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Rapid discrimination of fungal species by the colony fingerprinting

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ABSTRACT

The contamination of foods and beverages by fungi is a severe health hazard. The rapid identification of fungi species in contaminated goods is important to avoid further contamination. To this end, we developed a fungal discrimination method based on the bioimage informatics approach of colony fingerprinting. This method involves imaging and visualizing microbial colonies (referred to as colony fingerprints) using a lens-less imaging system. Subsequently, the quantitative image features were extracted as discriminative parameters and subjected to analysis using machine learning approaches. Colony fingerprinting has been previously found to be a promising approach to discriminate bacteria. In the present proof-of-concept study, we tested whether this method is also useful for fungal discrimination. As a result, 5 fungi belonging to the *Aspergillus*, *Penicilium*, *Eurotium*, *Alternaria*, and *Fusarium* genera were successfully discriminated based on the extracted parameters, including the number of hyphae and their branches, and their intensity distributions on the images. The discrimination of 6 closely-related *Aspergillus* spp. was also demonstrated using additional parameters. The cultivation time required to generate the fungal colonies with a sufficient size for colony fingerprinting was less than 48 h, shorter than those for other discrimination methods, including MALDI-TOF-MS. In addition, colony fingerprinting did not require any cumbersome pre-treatment steps prior to discrimination. Colony fingerprinting is promising for the rapid and easy discrimination of fungi for use in the ensuring the safety of food manufacturing.

1. Introduction

The contamination of foods by microorganisms, including toxic bacteria and fungi, can lead to severe health hazards. Therefore, the detection and discrimination of the contaminating microorganisms is critically important to ensure the safety of foods and beverages. Contamination by fungi, in particular, can cause various problems, including foods spoilage, change in color, and a moldy odor (Schnurer et al., 1999). More harmful effects can appear if the fungi species produces secondary metabolites known as mycotoxins, such as aflatoxins (Richard, 2007). Because closely-related fungal species can exhibit very different toxicity, the discrimination of fungi at the species-level with a high accuracy in food safety tests is necessary. Conventionally, fungi discrimination is implemented by analyzing the morphological features of fungal cells. However, this laborious method requires a high level of expertise and a long cultivation time for an accurate discrimination.

Apart from the traditional method, several alternative approaches for the discrimination of fungi have been proposed. For example, the discrimination of fungi based on matrix-mediated laser assisted mass spectrometry (MALDI-TOF-MS) has been well-studied in the past decade, and several platforms are currently commercially available (Cassagne et al., 2016). MALDI-TOF-MS had been reported to reveal chemical fingerprints, mainly derived from the extracted proteins of the fungi of interest. However, this method requires fully-grown colonies of fungi cultured in strictly controlled conditions for several days or weeks (Packeu et al., 2013) in order to perform a highly accurate identification. Furthermore, the extraction of proteins from the fungal cells, surrounded by robust cell walls, required pre-treatments to obtain mass spectra reflecting species-specific chemical fingerprints. In addition, it would be difficult for small-sized food manufacturers to set up and install the expensive platforms required for MALDI-TOF-MS. In addition to MS-based chemical fingerprinting, other optical fingerprinting methods exist, such as surface-enhanced Raman spectroscopy (SERS) (Dina et al., 2018), and Fourier transform infrared spectroscopy (FTIR)

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(Lecellier et al., 2015), which have been proposed for use in fungal discrimination. However, these methods also require relatively long assay times and cumbersome operation steps.

To address these issues, in the present study, we propose an alternative method for the discrimination of fungi based on a bioimage informatics approach, denoted as "colony fingerprinting" (Maeda et al. 2017, 2018; Tanaka et al., 2019). In colony fingerprinting, the growth of micro-sized colonies can be visualized using a unique imaging technique, namely lens-less imaging (Jung and Lee, 2016; Ozcan and Demirci, 2008; Saeki et al. 2014, 2015; Tanaka et al., 2010). Lens-less imaging takes advantage of a wide field-of-view, although this is counteracted by the fact that its resolution is low compared to microscopy. However, the wide field-of-view allows for the simultaneous observation of a number of colonies over time with a high resolution. The colony images obtained using lens-less imaging are referred to as "colony fingerprints," from which a set of quantitative parameters useful for the discrimination of microbial species can be extracted. Subsequent multivariate analyses using machine learning approaches allow us to discriminate the microbial species. In a recent study, we successfully demonstrated the discrimination of up to 20 species of bacteria, including closely-related species (Tanaka et al., 2019). However, it is still unclear whether colony fingerprinting will be useful in the discrimination of fungi, which may exhibit colony fingerprints that are much different fromthose of bacteria.

