INTRODUCTION

The bedding is the place where people spend most of the day and have long hours of contact with human skin. Therefore, if the bedding is contaminated with some kinds of substances, exposure to the contaminants continues while asleep. Exposure to such contaminants could adversely affect sleeper’s health. For example, the house dust mite (Genus Dermatagophides) is almost 100% detected in bedding (Kawakami et al., 2016), and it is known that the higher the contamination level, the higher the risk of developing illness such as asthma and atopic dermatitis (Platts-Mills, 1992; Platts-Mills et al., 1997). Since the fungi also contaminate bedding as well as house dust mites, there are several researches about them (Ohsuna et al., 1997; Akiyama and Taniguchi, 2009; Nambu et al., 2009). However, not many researches detail the fungal flora in bedding. While the authors also compared the fungi in bedding with on floors in the past and suggested that yeasts, in particular, had more significant numbers in bedding, genera and species of them could not be examined (Kawakami et al., 2016). Therefore, in this study, yeasts isolated from bedding were identified by genetic analysis to examine the main species of isolates. In addition, the fungal flora in bedding, which was shown only by the isolation rate in previous reports, was clarified by showing the detailed statistical charts and the mean values of filamentous fungi by each genus or species.

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MATERIALS AND METHODS

Fungal sampling method

Between July and September in 2016, dust was collected from beddings used in 50 houses in Japan. The beddings consisted of 32 beds, 16 traditional Japanese bedding called futons, and two others. The vacuum cleaner (Dyson V6 mattress+; Dyson Technology Limited, UK) was used to collect dust by sucking the surface of beddings with the area of 180 × 90 cm for one minute. After taking out the dust from the vacuum cleaner, weighing it, and removing as much hair and large crumbs as possible from it, 50 to 70 mg of dust was used to isolate the fungi. Isolation was conducted within two weeks from the dust collection. Each dust was suspended in 10 mL of phosphate-buffered saline (PBS) and vortexed for one minute. Each suspension was serially diluted to 1000 times, and each diluted solution (0.5 mL) was inoculated to a DG18 (Dichloran 18% Glycerol agar; Merck, Germany) plates. Fungi collected on DG18 plates were cultured at 25°C for 7 days for subsequent counting. 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., 2004) and M40Y agar (Malt yeast 40% sucrose agar; Samson et al., 2004) media. Genus Aspergillus was identified based on the description of Raper and Fennell (1965) and Klich (2002). The other fungi were identified based on the description of Samson et al. (2004).

Identification of yeasts by genetic analysis

When the yeasts were detected, the colonies were extracted to produce the pure culture in new DG18 culture media. However, the target was set to be white or cream colonies, and red yeasts (pink or red colonies, most of them are presumed to be Rhodotorula spp.) were excluded. Isolates from one plate medium were three colonies at the maximum, and when multiple colonies presumed to be the same morphology were isolated, one colony was selected and purely cultured. Yeast isolates were identified by D1/D2 region of the 26S rRNA gene analysis, as described by Hashimoto et al. (2019). DNA was extracted using the commercially available kit (Cica Geneus® DNA Extraction Reagent ST; Kanto Chemical Co., Inc., Japan) while following the instructions. Amplification of the D1/D2 region was performed using the primers ITSS (5’-GGAAGTAAAGTCTGTTTTGACG-3’) and NL4 (5’-GGTCCGTGTTTTCAAGACG-3’) (White et al., 1990) and illustrate Hot Start Mix RTG (GE Healthcare, UK). The PCR cycling conditions were 94°C for 3 min, followed by 35 amplification cycles (94°C for 30 sec, 52°C for 40 sec, and 72°C for 1.5 min), and a final extension step at 72°C for 5 min. The amplified DNA strands were sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and ABI 3130xl genetic analyzer. Evolutionary and phylogenetic tree 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.

