Genetic Identification of Dematiaceous Fungi Isolated from Washing Machine in Japan, and Considering of Fungal Removal Methods

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In 2017, 2 household washing machines which were used at a house in Saitama prefecture were tested for fungal flora sampling. The fungi were identified in detail via genetic analysis of the ITS region. The number of fungi which were isolated were $8.7 \times 10^6$ to $6.1 \times 10^7$ cfu/100cm$^2$ and dematiaceous fungi such as Ochroconis musae, Exophiala, Cyphellophora, Knufia epidermidis were dominant. As for Knufia epidermidis, this is the first report for identification from a Japanese household. The number of isolated Cladosporium, which is common in the environment, was fewer compared with the above 4 genera. The identified fungi were Cladosporium halotolerans, which is recently being recognized as the main species of Cladosporium. In our research, we have cleaned the 2 washing machines with different bleaches. Residual fungi was detected in washing machine A, which was cleaned with an oxygen bleach. On the other hand, no fungi was detected in washing machine B, which was cleaned with a chlorine bleach.

Key words: Ochroconis musae / Knufia epidermidis / Exophiala / Cyphellophora europaea / Cladosporium halotolerans.

INTRODUCTION

Of all household electronic appliances, washing machines are the most prone to fungal infestation. Currently, many users are dealing with this problem. Research on fungal infestation of washing machines are reported in detail by Hamada (2002a; 2002b; 2004; 2005a; 2006; 2010). However, there are not many other reports on this topic. One reason for this is because washing machines are difficult to dismantle, and the fungal samples are not easy to collect. In this research, we had the opportunity to collect fungi from 2 washing machines. We have identified the dematiaceous fungi, which are the dominant fungi of washing machines, and obtained data of the fungal flora in detail using genetic analysis. We have cleaned the 2 washing machines using different types of bleaches, and confirmed that cleaning with a chlorine bleach was effective among them.

MATERIALS AND METHODS

Washing machines

In September 2017, 2 washing machines were obtained from a collective residence in Saitama prefecture, Japan. The collective residence was a company dormitory for single employees, and the washing machines were used at different homes.

Fungal sampling

The washing machines were dismantled and the inner-tub of the washing tubs were removed. Samples were obtained from areas on the outer-tub and on the pulsator where a large amount of blackish-brown substance was seen. As more fungi grow at the upper end of washing tubs (Hamada, 2004), the area 20 cm below the top of the outer-tub was swabbed for microbial testing using a swab kit (PF2002; Eiken, Japan). An area of 100 cm$^2$ was swabbed and vortexed for 1
Each suspension was serially diluted to 10000 times and each diluted solution was inoculated to a chloramphenicol-added PDA (Nissui, Japan) plates. The culture media were incubated for 7 days at 25°C. Fungal colonies that grew on the media were classified by morphology and counted. From each type of classifica-

### TABLE 1. Reference strains used for drawing the phylogenetic tree.

| Strain No. | Species                         | GenBank accession No. | Status    |
|------------|---------------------------------|-----------------------|-----------|
| NBRC* 4460 | Cladosporium halotolerans       | 00446001*             |           |
| NBRC 31678 | Cladosporium halotolerans       | 03167801*             |           |
| CBS 19354  | Cladosporium sphaerospermum     | AY361958              | Neotype   |
| NBRC 6348  | Cladosporium sphaerospermum     | 00634803*             | Neotype   |
| CBS 112388 | Cladosporium cladosporoides     | HM148003              |           |
| CBS 38664  | Cladosporium colosacae          | HM148067              | Isotype   |
| CBS 101466 | Cyphellophora europaea          | JQ766443              | Holotype  |
| CBS 124186 | Cyphellophora europaea          | KF928471              |           |
| CBS 21878  | Cyphellophora europaea          | JQ766441              |           |
| CBS 66682  | Cyphellophora europaea          | JQ766442              |           |
| CBS 28685  | Cyphellophora pluriseptata      | JQ766429              |           |
| CBS 19061  | Cyphellophora laciniata         | JQ766423              | Holotype  |
| CBS 22886  | Cyphellophora vermispora        | JQ766426              | Holotype  |
| CBS 52082  | Exophiala alcalphila            | NR_111624             | Type      |
| CBS 122256 | Exophiala alcalphila            | JF747044              |           |
| CBS 20735  | Exophiala dermatitidis          | AF050269              | Isotype   |
| NBRC 8193  | Exophiala dermatitidis          | 00819303*             |           |
| CBS 50790  | Exophiala jeanselmi             | NR_111129             | Type      |
| CBS 120353 | Knufia epidermidis              | NR_111330             | Holotype  |
| CCFEE 5813 | Knufia epidermidis              | JX681055              |           |
| CBS 88595  | Knufia perforans                | JN040506              |           |
| CBS 140325 | Phialophora verrucosa           | NR_146242             | Epitype   |
| NBRC 9389  | Phialophora verrucosa           | 00938901*             |           |
| CBS 21153  | Ochroconis constricta           | NR_145365             | Type      |
| CBS 116655 | Ochroconis humicola             | HQ667521              | Type      |
| CGMCC 3.14990 | Ochroconis musae       | JQ364738              |           |
| CBS 72995  | Ochroconis musae                | KF156029              |           |
| NBRC 32054 | Ochroconis musae                | 03205402*             |           |
| CBS 116659 | Ochroconis musae                | HQ667537              |           |
| CBS 12465  | Ochroconis musae                | HQ667532              |           |
| CBS 113948 | Ochroconis musae                | HQ667530              |           |