Similarly, another method for microbial discrimination based on a bioimage informatics approach was proposed by Bhunia's group. In this method, referred to as BActerial Rapid Detection using Optical scatter Technology (BARDOT), the microbial colonies were irradiated using lasers, and the generated light scattering patterns were analyzed. The images of the light scattering patterns correspond to the three-dimensional (3D) structures of the colonies (Bae et al., 2007; Doh et al., 2019). Although BARDTOT can be used for the discrimination of various bacteria (Banada et al. 2007, 2009; Huff et al., 2012; Tang et al., 2014), it has not yet been utilized for the discrimination of fungi.

The aim of this study was to confirm whether colony fingerprinting is applicable to rapid fungal discrimination. As a proof-of-concept study, we attempted to discriminate 5 fungal species belonging to different genera, namely *Aspergillus*, *Penicilium*, *Eurotium*, *Alternaria*, and *Fusarium*, which are frequently detected in food, by colony fingerprinting. A total of 5 discriminative parameters, including the number of hyphae and their branches, were calculated from the images of the fungal colonies, before being subjected to machine learning for species discrimination. We further analyzed 6 closely-related species belonging to the identical genus *Aspergillus* with additional 2 parameters. The results obtained in this study suggest that colony fingerprinting is useful to easily and rapidly discriminate fungi.

2. Experimental details

2.1. Fungal strains

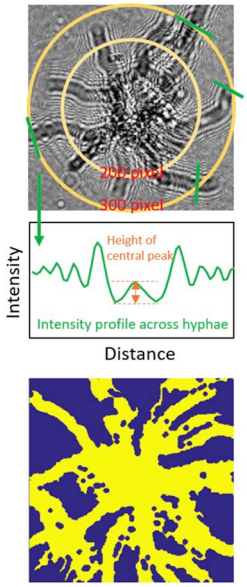
The fungi used in this study were obtained from the culture collection of the National Institute of Technology and Evaluation (NITE) Biotechnology Research Center (NBRC). We analyzed 10 fungal species, as summarized in Supplementary Table S1. The genus names of microorganisms are usually abbreviated with one capital letter, according to general nomenclature (e.g. *A. oryzae* for *Aspergillus oryzae,* and *A. alternata* for *Alternaria alternata*). However, in this study, the genus name of *Alternaria alternata* was abbreviated into two letters *Al*, i.e. *Al. alternata*, to distinguish *Aspergillus* and *Alternaria*. The fungi were maintained on potato dextrose agar medium at room temperature (Supplementary Fig. S1), and transferred to a new agar medium every 2 months.

2.2. Lens-less imaging system

The imaging system used in this study is composed of a complementary metal oxide semiconductor (CMOS) image sensor (2048 × 1536 pixels, pixel size: 3.2 μm, imaging area: 6.55 × 4.92 mm^2, DFK61BUC02; The Imaging Source, Bremen, Germany), a pinhole (φ100 μm; Sigmakoki Co., Saitama, Japan), and a blue light-emitting diode (LED, λ = 465 nm, located at least 6 cm above the sensor) (Fig. S2). The fungi were first cultured on potato dextrose agar medium (approximately 20 ml, Difco Laboratories, USA) in a plastic culture dishes for at least 1 week. The cells were then collected from the agar medium using a wooden toothpick and suspended in phosphate buffered saline (PBS) at pH 7.4. The concentrations of the spores were determined using a hemocytometer and a microscope. Subsequently, a lysogeny broth (LB) agar medium (2 ml) containing 1.5% (w/v) agarose with a low-melting point (Invitrogen, Thermo Fisher Scientific K.K., Tokyo, Japan) was prepared in plastic culture dishes (internal diameter: 35 mm; IWAKI, AGC Techno Glass Co., Ltd., Tokyo, Japan). The spore suspension was diluted to a concentration of 500 spores/ml in PBS. Then, 100 μl of the diluted spore suspension was spread onto the LB agar medium by shaking the culture dishes. Culture dishes were fixed on CMOS image sensors using double-stick tape (thickness: 300 μm; Thermo Fisher Scientific K.K., Tokyo, Japan), irradiated by blue LED through a pinhole, and incubated at room temperature. The lid of the culture dish was connected to digital micro-servomotor SG90 (Tower Pro Pte Ltd, Singapore) and opened for 1 min under the control of a microcomputer (Arduino Uno compatible board; Marutsuelec Co., Ltd., Chiyoda, Japan). After the lid was opened, an image of the culture dish was captured using the CMOS image sensor (exposure time: 1/18 s) under the control of IC Capture 2.2 software (The Imaging Source Europe GmbH, Bremen, Germany). The lid was then closed for 14 min. This cycle was repeated for at least 19 h. If the lid of the dish was not opened, water condensation at the surface of the lid occurred, which hampered imaging during incubation. This problem was solved by opening the lid using an automatic motor-control system. The entire system, including the CMOS image sensors, LED, culture dishes, micro-servomotors, and microcomputers, was enclosed in a desktop-type light shielding box.

