Household air conditioners (hereinafter referred to as ACs) are household appliances where the fungi tend to grow easily, and many users still suffer from the fungus contamination of ACs. Although a wide variety of fungi contaminates ACs, the dematiaceous fungi such as genus \textit{Toxicocladosporium} and genus \textit{Cladosporium} are particularly grown a lot in the blowers inside household ACs according to Hamada and Abe (2016). The authors examined fungal contamination of ACs in nearly 20 cases and often encountered cases in which large numbers of white filamentous fungal colonies presumed to be of the family Cordycipitaceae were isolated. Therefore, in this study, we attempted to identify the fungal species by identifying the colonies isolated from the four household room ACs, which were highly contaminated with Cordycipitaceae. In addition, since the \textit{Toxicocladosporium} could be isolated together with Cordycipitaceae, the species was also identified by the molecular phylogenetic analysis.

Household air conditioners are known to be contaminated with dematiaceous fungi such as genus \textit{Toxicocladosporium} and genus \textit{Cladosporium}. We frequently encounter cases in which a large amount of fungi, which are presumed to belong to the family Cordycipitaceae, are isolated from the blowout air of the household air conditioners. Therefore, the Cordycipitaceae isolated in the survey of four cases of the air conditioners were identified by genetic analyses. As a result, all of them were found to be \textit{Simplicillium sympodiophorum}. The concentration of airborne fungi, \textit{S. sympodiophorum} in the blowout air was high ($> 10^4$ cfu/m$^3$) as exceeding the upper limit of quantification in three of four cases, and 5,000 cfu/m$^3$ in one case. This study revealed that \textit{S. sympodiophorum} contaminated multiple air conditioners. Genus \textit{Toxicocladosporium} was also isolated from the two air conditioners, and it was found to be \textit{Toxicocladosporium irritans} by the genetic analysis.

\textbf{Key words}: Airborne fungi / \textit{Aspergillus} / \textit{Penicillium} / Air conditioning / Mold contamination.

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Fungi collected on DG18 plates were cultured at 25°C for seven days for subsequent counting. Feller’s formula (Feller, 1950; Karwowska, 2005) was used to correct for coincidence loss in the number of isolated colonies. Isolates were identified based on their colony and microscopic characteristics after subculturing on PDA (potato dextrose agar; Nissui, Japan), MEA (malt extract agar; Difco, USA), CYA (czapek yeast extract agar; Samson et al., 2019) plates. Isolation was identified based on the description of Samson et al. (2019). When large numbers of colonies of the same species presumed to be major contaminated fungi were isolated, one

| Strains isolated in this study | GenBank accs. No. | Air conditioner |
|-------------------------------|------------------|----------------|
| NBRC 114579 Simplicillium sympodiophorum | LC545405 | Case 1 |
| FCG 2097 Simplicillium sympodiophorum | LC545406 | Case 2 |
| NBRC 114578 Simplicillium sympodiophorum | LC545404 | Case 3 |
| FCG 1835 Simplicillium sympodiophorum | LC545403 | Case 4 |
| FCG 2024 Toxicocladosporium irritans | LC545408 | Case 1 |
| NBRC 114583 Toxicocladosporium irritans | LC545407 | Case 3 |

**Table 1.** Strains used for drawing the phylogenetic tree (ITS region).

| Strains isolated in this study | GenBank accs. No. | Air conditioner |
|-------------------------------|------------------|----------------|
| NBRC 114579 Simplicillium sympodiophorum | LC545405 | Case 1 |
| FCG 2097 Simplicillium sympodiophorum | LC545406 | Case 2 |
| NBRC 114578 Simplicillium sympodiophorum | LC545404 | Case 3 |
| FCG 1835 Simplicillium sympodiophorum | LC545403 | Case 4 |
| FCG 2024 Toxicocladosporium irritans | LC545408 | Case 1 |
| NBRC 114583 Toxicocladosporium irritans | LC545407 | Case 3 |