RESULTS AND DISCUSSION

The isolated fungi

Fig. 1 showed the isolation rate of the main fungi isolated from bedding dust in 50 houses in Japan. Yeasts were distinguished between red yeasts (pink/ red colonies) and other yeasts (white/cream colonies), and the breakdown of each yeast was shown. For Aspergillus spp., the breakdown was shown by species. In addition, the number of fungi per gram (cfu/g) of each dust sample was grouped by genera or species and plotted in Fig. 2 and 3. The fungi were isolated from 49 of 50 houses (98%), and the mean, median, and maximum values were 1.7×10^5 cfu/g, 1.5×10^5 cfu/g, 5.7×10^5 cfu/g respectively. There was a sample without any fungus detection (the lower detection limit was 380 cfu/g). The isolation rate of yeasts was 84% (mean 2.7×10^5 cfu/g, median 1.5×10^5 cfu/g, max 6.9×10^5 cfu/g), and it exceeded the isolation rate of Cladosporium spp. (80%, mean 1.3×10^5 cfu/g, median 1.2×10^5 cfu/g, max 5.0×10^5 cfu/g) and Aspergillus spp. (66%, mean 1.1×10^5 cfu/g, median 3.7×10^5 cfu/g, max 5.6×10^5 cfu/g) which were recognized as the most commonly exist fungi in the living environment (Samson et al., 2004). Although there is very little fungal flora data regarding bedding dust, several cases such as Ohnsen et al. (1997), Akiyama and Taniguchi (2009), Nambu et al. (2009), and Kawakami et al. (2016) are found in Japan. In each Japanese case, yeasts were isolated with relatively high percentages, and similar results were obtained in this study, which could confirm the conclusion that Japanese bedding dust is high in yeasts. Also, the isolation rate of Alternaria was 66% (mean 1.8×10^5 cfu/g, median 4.0×10^5 cfu/g, max 1.8×10^6 cfu/g), and it tied with Aspergillus (Fig. 1). According to Ohnsen et al. (1997), Akiyama and Taniguchi (2009), Nambu et al. (2009), and Kawakami et al. (2016), the isolation rate of Alternaria was 17%, 32%, less than 5%, and 23% respectively. Compared to these previous studies, the isolation rate of Alternaria in this study was high. Alternaria is closely related to asthma and is one of the few fungi that clarified its clinical significance as an inhaled allergen (Fukutomi and Taniguchi, 2015). In particular, many studies are pointing out the
The association between Alternaria floating outdoors and asthma exacerbation. It is thought that Alternaria related asthma attacks and deaths increase between June and September when outdoor airborne fungal level arises (Denning et al., 2006; Black and Brodie, 2000). It is known that the massive dispersal of Alternaria by the thunderstorm wind causes the deterioration of asthma symptoms (Thunderstorm asthma) (Pulimood et al., 2007). On the other hand, few reports indicate an association between fungal allergens and asthma since it is difficult to quantify them indoors. However, in recent years, the association between infants’ asthma and the dust borne Alternaria has been reported (Behbod et al., 2013), and it has been indicating the possibility of involvement of indoor Alternaria as an allergen (Fukutomi and Taniguchi, 2015). The result of the high isolation rate of Alternaria from the bedding where people spend a long time is considered to be interesting as the source of Alternaria exposure indoors. Compared to floors, fungi tend to be detected in bedding, which is affected by body temperature and sweat of residents throughout the year (Kawakami et al., 2016).

**TABLE 1.** Reference strains used for drawing the phylogenetic tree (D1/D2 region).

| Strain No. | Species                        | GenBank accession No. | Status                                      |
|------------|--------------------------------|-----------------------|---------------------------------------------|
| CBS 926    | Naganishia diffluens           | AF181542              | Type of Cry. albidus var. diffluens          |
| CBS 160    | Naganishia diffluens           | AF075502              | Holotype                                    |
| CBS 968    | Naganishia liquefaciens        | AF181515              | Type                                        |
| IGC 2406   | Naganishia liquefaciens        | AF181513              |                                             |
| CBS 142    | Naganishia albidus             | AF075474              | Holotype                                    |
| CBS 1926   | Naganishia albidus             | AF137602              | Type of Cry. albidus var. ketzingii          |
| NBRC 0434  | Naganishia albidus             | 00043401              | Type                                        |
| CBS 140    | Filobasidium magnum            | AF181851              | Type                                        |
| CBS 1896   | Cutaneotrichosporon debeurmannianum |                | Type                                        |
| CBS 2479   | Trichosporon asahii var. asahii | EU559350              | Type                                        |
| CBS 331    | Saitozyma flav                 | AF075497              | Type                                        |
| CBS 8933   | Vishniacozyma heimaeyensis     | DQ000317              | Type                                        |
| CGMCC 2.2471 | Vishniacozyma folicola         | AY557599              | Type                                        |
| NBRC 10058 | Clavispora lusitaniae          | 01005802              | Type of Candida obtusa                      |
| NBRC 10059 | Clavispora lusitaniae          | 01005901              | Type of Candida obtusa                      |
| ATCC 22019 | Candida parapsilosis           | KJ463413              | Type                                        |
| ATCC 18804 | Candida albicans var. albicans | HQ876051              | Type                                        |
| ATCC 18824 | Saccharomyces cerevisiae        | KC881066              | Type                                        |
| NBRC 0622  | Candida glabrata               | 00062201              | Type of Candida obtusa                      |
| NBRC 0083  | Debaryomyces hansenii var. hansenii | 00008301       | Type of Candida obtusa                      |
| CBS 1795   | Candida famata var. famata     | AJ508559              | Type, anamorph of D. hansenii              |
| CBS 566    | Meyerozyma guillermondii       | AJ508562              | Type                                        |
| NBRC 10106 | Pichia guillermondii           | 01010601              | Type, anamorph of M. guillermondii          |