2.3. Extraction of discriminative parameters from the images

We extracted 7 discriminative parameters from the colony fingerprints (Fig. 1), namely: (i) the number of hyphae (fungal filaments) intersecting with the circle with a diameter of 200 pixels (i.e. 640 μm), where the center of the circle was determined to be a center of the spore that was visualized at the initial stage of the incubation; (ii and iii) the number of branches of hyphae in the circle with a diameter of 200 and 300 pixels, respectively; (iv) the average height of the central peaks of the intensity prolife across the hyphae on the circle with a diameter of 300 pixels; (v, vi, and vii) the average intensities of the hyphae within the 300, 200, and 100 pixel squares, respectively. The number of hyphae and branches (parameters 1–3) were manually counted by observing the extension and branching behaviour of the hyphae over time. This was possible since the colony fingerprints were obtained in a time-series manner (every 15 min). The intensity prolife across the hyphae was obtained using an image analysis tool, Image J (Schneider et al., 2012). The lens-less images of the hyphae were typically outlined by lines with low intensity. The center of the hyphae, between the low intensity lines, showed a relatively high intensity. The height of the central peak was defined as the intensity difference between the maximum intensity of the center region and the minimum intensity of low intensity lines (Fig. 1). The average value of the heights of the central peaks of the hyphae was used as the discrimination parameters of a certain colony. To extract the hyphae region in the image, the raw image was trimmed into certain sizes (300, 200, or 100 pixel squares), and subjected to binarization, noise removal (removing objects smaller than 50 pixels), smoothing, and filling-up processes using the image analysis software



(1) Number of hyphae intersecting with the circle with a diameter of 200 pixel
(2) Number of branches in a circle with a diameter of 200 pixels
(3) Number of branches in a circle with a diameter of 300 pixels
(4) Average height of central peaks of intensity profile across the hyphae on a circle of 300 pixels
(5) Average intensity of hyphae within a 300 pixel square
(6) Average intensity of hyphae within a 200 pixel square
(7) Average intensity of hyphae within a 100 pixel square

Fig. 1. Discriminative parameters extracted from the colony fingerprints of fungi for species discrimination. Intensity profiles were obtained across the green lines shown in the image of fungal colony.

MATLAB (The MathWorks, Inc., Natick, MA, USA). The average intensities (parameters 5–7) of the extracted hyphae regions were computed using MATLAB. These parameters were computed from the colony fingerprints of at least 10 colonies of each species using MATLAB. The colonies were selected randomly from culture dishes. However, we did not select any colonies that overlapped with other colonies at the early stage, since the overlapping of colonies at the early stage hampers the accurate counting of the number of hyphae and branches.

Discrimination analysis was performed using R version 3.1.2 (R Foundation for Statistical Computing, Vienna, Austria) (R Development Core Team, 2013). Support vector machine (SVM) and random forest (RF) were operated using the svmRadial and rf methods in the caret package, respectively. Leave-one-out cross-validation was employed to assess the generalizable discrimination accuracy of the models.

2.4. Confocal microscopy

Each fungus was cultivated on LB agar medium for approximately 24 h, as described above. Subsequently, 100 µl of aqueous solution of FM 1–43 Dye (N-(3-Triethylammoniumpropyl)-4-(4-(Dibutylamino) Styryl) Pyridinium Dibromide, 5 µg/ml; Thermo Fisher Scientific K.K., Tokyo, Japan) was added to the fungi on the culture dish (Fischer-Parton et al., 2000). The culture dish was incubated with the dye at room temperature, then observed using a confocal microscope Fluoview FV1000 (Olympus Corporation, Tokyo, Japan). The microscopic data were analyzed using the image analysis software, Volocity version 6.0 (PerkinElmer, Waltham, MA, USA).

2.5. Phylogenetic analysis

We obtained the DNA sequences of the internal transcribed space (ITS) and 26/28S rDNA-D1/D2 of the fungi from the NBRC website. These sequences have long been employed to identify fungal species (Makimura et al. 1998, 2000). The neighbour joining and maximum likelihood methods were employed using MEGA 7 software. Bootstrap trials were replicated 1000 times for the neighbour joining method. Gaps were treated as pairwise deletions.