| Reference strains | Species | GenBank accs. No. | Status |
|-------------------|---------|------------------|--------|
| JCM 18184 Simplicillium sympodiophorum | NR111027 | Type |
| IFM 63509 Simplicillium sympodiophorum | LC317790 | |
| MAFF 242282 Simplicillium sympodiophorum | 242282 a | |
| CGMCC 3.17943 Simplicillium calcicola | KU746706 | Holotype |
| CBS 116.25 Simplicillium lamellicola | NR111098 | Type of Cephalosporium lamellicola |
| CBS 311.74 Simplicillium obclavatum | NR111099 | Type of Acremonium obclavatum |
| JCM 18167 Simplicillium agashimaense | AB604002 | Type |
| JCM 18180 Simplicillium subtrropicum | NR111024 | Type |
| JCM 18169 Simplicillium cylindrosporum | NR111023 | Type |
| GY 11011 Simplicillium cicadellidae | MN006249 | Holotype |
| CBS 962.72 Simplicillium lanosoniveum | EF641862 | |
| GY 29131 Simplicillium lepidopterorum | MN006251 | Holotype |
| JCM 18176 Simplicillium minatense | AB603992 | Type |
| COAD 2057 Simplicillium coffeanum | MF066034 | Holotype |
| URM 7918 Simplicillium filiforme | MH979338 | Holotype |
| CBS 102067 Lecanicillium lecanii | MH862778 | Neotype of Cephalosporium lecanii |
| CBS 504.83 Parenzyodontium album | LC092880 | Epitype |
| CBS 124157 Toxicocladosporium chlamydosporum | FJ790283 | Holotype |
| CBS 124159 Toxicocladosporium velox | NR155890 | Type |
| CPC 23639 Toxicocladosporium pini | KJ869160 | Holotype |
| CBS 126499 Toxicocladosporium protearum | HQ599586 | Holotype |
| CBS 128775 Toxicocladosporium pseudovelox | NR137774 | Holotype |
| CPC 21282 Toxicocladosporium ficiniae | KF777190 | Holotype |
| CBS 128215 Toxicocladosporium banksiae | HQ599598 | Holotype |
| URM 7489 Toxicocladosporium cacti | KY752806 | Holotype |
| CBS 185.58 Toxicocladosporium irritans | EU040243 | Holotype |
| CBS 140694 Toxicocladosporium hominis | LN834444 | Holotype |
| CBS 132535 Toxicocladosporium strelitziae | NR111765 | Holotype |
| CPC 19305 Toxicocladosporium posoqueriae | NR121555 | Holotype |
| URM 7491 Toxicocladosporium immaculatum | KY752815 | Holotype |
| CBS 119416 Cladosporium halotolerans | DQ780364 | Holotype |

a Sequence ID of MAFF strains; data obtained from MAFF website (https://www.gene.affrc.go.jp/databases-micro_search.php).
strains per one AC was extracted and stored.

Stock cultures of fungi were identified by nuclear ribosomal internal transcribed spacer (ITS) region analysis, as described by Hashimoto et al. (2019). Molecular phylogenetic analysis was conducted in MEGA X (Kumar et al., 2018). A list of reference strains for drawing phylogenetic trees is shown in Table 1. The base sequence of each isolate was registered to GenBank, and some of the isolates were deposited and preserved at the NBRC.

Table 2 showed the changes in airborne fungal concentration before and after the operation of the ACs. In all cases, the airborne fungal concentration clearly increased after the operation, and three of them exceeded the maximum limit of quantitation. Cordycipitaceae, which were isolated in large quantities from four ACs, were identified as *Simplicillium sympodiophorum* by the ITS region of genetic analysis (Fig. 1 and Table 2). The four strains obtained from the air from each AC all corresponded to the type strain of *S. sympodiophorum* with the bootstrap probability (BP value) of 96% (Fig. 2). The concentration of *S. sympodiophorum* in the air before the operation of the ACs was 540 cfu/m³ in Case 1, whereas *S. sympodiophorum* was not detected in Cases 2, 3, and 4 (Fig. 1). The concentration of *S. sympodiophorum* sharply increased in the blowout air after the operation, and it exceeded the upper limit of quantification (>10⁴ cfu/m³) in Cases 1, 2, and 3, and was 5,000 cfu/m³ in Case 4 (Fig. 1).

Also in the ACs of Cases 1 and 3, mixing with a large amount of *S. sympodiophorum*, multiple colonies of dematiaceous fungi presumed to be the same species were isolated. As a result of molecular phylogenetic analysis, dematiaceous fungi isolated from the two ACs were all corresponded to *Toxicocladosporium irritans* with the BP value of 99% (Fig. 3). *T. irritans* was not detected in air before the operation of the ACs in Cases 1 and 3 (Fig. 1). The concentration of *T. irritans* increased in the blowout air after the operation, and it was 370 cfu/m³ and 14,000 cfu/m³ in Cases 1 and 3, respectively (Fig. 1).

*Simplicillium* belongs to the family Cordycipitaceae known as entomopathogenic fungi, and is mostly made up of anamorph species (Sung et al., 2007). *Simplicillium sympodiophorum* (Fig. 4) detected in large quantities in this study, was the new species proposed in 2013 (Nonaka et al., 2013). Its characteristics and distribution were not yet revealed due to its small numbers of isolated cases. The first case of its isolation was from the soil in Aogashima, Tokyo (Nonaka et al., 2013), and the second case was isolated from woodlice *Armadillidium vulgare* (Jaber et al., 2016). Since then, there has been a report, and this study revealed the fact that *S. sympodiophorum* contaminate multiple household room ACs for the first time. Although in common ACs, *Cladosporium, Toxicocladosporium, Penicillium*, and *Aspergillus* (Hamada and Abe, 2016; Moustafa, 2019) are found in large numbers, a much higher concentration of *S. sympodiophorum* was blown out in four cases this time.