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*d* China General Microbiological Culture Collection Center.  
*e* American Type Culture Collection.  
*f* Sequence ID of NBRC strains; data obtained from NBRC web site (http://www.nbrc.nite.go.jp/NBRC2/NBRCDispSearchServlet?lang=ja).
be exposed to and affected by *Alternaria* in bedding throughout the year. However, its seasonal variation in the amount is not clear.

The possible cause of the higher isolation rate of *Alternaria* compared to the previous researches is the larger amount of inoculated dust per medium. The author leveraged the results of previous research (Kawakami et al., 2016) and increased the amount of inoculated dust fivefold per medium. As a result, in addition to *Alternaria*, the detection rates of major fungi such as *Cladosporium*, *Aspergillus*, *Penicillium*, and yeast were increased overall compared to the previous report (Kawakami et al., 2016). We consider that increasing the amount of inoculated dust is important for obtaining favorable isolation results. Ohsuna et al. (1997) and Nambu et al. (2009) used sticky tape to collect dust, which lowers the detection rate compared to the use of the vacuum cleaner. The detection rate may become higher by further increasing the amount of inoculated. However, since there is a limit to increase the amount of inoculated, next-generation sequencing (NGS) analysis can also be used as one of the effective methods to detect DNA to investigate trace amounts of fungi.

The isolation rate and the number of *A. penicillioides*, *A. restrictus*, *Eurotium*, and *Wallemia* known as xerophilic fungi, were not so high (Fig. 1, 2 and 3). Usually, these xerophilic fungi often have a higher number in the house dust on the floor (Takatori et al., 1992; Hamada, 2007). In particular, *Aspergillus* section *Restricti*, to which *A. penicillioides* and *A. restrictus* belong, is the fungi having

![FIG. 1. The isolation rate of each fungus isolated from bedding dust.](image)
the largest numbers in house dust on the floor and indoor air (Hamada, 2007; Hashimoto and Kawakami, 2018). Nevertheless, the study showed that the non-xerophilic fungi such as the yeasts, Cladosporium, and Alternaria have larger numbers and xerophilic fungi have less in bedding dust. Even if lumping A. penicillioides and A. restrictus together as A. section Restricti, the isolation rate was 34% (mean $1.1 \times 10^2$ cfu/g, max $5.6 \times 10^3$ cfu/g), which was lower than that of Alternaria, Penicillium, or Pseudopithomyces. It is assumed that the reason for the larger numbers of non-xerophilic fungi in bedding compared to them on the floors is that bedding contains a lot of moisture due to the sweat of users during sleep. Akiyama and Taniguchi (2009) also showed similar results, which A. restrictus and Wallemia were detected a lot on floors were not found in bedding at all. In this study, although A. restrictus (16%), and Wallemia (8%) were isolated, the results were different from that of Akiyama and Taniguchi (2009). As described above, the reason for such differences was attributable to the larger amount of inoculated dust.