2.6. Statistical analysis

One-way analysis of variance (ANOVA) was used to assess the statistically significant difference of each discriminative parameter extracted from fungi, with an α-level of 0.05.

3. Results

3.1. Analysis of fungal growth using the lens-less imaging system

Fig. 2 shows a colony fingerprint observed using lens-less imaging and a microscopic image of an *A. nomius* colony. In the microscopic image, some hyphae were observable at the focal plate, while others became observable after focusing on different planes (Fig. 2 (a)), suggesting that the hyphae extended not only across a two dimensional (2D) surface, but also in three-dimensions (3D), even at the early stages of micro-scale colonies. Indeed, we confirmed the substantial growth of fungi in the 3D direction using confocal microscopy (Supplementary Fig. S3). By contrast, these hyphae, which were visualized at different focal planes in microscopy, were simultaneously observable using lens-less imaging as expected (white arrows in Fig. 2 (b)). In principle, lens-less imaging systems have the advantage of not only a wide-field-of-view on the x-y plane but also long depth along the z-axis of the observable space. For example, a similar lens-less imaging platform showed millimeter-scale depth-of-field (Su et al., 2012), which is long enough to visualize the microcolonies of fungi (Supplementary Fig. S3). This feature allowed us to visualize the hyphae distributing in 3D space. As a result, the 3D information of the fungal colonies was included in the colony fingerprints.

We monitored the growth of 6 *Aspergillus* spp. and *P. citrinum*, *F. solani*, *E. amstelodami*, and *Al. alternata* (*Al.* stands for *Alternaria*) using lens-less imaging (Supplementary Fig. S4). Fig. 3 shows the time-course

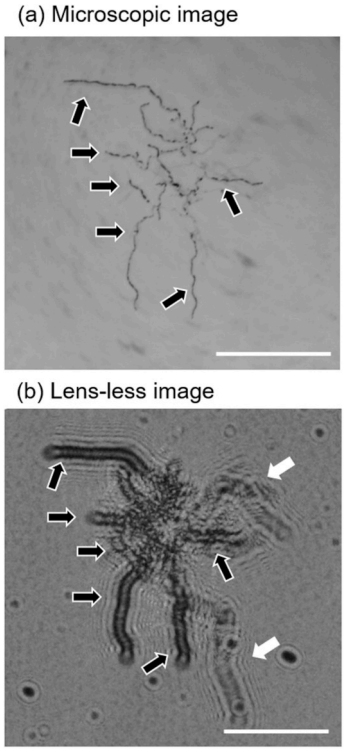


Fig. 2. Comparison of the microscopic image (a) and lens-less image (b) of an identical colony of *Aspergillus nomius*. Black arrows indicate the hyphae clearly observed in both images, while white arrows indicate those which were found only in the lens-less image. (scale bar = 400 µm, the scale bar of the lens-less image was defined from the pixel size).