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| Species | GenBank accession No. | Status    |
|---------|-----------------------|-----------|
| Cladosporium halotolerans | 00446001* | Neotype   |
| Cladosporium sphaerospermum | AY361958 | Neotype   |
| Cladosporium cladosporoides | HM148003 | Neotype   |
| Cladosporium colosacae | HM148067 | Isotype   |
| Cyphellophora europaea | JQ766443 | Holotype  |
| Cyphellophora europaea | KF928471 |           |
| Cyphellophora europaea | JQ766441 |           |
| Cyphellophora europaea | JQ766442 |           |
| Cyphellophora pluriseptata | JQ766429 |           |
| Cyphellophora laciniata | JQ766423 | Holotype  |
| Cyphellophora vermispora | JQ766426 | Holotype  |
| Exophiala alcalphila | NR_111624 | Type      |
| Exophiala alcalphila | JF747044 |           |
| Exophiala dermatitidis | AF050269 | Isotype   |
| Exophiala dermatitidis | 00819303* |           |
| Exophiala jeanselmi | NR_111129 | Type      |
| Knufia epidermidis | NR_111330 | Holotype  |
| Knufia epidermidis | JX681055 |           |
| Knufia perforans | JN040506 |           |
| Phialophora verrucosa | NR_146242 | Epitype   |
| Phialophora verrucosa | 00938901* |           |
| Ochroconis constricta | NR_145365 | Type      |
| Ochroconis humicola | HQ667521 | Type      |
| Ochroconis musae | JQ364738 |           |
| Ochroconis musae | KF156029 |           |
| Ochroconis musae | 03205402* |           |
| Ochroconis musae | HQ667537 |           |
| Ochroconis musae | HQ667532 |           |
| Ochroconis musae | HQ667530 |           |
Dermatophytes were isolated from the washing machine. A genetic tree analysis was conducted in MEGA7 (Kumar et al., 2016). A list of reference strains for drawing phylogenetic trees is shown in Table 1.

By comparison with the morphologic characteristics and past literature (Feng et al., 2014; Hao et al., 2013; Li et al., 2008; Nishimura et al., 1987; Samerpitak, 2016), each colony was confirmed to correspond with the results from genetic analysis. The base sequence of each colony was registered to GenBank, and some of the colonies were deposited and preserved at the NBRC. As the Phoma genus are difficult to identify by genetic markers such as ITS region, we have limited identification of this genus to confirming Phoma sp. from morphology (Boerema et al., 2004).

A Fusarium oxysporum species complex was suspected from the morphological characteristics of a few white colonies which were isolated (Nelson et al., 1983), and were identified from analyzing the \( \beta \)-tubulin gene. Amplification of the \( \beta \)-tubulin gene was performed using the primers Btu-FuF01 (5'-CAGACCGGTCAGTACGCT-3') and Btu-FuR01 (5'-TTGGGGTGAAACATCGTACGCT-3') (Watanabe et al., 2011). The reference sequence was obtained and used from GenBank PopSet: 959098730 to create a phylogenetic tree. The procedure of gene analysis is the same as above.