This time, the isolation rate of Pseudopithomyces (synonym: Pithomyces) was 38% (mean $7.3 \times 10^2$ cfu/g, max $1.7 \times 10^3$ cfu/g), and it was ranked relatively high among all fungi (Fig. 1). This is a high value following Penicillium (44%, mean $2.0 \times 10^3$ cfu/g, max $6.6 \times 10^3$ cfu/g), which is recognized as a common fungus in the living environment. Pseudopithomyces is the fungus
mainly distributed in soil and plants (Tóth et al., 2007). While it is isolated from house dust and indoor air, it is very rare (Flannigan et al., 2002; Toyazaki, 2002; Sakai et al., 2003). *Pseudopithomyces* is slow in producing conidia on media and has no distinctive morphological features of colonies (Tóth et al., 2007). Therefore, when the inspector cannot recognize morphological features of colonies in the early stage of the culture, resulting in the oversight of them. This could be the reason for little isolation cases of *Pseudopithomyces*. In recent years, the study of dust in the office using the clone library analysis showed that the detection rate of DNA of *Pseudopithomyces* was reached to 72.7% (16 of 22) (Park et al., 2018) and it indicated that *Pseudopithomyces* very commonly existed in the dust. Even though the distribution in ordinary houses was not clear, the result of this study clarified that *Pseudopithomyces* was very common, at least in bedding. This is the point the study desire to emphasize. Additionally, many of the isolates obtained this time were *Pseudopithomyces chartarum* (synonym: *Pithomyces chartarum*). While this species is known to cause facial eczema in sheep and cattle (Di Menna et al., 2009), the effects on humans are not clear.

### TABLE 2. Yeasts identified by D1/D2 region analysis and the strain numbers.

| Species (synonym) | Number of isolates (%) | Isolates ID (GenBank accession No.) |
|-------------------|------------------------|--------------------------------------|
| *Naganishia diffluens* species complex (Cryptococcus diffluens species complex) 13 (32%) | FCGNE:48 (LC529193) FCGNE:17 (LC529169) |
|                    |                        | FCGNE:42 (LC529190) FCGNE:22 (LC529175) |
|                    |                        | FCGNE:39B (LC529186) FCGNE:25C (LC529178) |
|                    |                        | FCGNE:20A (LC529172) FCGNE:29A (LC529181) |
|                    |                        | FCGNE:8A (LC529163) FCGNE:25B (LC529177) |
|                    |                        | FCGNE:15B (LC529167) FCGNE:35 (LC529184) |
|                    |                        | FCGNE:54A (LC529196) |
| *Filobasidium magnum* (Cryptococcus magnus) 13 (32%) | FCGNE:4B (LC529161) FCGNE:39A (LC529185) |
|                    |                        | FCGNE:2 (LC529158) FCGNE:40 (LC529188) |
|                    |                        | FCGNE:6 (LC529162) FCGNE:41 (LC529189) |
|                    |                        | FCGNE:15A (LC529166) FCGNE:43A (LC529191) |
|                    |                        | FCGNE:18A (LC529170) FCGNE:47 (LC529192) |
|                    |                        | FCGNE:21 (LC529174) FCGNE:55 (LC529198) |
|                    |                        | FCGNE:30 (LC529183) |
| *Candida parapsilosis* 5 (12%) | FCGNE:54B (LC529197) |
|                    |                        | FCGNE:39C (LC529187) |
|                    |                        | FCGNE:27 (LC529180) |
|                    |                        | FCGNE:4A (LC529160) |
|                    |                        | FCGNE:3 (LC529159) |
| *Meyerozyma guilliermondii* (Candida guilliermondii) 3 (7.3%) | FCGNE:20B (LC529173) |
|                    |                        | FCGNE:12 (LC529165) |
|                    |                        | FCGNE:26 (LC529179) |
| *Candida glabrata* 1 (2.4%) | FCGNE:49 (LC529194) |
| *Clavispora lusitaniae* (Candida lusitaniae) 1 (2.4%) | FCGNE:19 (LC529171) |
| *Debaryomyces hansenii* (Candida famata var. famata) 1 (2.4%) | FCGNE:16 (LC529168) |
| *Saccharomyces cerevisiae* 1 (2.4%) | FCGNE:25A (LC529176) |
| *Saitozyma flavus* (Torula flavus) 1 (2.4%) | FCGNE:51 (LC529195) |
| *Vishniacozyma* aff. folicola 1 (2.4%) | FCGNE:9 (LC529164) |
| *Cutaneotrichosporon debeurmannianum* (Trichosporon debeurmannianum) 1 (2.4%) | FCGNE:29C (LC529182) |
| **Total** 41 (100%) | **Total** 41 (100%) | **Total** 41 (100%) |
FIG. 4. Phylogenetic tree of yeasts identified by D1/D2 region analysis. The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model (Kimura, 1980). A discrete Gamma distribution was used to model evolutionary rate differences among sites. The bootstrap values (1000 replicates) over 50% are displayed on the nodes. The strain numbers marked by black rhombus are the strains obtained in this study. T indicates a type strain.
Yeasts identified by genetic analysis