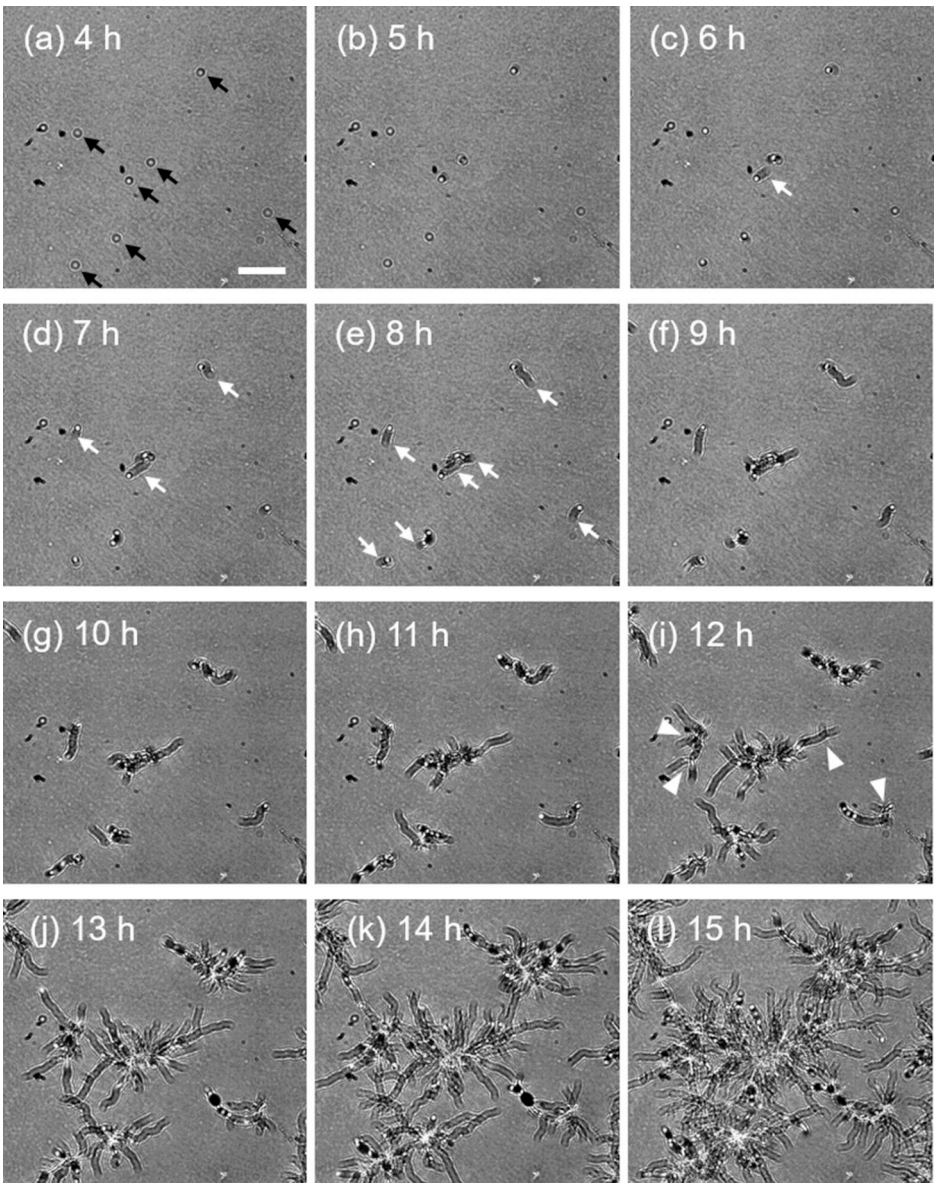


Fig. 3. Time-course variation of colony fingerprints of *Aspergillus oryzae* (scale bar = 400 μm). The time shown in the figures denotes the cultivation time. The black arrows in (a) indicate the spore before hyphae extension. The white arrows in (c), (d), and (e) indicate the extending hyphae. The white arrowheads in (i) indicate the branches.

variation of colony fingerprints of a representative fungus, *A. oryzae*. From the spores (black arrows in Fig. 3 (a)), we confirmed the extension of hyphae (fungal filaments) (white arrows in Fig. 3 (c ~ e)), as well as the branching points of the hyphae (white arrowheads in Fig. 3 (i)). *Aspergillus* spp. and *P. citrinum* extended their hyphae in arbitrary directions. *F. solani* first extended a relatively long single hypha, and subsequently, several branches appeared across the hypha. The hyphae of *E. amstelodami* generated two-pronged branches during the branching events. *Al. alternata* extended relatively thick hyphae with high contrast. *Aspergillus* spp., including *A. oryzae*, showed similar colony fingerprints, whereas some of the image features, such as the intensity and number of hyphae, around the initial spore looked different. We assumed that these features would be useful to discriminate fungi based on colony fingerprinting, and analyzed them quantitatively in subsequent experiments.

3.2. Discrimination of fungi belonging to different genera

Next, we attempted to discriminate 5 fungi belonging to different genera, namely *A. oryzae*, *P. citrinum*, *F. solani*, *Al. alternata* and *E. amstelodami*. Five discriminative parameters (parameters 1–5 shown in Fig. 1) were extracted from the colony fingerprints of each fungi. The representative fungal colony fingerprints and the corresponding parameter values are shown in the Supplementary Information (Supplementary Fig. S5; a list of the parameter values is also available as the Supplementary Table S2). Subsequently, fungi discrimination was performed using SVM with the 5 discriminative parameters. As a result, a discrimination accuracy of 100% was obtained (Supplementary Table S3), indicating that the discrimination of fungi by colony fingerprinting was successfully demonstrated, while a set of discriminative parameters different from those used for bacterial discrimination should be employed (Maeda et al. 2017, 2018; Tanaka et al., 2019).