**RESULTS**

The results of the identified dermatophytes from the 2 washing machines are shown in Table 2. The dermatophyte fungi were classified into 5 genera and 7 species. The estimated phylogenetic tree is shown.
FIG. 1. Phylogenetic tree of dematiaceous fungi identified by ITS region analysis. The evolutionary history was inferred using the Maximum Likelihood method based on the 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 circles are the strains obtained in this study. T indicates a type strain.
in Fig. 1. The 12 strains were each classified into one of the clusters with high bootstrap values. Cladosporium halotolerans (3 strains; 91%), Cyphellophora europaea (1 strain; 98%), Exophiala alcalophila (1 strain; 100%), Exophiala dermatitidis (2 strains; 99%), Ochroconis musae (2 strains; 97%). Species identification was deferred for 2 strains - strain no. NBRC 113468 and NBRC 113472 - which were classified into the Cyphellophora genus cluster because they developed a different monophyly from C. europaea. Also, the only white colony of fungus, strain FCG 1834 (GenBank accession No. LC414363) was identified as Fusarium oxysporum from the phylogenic tree which was estimated from a β-tubulin genetic sequence (the phylogenetic tree is omitted).

The type and number of isolated fungi is shown in Table 3. Approximately the same number of E. alcalophila, O. musae, and Cyphellophora sp. were the most isolated from the outer-tub of washing machine A and reached $10^6$cfu/100 cm$^2$ each. From the pulsator of washing machine A, E. alcalophila was most identified at $4.1 \times 10^3$cfu/100 cm$^2$, and took up 68% of all species isolated. From the outer-tub of washing machine B, both K. epidermidis and Rhodotorula were $2.7 \times 10^6$cfu/100 cm$^2$ and took up a large part. From the pulsator of washing machine B, O. musae was most isolated at $2.2 \times 10^5$cfu/100 cm$^2$ and took up 86% of all isolated species. After washing with oxygen bleach, washing machine A had fewer fungi in both the outer-tub and pulsator, but still much fungi remained. On the other hand, washing machine B was completely free of fungi after washing with chlorine bleach.

### DISCUSSIONS

Research on fungal flora of dematiaceous fungi in house hold water sections were stagnant in recent years, however, this research shows a new example of fungal flora based on the latest classification method.

Ochroconis musae which was predominant at the outer-tub of washing machine A and the pulsator of washing machine B is the most common species of the Ochroconis genus which is associated with infection on vertebrates (Samerpitak et al., 2015). This fungus was recorded as a new species in 2013 and was named Scolecobasidium musae at first (Hao et al., 2013). Immediately after this, Ochroconis genus and Scolecobasidium genus, which are related were classified by genetic analysis and most of the Scolecobasidium genus was moved to the Ochroconis genus. With this, S. musae was revised as O. musae (Samerpitak et al., 2015). At a fungal flora research of washing machines conducted at Osaka, the Scolecobasidium genus and the Exophiala genus were excessively predominant (Hamada, 2002a; 2004; 2005a; 2010, Abe and Hamada, 2011). From these reports, the fungus identified as Scolecobasidium genus and the fungus identified as O. musae in this study are considered the same. Abe and Hamada (2011) identified the Scolecobasidium genus isolated from moist environments such as inside
washing machines or bathrooms, as *Scolecosasidium humicola* from genetic analysis. In their study, they used strain NBRC 32054 (S. humicola), as a reference. We reidentified this strain based on the current classification as *O. musae*. The type strain (CBS 116655) of *S. humicola* was classified as a completely different cluster. NBRC 32054 and the 2 strains isolated in this study were in the same cluster as *O. musae* (Fig. 1). *O. musae* were predominantly isolated from both washing machines and this result is consistent with the research done in Osaka (Hamada, 2002a; 2004; 2010). According to Hamada (2005a, 2006), *Scolecosasidium* are predominant in washing machines where synthetic detergent is used, because their growth is not inhibited by non-ionic surfactants. In Italy, *O. musae* is isolated from soap dispensers for washing machines (Isola, 2103). The infestation of washing machines by this dematiaceous fungi is not a case only in Japan.

From washing machine A, 2 species of the *Exophiala* genus *E. alcalophila* and *E. dermatitidis* were isolated, and *E. alcalophila* was especially predominant. According to Hamada (2005a; 2005b; 2006), the growth of *Exophiala* is also not inhibited by synthetic detergent as is *Scolecosasidium*, and becomes predominant in washing machines. However, sensitivity to non-ionic surfactants differ with species, and *E. alcalophila* is presented as a species capable of growth (Hamada, 2005a). This matches the results of our study and indicates the high affinity of *E. alcalophila* in washing machines.

As discussed above, the major fungi known to exist in washing machines are *O. musae* and *Exophiala*, however, in our study it is notable that we have isolated just as many *Cyphellophora* and *Knufia epidermidis*.