*Simplicillium* have species that have entomopathogenic and plant-parasitic, but there is no clear case showing its pathogenicity to humans (Wei et al., 2019). Allergy is one of the diseases caused by airborne fungi in humans. Only a few fungi, such as *Alternaria* and *Aspergillus fumi
gatus*, were identified as important inhaled allergens, and many aspects of other fungi are little known as allergen factors (Fukutomi and Taniguchi, 2015). However, inhalation of high concentration of airborne fungi, even if they are non-pathogenic, over medium to long term may cause allergic symptoms (Oshikaka et al., 2017). Some reports showed the ACs used by people with symptoms of allergic bronchitis and the asthmatic response had a higher number of fungi attached to the inside of them than ACs used by healthy people (Hamada and Abe, 2016). Since *S.

**TABLE 2.** Four air conditioners analyzed in this study and total concentration of fungi in the blowout air before and after the operation.

| Prefecture | Fungal conc. [cfu/m³] | Measurement date |
|------------|-----------------------|------------------|
|            | ACs off | Immediately after ACs on |
| Case 1     | Hyogo      | 660 | >10⁵ | Nov. 2018 |
| Case 2     | Hyogo      | 40  | >10⁵ | Jul. 2019 |
| Case 3     | Osaka      | 920 | >10⁴ | Jul. 2019 |
| Case 4     | Kanagawa   | 330 | 5,500 | Jun. 2017 |

>10⁵ and >10⁴ show that the values exceeded the upper limit of quantitation.

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Fig 1, Fig 2, Fig 3, Fig 4
FIG. 1. The change of fungal concentration of five fungi in the blowout air before and after the operation of the ACs. nd: not detected.
**sympodiophorum** has been a blind spot as fungi, which has a possibility to highly exposure in indoor environment, this study would like to pay attention to them.

According to the report of household AC study conducted in recent years, genus *Toxicocladosporium* was the most common fungi that adhered to internal blowers (Hamada and Abe, 2016). This time, our study showed that these species are highly likely to be *Toxicocladosporium irritans*. *Toxicocladosporium* was newly created in 2007, and *T. irritans* was proposed as a type species at the same time (Crous et al., 2007). The various isolation sources of *T. irritans*, such as paints, ancient laid-paper, and some plants, were reported (Bezerra et al., 2017). In addition, it was also isolated from the devices inside the International Space Station or the Space Shuttle (Satoh et al., 2016), and it is presumed to widely distributed in the environment. Although there are not many cases of isolation of the *Toxicocladosporium* in Japan, it has been revealed that they commonly exist in indoor environment in Japan as DNA was detected by the next generation sequencing (NGS) analysis in ACs ducts (Yanagi, 2020).
FIG. 3. Phylogenetic tree for the isolates and related species of *Toxicocladosporium* constructed based on the analysis of the ITS region. The method of drawing a phylogenetic tree is the same as of Fig 2. The strain numbers marked by black circles are the strains obtained in this study. T indicates a type strain.

FIG. 4. *Simplicillium sympodiophorum* (NBRC 114579)
A. Colonies cultivated on PDA at 25 °C for 14 days, form white floccose aerial mycelium.
B. Conidiophores.
house dust (Hasegawa, 2020) recently. Furthermore, *Toxicocladosporium* were also detected by NGS analysis of human skin (Sugita et al., 2013), and it was reported that they involve in atopic dermatitis (Zhang et al., 2011).

Hamada (2016) reported that the number of fungi increased when frequency of air conditioners usage were high or length of time per usage were long, and the reason was predicted that the condensation inside the ACs becomes chronic. Abe (1998) demonstrated that inside of ACs were high humidity environment where hygrophilous fungi can grow easily. However, since *S. sympodiophorum* and *T. irritans* are not isolated in bathrooms and washing machines containing much moisture (Hamada and Abe, 2008; Hashimoto et al., 2019), it cannot conclude that they are likely to grow if the humidity is simply high. Although bedding, which exist in the same environment (bedrooms) as ACs, are also contamination sources of fungi, *S. sympodiophorum* and *T. irritans* are not isolated, probably because of relatively low in water (Hashimoto et al., 2020). Some factors in ACs may be facilitating the growth of these fungi. We would like to clarify the mechanism of its occurrence in the future.

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