Yeasts other than red yeasts were detected in 35 out of 50 dust samples (isolation rate 70%, mean 1.0×10^6 cfu/g, median 8.5×10^4 cfu/g, max 2.0×10^6 cfu/g), and one to three strains of yeasts were isolated from each sample, which came to a total of 41 strains and stored. Table 2 and Fig. 4 showed the identification results of the D1/D2 region of 41 strains performed by the generic analysis. 13 out of the 41 strains (32%) matched the Naganishia diffluens species complex, and 13 strains (32%) matched the Filobasidium magnum. The total value of these two clades reached to 63%, which clearly showed they were dominant species of the isolated yeasts. Other results having the larger numbers were five strains (12%) of Candida parapsilosis and three strains (7.3%) of Meyerozyma guilliermondii (synonym: Candida guilliermondii).

The respective bootstrap values in Naganishia diffluens (synonym: Cryptococcus diffluens) and Naganishia liquefaciens (synonym: Cryptococcus liquefaciens) used for reference sequences were not enough, which resulted in the failure of dividing them into the monophyly (Fig. 4). Therefore, the clade of N. diffluens and N. liquefaciens were integrated as N. diffluens species complex in the study. Although they were used to be Cryptococcus albidus before, subdivided into 12 species in 2000 (Fonseca et al., 2000), and transferred from Genus Cryptococcus to Genus Naganishia in 2015 (Liu et al., 2015). N. diffluens and N. liquefaciens are known to be detected in the skin of atopic patients (Sugita et al., 2003; Zhang et al., 2011; Chander, 2017). Additionally, N. diffluens and N. liquefaciens have common antigens, which are thought to act as allergens and have the possibility to associate the cause of atopic dermatitis (Kato et al., 2007). The strains isolated in the study were thought to be derived from humans. Meanwhile, N. diffluens was said to be the most dominant in the study of yeasts in house dust on the floor in Moscow (Glushakova et al., 2004). Combined with our results, N. diffluens could be considered the dominant yeast in house dust. Nevertheless, at this point, it is difficult to confirm whether the isolated N. diffluens is human dependent or dust dependent.

On the other hand, there are few isolation cases of Filobasidium magnum in the study of fungal flora in the skin of atopic patients and house dust, although they were highly likely to exist. According to the study of the fungal microbiome of the patients’ skin with seborrheic dermatitis by NGS analysis, the component ratio of F. magnum in the microbiome was 1.7%, which was much less than Can. parapsilosis (11%) and M. guilliermondii (5.8%) (Tanaka et al., 2014). Furthermore, F. magnum was not isolated in the study of house dust on floors in Moscow (Glushakova et al., 2004). This is the first case of study that showed F. magnum was isolated from bedding with relatively high frequency, suggesting that the species may be the specific yeasts in bedding. Still, it is unclear at this point whether this trend is common worldwide or unique to Japan. Additionally, there were some cases that F. magnum was detected with high frequency in kitchens and bathrooms in homes by using NGS analysis (Adams et al., 2017). Also, some studies found many F. magnum in the nasal cavity of the cat (Danesi et al., 2014). Furthermore, these isolates are likely to be Papiliotrema laurentii, a genetically distinct species from F. magnum, although there were some reports that Cryptococcus laurentii, which were once considered to be basionym, were isolated from house dust in China and South Korea (Lee et al., 1994; Beguin, 1995; Beguin and Nolard, 1996). The next highest was five strains of Can. parapsilosis (12%) and three strains of M. guilliermondii (7.3%). These yeasts are also often isolated from lesions of the skin such as atopic skins as well as N. diffluens species complex (Zhang et al., 2011; Tanaka et al., 2014), and it is thought that the fungus flora of human skin links to that of bedding. Sugita et al. (2013) evaluated that four species of Candida albicans, Can. parapsilosis, N. diffluens, and N. liquefaciens were specific to atopic patients, which was consistent with the top three species isolated in this study. Nevertheless, Can. albicans was not detected at all this time. As mentioned above, it is currently unknown whether these yeasts depend on humans or dust. A comprehensive study of the atopic status of residents, fungal skin flora, and house dust flora might be required.

In this study, the Malassezia species were not the object of study. On human skin, the largest number of yeasts adhering to are the yeasts of Genus Malassezia such as Malassezia restricta and Malassezia globosa (Zhang et al., 2011; Tanaka et al., 2014). Although DG18 is considered to be the most suitable media for isolation of fungi in the living environment (Samson et al., 2019), Dixon’s agar or Leeming and Notman Agar (LNA) are required to detect Malassezia.

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