The distributions of the 5 extracted parameters are shown in Supplementary Fig. S6. These parameters were examined using one-way ANOVA ($\alpha = 0.05$), and significant differences were found in all parameters (Supplementary Table S4), suggesting that all of these parameters could contribute to successful discrimination. A particularly significant difference was found in the intensity of the hyphae (parameter 5), where the lowest p-value was attributed to differences in the

light absorption characteristics of the fungi analyzed. The branching behaviour of the hyphae also exhibited significant differences among the fungi analyzed in this study. It was reported that intrinsic features, including intercellular vesicle trafficking and the formation of the septum (a wall structure dividing the hypha cells), were involved in the branching phenotypes of the fungal hyphae (Harris, 2008). In addition, it was previously reported that the height of the central peaks reflected the actual size of the objects (i.e., size of hyphae) visualized with lens-less imaging (Roy et al., 2015). These species-specific features could contribute to the differentiation of the fungal colony fingerprints. As mentioned above, the 3D information of the hyphae is likely to be included in the colony fingerprints, such that the positions of the hyphae along the z-axis may affect the values of the discriminative parameters. However, such fluctuating effects are likely to be minor compared to the differences between fungal species.

We then examined whether there existed any correlations between the colony fingerprints and the taxonomic relationships of these fungi. We compared the results of the hierarchical clustering based on the 5 discriminative parameters and the phylogenetic tree based on the DNA sequences of ITS and 26/28S rDNA-D1/D2 regions (Supplementary Fig. S7 (a)). In hierarchical clustering, the separation of *E. amstelodami* from other 4 species was confirmed (Supplementary Fig. S7 (b)). This separation was also supported by principal component analysis (Supplementary Fig. S7 (c)). However, such a separation was not confirmed with the phylogenetic analysis based on DNA sequences (Supplementary Fig. S7 (a)). Meanwhile, at least, a part of the hierarchical clustering result was consistent by the phylogenetic analysis; e.g. *A. oryzae* and *P. citrinum,* both of which belongs to the same family Trichocomaceae, were positioned closely. These results suggest that only 5 image features extracted from the colony fingerprints did not fully represent the taxonomic relationships, but could partially highlight the tendency that relatively close species have similar colony fingerprints.

3.3. Discrimination of closely-related fungi belonging to an identical genus

The next challenge was to discriminate between closely-related species belonging to the same genus. The aforementioned 5 discriminative parameters were extracted from the colony fingerprints of the 6 closely-related *Aspergillus* spp., namely *A. oryzae*, *A. niger*, *A. nomius*, *A. flavus*, *A. awamori*, and *A. versicolor* (Supplementary Fig. S8 (a-e)). ANOVA was used to determine the significant differences in all 5 parameters among the 6 species (Supplementary Table S4). In particular, it was confirmed that the average intensity of the hyphae of *A. niger* was significantly lower than those of the other fungi (Supplementary Fig. S8 (e)). The low intensity of the hyphae of *A. niger* detected by lens-less imaging could be attributed to the black melanin pigments in these cells (Jorgensen et al., 2011).

We attempted to discriminate 6 *Aspergillus* spp. by SVM with these 5 parameters. However, the accuracy of the discrimination was only 86.7% (Fig. 4 (a)), indicating that species-level discrimination is more difficult than genus-level discrimination, as predicted. Therefore, we added 2 discriminative parameters, namely the average values of hyphae intensity within 100 and 200 pixel squares (parameters 6 and 7 in Fig. 1). These parameters also showed statistically significant differences

(a) SVM with 5 discriminative parameters

Predicted \ Actual	*A. oryzae*	*A. niger*	*A. nomius*	*A. flavus*	*A. awamori*	*A. vercicolor*
A. oryzae	18	3	0	2	3	0
A. niger	0	17	2	0	0	0
A. nomius	0	0	18	0	0	0
A. flavus	2	0	0	15	1	0
A. awamori	0	0	0	3	16	0
A. vercicolor	0	0	0	0	0	20

(b) SVM with 7 discriminative parameters

Predicted \ Actual	*A. oryzae*	*A. niger*	*A. nomius*	*A. flavus*	*A. awamori*	*A. vercicolor*
A. oryzae	19	0	0	1	1	0
A. niger	0	20	0	0	0	0
A. nomius	0	0	19	0	0	0
A. flavus	1	0	0	18	1	0
A. awamori	0	0	0	1	18	0
A. vercicolor	0	0	1	0	0	20

(c) Random forest with 7 discriminative parameters

Predicted \ Actual	*A. oryzae*	*A. niger*	*A. nomius*	*A. flavus*	*A. awamori*	*A. vercicolor*
A. oryzae	20	0	0	0	0	0
A. niger	0	20	0	0	0	0
A. nomius	0	0	20	0	0	0
A. flavus	0	0	0	20	0	0
A. awamori	0	0	0	0	20	0
A. vercicolor	0	0	0	0	0	20

