiguro et al., "New clinical diagnostic criteria for allergic bronchopulmonary aspergillosis/mycosis and its validation," *The Journal of Allergy and Clinical Immunology*, vol. 147, no. 4, pp. 1261–1268.e5, 2021.

[23] T. J. White, T. Bruns, S. Lee, and J. Taylor, "Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics," in *PCR Protocols*, M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, Eds., pp. 315–322, Academic Press, San Diego, 1990.

[24] M. Ikenaga, M. Tabuchi, T. Kawauchi, and M. Sakai, "Application of locked nucleic acid (LNA) primer and PCR clamping by LNA oligonucleotide to enhance the amplification of internal transcribed spacer (ITS) regions in investigating the community structures of plant-associated fungi," *Microbes and Environments*, vol. 31, no. 3, pp. 339–348, 2016.

[25] E. Bolyen, J. R. Rideout, M. R. Dillon et al., "Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2," *Nature Biotechnology*, vol. 37, no. 8, pp. 852–857, 2019.

[26] B. D. Ondov, N. H. Bergman, and A. M. Phillippy, "Interactive metagenomic visualization in a web browser," *BMC Bioinformatics*, vol. 12, no. 1, 2011.

[27] M. Hamady, C. Lozupone, and R. Knight, "Fast UniFrac: facilitating high-throughput phylogenetic analyses of microbial communities including analysis of pyrosequencing and PhyloChip data," *The ISME Journal*, vol. 4, no. 1, pp. 17–27, 2010.

[28] C. Lozupone and R. Knight, "UniFrac: a new phylogenetic method for comparing microbial communities," *Applied and Environmental Microbiology*, vol. 71, no. 12, pp. 8228–8235, 2005.

[29] C. Lozupone, M. E. Lladser, D. Knights, J. Stombaugh, and R. Knight, "UniFrac: an effective distance metric for microbial community comparison," *The ISME Journal*, vol. 5, no. 2, pp. 169–172, 2011.

[30] D. McDonald, Y. Vazquez-Baeza, D. Koslicki et al., "Striped UniFrac: enabling microbiome analysis at unprecedented scale," *Nature Methods*, vol. 15, no. 11, pp. 847-848, 2018.

[31] D. W. Li and B. Kendrick, "Indoor aeromycota in relation to residential characteristics and allergic symptoms," *Mycopathologia*, vol. 131, no. 3, pp. 149–157, 1995.

[32] A. M. Caliendo, D. N. Gilbert, C. C. Ginocchio et al., "better tests, better care: improved diagnostics for infectious diseases," *Clinical Infectious Diseases*, vol. 57, supplement 3, pp. S139–S170, 2013.

[33] S. Goodwin, J. D. McPherson, and W. R. McCombie, "Coming of age: ten years of next-generation sequencing technologies," *Nature Reviews. Genetics*, vol. 17, no. 6, pp. 333–351, 2016.

[34] E. Acerbi, C. Chenard, D. Miller et al., "Ecological succession of the microbial communities of an air-conditioning cooling coil in the tropics," *Indoor Air*, vol. 27, no. 2, pp. 345–353, 2017.

[35] A. Bakker, J. A. Siegel, M. J. Mendell, and J. Peccia, "Building and environmental factors that influence bacterial and fungal loading on air conditioning cooling coils," *Indoor Air*, vol. 28, no. 5, pp. 689–696, 2018.

[36] A. Bakker, J. A. Siegel, M. J. Mendell, A. J. Prussin II, L. C. Marr, and J. Peccia, "Bacterial and fungal ecology on air conditioning cooling coils is influenced by climate and building factors," *Indoor Air*, vol. 30, no. 2, pp. 326–334, 2020.

[37] X. Fu, Z. Ou, and Y. Sun, "Indoor microbiome and allergic diseases: from theoretical advances to prevention strategies," *Eco-Environment & Health*, vol. 1, no. 3, pp. 133–146, 2022.

[38] Y. Sun, Y. Meng, Z. Ou et al., "Indoor microbiome, air pollutants and asthma, rhinitis and eczema in preschool children - a repeated cross-sectional study," *Environment International*, vol. 161, p. 107137, 2022.