Many of the *Cyphellophora* genus previously belonged to the *Phialophora* genus. Due to genetic analysis in recent years, many *Phialophora* were included into the *Cyphellophora* genus (Réblová et al, 2013). There have been several reports that *Cyphellophora europaea*, which is known to be a cause of chromomycosis (de Hoog, 2000; Li et al., 2008), were isolated from bathrooms (Hamada and Abe, 2009; Lian and de Hoog, 2010). These species (*Phialophora europaea*) are also capable of growth under the presence of detergents according to Hamada and Abe (2009). Both strains NBRC 113468 and NBRC 113472 needed further evaluation before confirming them as *C. europaea*, therefore they were referred to as *Cyphellophora* sp. and species identification was deferred (Fig. 1). However, many strains of fungi were isolated from the 2 washing machines that it can be expected that they have a similar ability of survival and growth as *C. europaea*.

As for *Knufia epidermidis* there is not much information on their distribution in household environments. The *Knufia* genus have been considered an environmental fungi creating black spots on the surface of plants and rocks (De Leo et al., 1999; Hyde et al., 2002; Sterflinger et al., 1997; 2001). However, recently, *K. epidermidis* was isolated from a patients skin with chromomycosis and the presence of a pathogenic species was recognized (Li et al., 2008; Tsuneda et al., 2011). There is only one report on isolation of this species from a household environment, which is from a kitchen in Slovenia (Zupančič et al., 2016). Although this report is the first example of isolation of this species from a household environment in Japan, they may have been overlooked due to the fact that *K. epidermidis* has a low productivity of conidiospores (Li et al., 2008). Many have been isolated from both washing machines A and B, and it is predicted that they are widely distributed in the Japanese household environment as is with *Ochrroconis, Exophiala* and *Cyphellophora*. Continued research is desired.

In washing machine A that were tested the 4 genera of fungal flora discussed above were most isolated, and was followed by *Cladosporium* which is the most common environmental fungi. Washing machine B also had less *Cladosporium* than other dematiaceous fungi.

*Cladosporium halotolerans* which was isolated from both washing machines is a hygrophil fungi isolated from various environments such as bathrooms and oceans, and were listed as a new species in 2007 (Zalar et al., 2007). Although C. cladosporioides and *C. sphaerospermum* were known as the main species of the *Cladosporium* genus (Samson et al., 2004), with the recent prevalence of genetic analysis, it has become recognized that *C. halotolerans* is the main *Cladosporium* in household environments (Sandoval-Denis et al., 2015; Bensch et al., 2018). In indoor environments in Japan, *C. halotolerans* is also a more dominant *Cladosporium* than *C. sphaerospermum*, and the fact *C. cladosporioides* sensu stricto barely exists in Japanese household environments, is gaining recognition (Kobayashi et al., 2012). As the 3 strains we have analyzed in this study are *C. halotolerans*, it can be estimated that there is a similar tendency also in washing machines. Generally, *Cladosporium* is one of the major fungi in household environments (Samson et al., 2004), however, in a research conducted in Osaka, it was suggested that *Cladosporium* was not predominant in washing machines (Hamada, 2002a; 2004). From fungal flora research the authors have conducted in households in Saitama and Tokyo, the dominant species in airborne fungi and house dust are overwhelmingly *Aspergillus* and *Cladosporium* (Hashimoto and Kawakami, 2015; 2018; Kawakami et al., 2016). However, in the washing machines in this study, the number of isolated *Cladosporium* was low compared to
other dermatiaceous fungi, and *Aspergillus* was not isolated at all. On the other hand, *O. musae*, *Exophiala*, *Cyphellophora*, *Knufia epidermidis* are presumed to exist in washing machines with a high affinity.

The 2 washing machines were cleaned with different bleaches. 48 % of fungi remained in the outer-tub and 8 % remained on the pulsator of washing machine A, which was washed with oxygen bleach. On the other hand, no fungi was isolated from both the outer-tub and the pulsator of washing machine B, which was washed with a chlorine bleach (Table 3). This indicates that the effect on fungi removal differs with the type of bleach used, although this cannot be confirmed due to limited sample numbers. Currently in Japan, the main products on the market are oxygen bleaches, and the majority of popular detergents on websites and magazines are oxygen bleaches as well. Chlorine bleaches are avoided due to their toxicity and corrosiveness on other products. However, if there is a dramatic difference on fungi removal as in the results of this study, this may be helpful information on Japanese consumers who are troubled by fungal infestation in their washing machines. Further continued research in this area is needed as well.

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