Fig. 4. Discrimination results of 6 closely-related *Aspergillus* spp. with colony fingerprinting. (a) Discrimination based on support vector machine (SVM) with 5 discriminative parameters (parameters 1–5, shown in Fig. 1). (b) Discrimination based on SVM with 7 discriminative parameters (parameters 1–7, shown in Fig. 1). (c) Discrimination based on random forest (RF) with 7 discriminative parameters (parameters 1–7, shown in Fig. 1). The number of colonies correctly and incorrectly discriminated are highlighted in dark grey and light grey, respectively.

(Supplementary Table S4). The discrimination accuracy with SVM after the addition of these two parameters (a total of 7 parameters) reached 95% (Fig. 4 (b)). For the discrimination with SVM using 7 parameters, the colony fingerprint of *A. oryzae* incorrectly classified to *A. flavus* possessed relatively few hyphae. By contrast, that of *A. flavus* incorrectly classified to *A. oryzae* possessed relatively fewer branches. To prevent incorrect classifications, we may need to develop more discriminative parameters to highlight the differences between closely-related *Aspergillus* spp. However, as an alternative, we attempted another machine learning approach, RF. This approach has previously exhibited a high performance in colony fingerprinting for bacteria, as reported in our previous study (Tanaka et al., 2019). Discrimination with RF using 7 parameters showed a discrimination accuracy of 100% (Fig. 4 (c)).

4. Discussion

In our previous study, we demonstrated that typical bacterial colonies generated radially symmetric colony fingerprints (Maeda et al., 2018; Tanaka et al., 2019). By contrast, the morphologies of fungal colony fingerprints were very different from those of bacteria (Figs. 2 and 3). Therefore, we did not use the same discriminative parameters employed for bacterial discrimination (i.e. roundness, solidity, and doughnutness (Maeda et al., 2018)). Instead, in this study, we developed a novel set of discriminative parameters, which represent the morphological features and intensity distributions of colony fingerprints, for fungi discrimination (Fig. 1).

In this study, SVM and RF were employed as representative machine learning approaches for the discrimination of fungal species using colony fingerprinting (Fig. 4). SVM is a classifier, used to determine the hyperplane to separate the classes. The hyperplane is defined as the margin between the classes and the hyperplane becomes maximum. SVM has been widely employed for discrimination of microorganisms due to its high performance. RF is an ensemble method in which multiple decision trees are developed. The final classification is the class with the most votes. The high performance of RF is attributable to a high number of decision trees, although the discrimination performance of each decision tree is not necessarily high. In addition to these, other methods can be potentially used, such as artificial neural network (ANN), which was employed in a previous study for bacterial discrimination (Maeda et al., 2018). Deep learning is also widely employed in the field of bioimage informatics (Esteva et al., 2017; Fuentes et al., 2017; Wang et al., 2016), as will be colony fingerprinting in future studies. Here, we demonstrated the discrimination of 5 fungi belonging to different genera, as well as the discrimination of 6 *Aspergillus* spp. as a proof-of-concept study. The use of these machine learning approaches and the development of new discrimination parameters in future studies will contribute to the expansion of this research towards more demanding goals, such as in the discrimination of hundreds of fungal species, as well as fungi discrimination at the strain level.

Table 1 summarizes the performance of fungal discrimination with colony fingerprinting presented in this study, as well as MALDI-TOF-MS, SERS, and FTIR. The discrimination of fungi using MALDI-TOF-MS has been widely studied, and its performance has been reviewed elsewhere (Cassagne et al., 2016). Four types of platforms are commercially available, all of which have been employed for the discrimination of tens of fungal species with high discrimination accuracies (Cassagne et al., 2016). However, using these methods, the extraction of proteins from fungal cells surrounded by robust cell walls is needed prior to analysis. This extraction process hinders the speed and simplicity of fungal discrimination using MALDI-TOF-MS. In addition, a relatively long cultivation time is required, ranging from several days (L'Ollivier et al., 2013; Lau et al., 2013) to weeks (Packeu et al., 2013). This long cultivation time can cause delays in treating the contamination of products by fungi.

In addition to chemical fingerprinting with MALDI-TOF-MS, fungi discrimination based on optical fingerprinting methods, such as SERS (Dina et al., 2018) and FTIR (Lecellier et al., 2015), has been reported. Compared to MALDI-TOF-MS, the optical methods require a relatively shorter cultivation time. However, cumbersome *in situ* synthesis of silver nanoparticles is required for signal enhancement in the SERS methods as a pre-treatment of the specimens before analysis. Another method, FTIR, requires less pre-treatment, however, using this method, fungal cells need to be washed and dried to remove nutrient salts and water to avoid any influence on the adsorption readings. Furthermore, all of these methods, namely MALDI-TOF-MS, SERS, and FTIR, only use snap-shot fingerprint data for discrimination, despite the fact that fungi produce different secondary metabolites, such as pigments, depending on the age of the colonies. Thus, fingerprints derived from chemical compounds contained in the cells can vary drastically. Indeed, even mass spectra obtained from identical colonies have been found to change over cultivation time (Coulibaly et al., 2011). Therefore, the cultivation time needs to be fixed when fingerprinting the samples.

In contrast to these methods, the cultivation time for colony fingerprinting was less than 48 h, which was shorter than that required for MALDI-TOF-MS, SERS, or FTIR, as it only required sub-millimeter scale micro-colonies. In colony fingerprinting, the image data is accumulated in the time-series manner, i.e. from the initial sporing stage to the filamentous hyphae extension stage, rather than in a snap-shot manner. This allows for the comparison of the fingerprints at similar growth stages. Moreover, it is possible to utilize the colony fingerprint data at multiple growth stages for discrimination, allowing us to extract more discriminative parameters towards a more accurate discrimination (Tanaka et al., 2019). It is also worth noting that the desktop platform used for colony fingerprinting (Fig. S1), consisting of an LED, pinhole, and CMOS image sensor, is more economic and compact than the MALDI-TOF-MS, SERS, or FTIR platforms. These advantages demonstrate that the colony fingerprinting method developed in this study is promising for the rapid and easy discrimination of fungi, which may be useful in a wide range of industries, including the manufacturing of foods, beverages, cosmetics, and pharmaceuticals. The purpose of this study was to demonstrate that colony fingerprinting can be applied to fungal discrimination. For its practical application, the discrimination of fungal species in real samples should be tested in future studies. In the present study, we performed leave-one-out cross-validation in which both the test data and the training data were the images of fungal colonies from a single source. Future studies should investigate whether colony fingerprinting allows for the discrimination of fungal species even if the test data and training data originate from different sources.

Table 1
Comparison of methods for discrimination of fungi.

Method	Cultivation time	Discrimination (Accuracy)	Pre-treatment	Reference
Colony fingerprinting	<48 h	5 species in different genera (100%) 6 species in identical genus (100%)	None	This study
MALDI-TOF-MS	5 days	91 species (88.9%)	Extraction of proteins	Lau et al. (2013)
SERS	Several hours ~ 2 days	3 species including 2 *Aspergillus* spp. (96.4–97.8%)	*In situ* synthesis of Ag nanoparticles	Dina et al. (2018)
FTIR	2 days	54 species, 105 strains (99.17% at genus level, 92.30% at species level)	Wash and dry the cells	Lecellier et al. (2015)

5. Conclusions

In this study, colony fingerprinting was used for the successful discrimination of 5 fungi belonging to different genera, as well as 6 closely-related fungi belonging to the same *Aspergillus* genus. Discriminative parameters, including the number of hyphae and their branches, and the intensity distributions in the colony fingerprints, were used for the discrimination of fungal species. Analyses using machine learning approaches, namely SVM and RF, allowed us to discriminate between different fungi. In particular, RF showed better results than SVM. It was possible to obtain the colony fingerprints needed for discrimination within 48 h. Moreover, no pre-treatment prior to analysis was needed. For the practical application of colony fingerprinting, further studies will be needed to performed fungi discrimination in real food samples using different machine learning approaches, such as deep learning. This study demonstrates that colony fingerprinting is a rapid and simple method for the discrimination of fungi, which could be potentially useful to ensure the safety of foods, cosmetics, and other products.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Yoshiaki Maeda: Methodology, Validation, Formal analysis, Writing - original draft, Visualization. **Yui Sugiyama:** Validation, Formal analysis, Investigation, Visualization. **Tae-Kyu Lim:** Formal analysis, Resources, Funding acquisition. **Manabu Harada:** Formal analysis, Resources, Funding acquisition. **Tomoko Yoshino:** Formal analysis, Writing - review & editing. **Tadashi Matsunaga:** Writing - review & editing, Formal analysis, Validation, Supervision. **Tsuyoshi Tanaka:** Conceptualization, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bios.2019.111747.

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