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MALDI-TOF MS-based identification of black yeasts of the genus *Exophiala*

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Abstract

In this study, we investigated the applicability of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) for the identification of *Exophiala* species. The analysis included a total of 110 *Exophiala* isolates, including 15 CBS strains representing 4 species, *Exophiala dermatitidis* (61), *E. phaeomuriformis* (36), *E. crusticola* (9), and *E. heteromorpha* (4), that had been previously identified based on internal transcribed spacer (ITS) regions. We also compared the relative efficacies of Sabouraud glucose agar (SGA) and Columbia agar (CA) for use in MALDI-TOF MS. Remarkably, we obtained a log-score value ≥2.0 by using either SGA or CA for all 15 CBS strains, indicating species-level identification. The remaining 95 *Exophiala* strains were identified to the genus or species levels, with identification rates of 96.8% and 90.5%, using SGA or CA, respectively. Most of the *E. dermatitidis* (100% and 92.9%), *E. phaeomuriformis* (80.6% and 83.9%), *E. crusticola* (50% and 100%), and *E. heteromorpha* (100% and 100%) isolates were correctly identified using SGA or CA, respectively. Furthermore, 58.9% and 26.3% of the strains had log-score values of ≥2.0 by using SGA and CA, respectively. Our results indicate that MALDI-TOF MS is a rapid and reliable technique with high rates of correct taxonomic identification.

Key words: black yeasts, database, ITS sequencing, MALDI mass spectrometry, species.

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Introduction

In the clinical laboratory, timely identification of black yeasts of the genus *Exophiala* is a major challenge due to the fact that they lack reproductive cells, which prevents definitive identification using morphological, physiological, and biochemical characteristics. Phenotypic parameters are unsuitable for the identification of most black yeast species because of the presence of alternating morphotypes during the pleomorphic life cycle and a low degree of morphological differentiation among the species [1]. Black yeasts belonging to the genus *Exophiala*, particularly thermophilic *Exophiala dermatitidis* and *E. phaeomuriformis*, are prevalent in two very different man-made environments, in other words, (i) creosote-treated oak wood railway sleepers [2–4] and (ii) hot and moist environments such as bathrooms [5], dishwashers [6], and steam baths [7]. Some black yeasts have been reported as agents of severe, potentially fatal infections in both immunocompetent and immunosuppressed individuals. Therefore, the establishment of rapid and reliable methods for the identification of black yeasts, particularly *Exophiala* species, is critically needed [1,8]. Currently, the preferred approach is based on sequencing of the ribosomal DNA (rDNA) internal transcribed spacer (ITS) regions followed by a BLAST search in dedicated databases with a sequence similarity cut-off of 98–99%, depending on the species in question. However, this methodology is relatively expensive, has a 2- to 3-day turnaround time, and is impractical for the analysis of large numbers of isolates [8].

In an elegant study, Heinrichs et al. [9] proposed the use of ITS-based short "barcode identifiers" (27–50 bp) for the rapid identification of black yeasts and their relatives. The identifiers were designed based on highly conserved motifs flanking the regions with high species-specific variability. Recently, Najafzadeh et al. [10] developed a rolling circle amplification (RCA) assay, which is a rapid, sensitive, specific, reproducible, and inexpensive isothermal DNA amplification method that can be used for the molecular identification of pathogenic fungi and does not involve sequencing. Using 5 padlock probes, the researchers successfully identified the most clinically relevant *Exophiala* species, i.e., *E. dermatitidis*, *E. oligosperma*, *E. jeanselmei*, *E. xenobiotica*, and *E. spinifera*.

During the last 5 years, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been introduced as a diagnostic technique in medical centers worldwide. This allows the use of peptide profiling in the identification and differentiation of microorganisms based on highly specific ribosomal "fingerprinting" [11,12]. Its general adoption has been attributed to low operational costs, universality and flexibility of detection, and specificity and speed of analysis [13]. Remarkably, MALDI-TOF MS is more easily implemented for the routine identification of bacteria, mycobacteria, and fungi than traditional diagnostic methods. The exception is viruses, which remain difficult to detect using MALDI-TOF MS owing to their relatively low protein content [13]. Besides identification of fungal species, MALDI-TOF MS has been successfully applied to the typing of strains, including varieties and hybrids [14] and to monitoring the emergence of antifungal drug resistance [15]. Importantly, in the clinical mycology laboratory, MALDI-TOF MS has a short turnaround time of approximately 10 minutes, compared to the hours and even days required for genotypic and phenotypic characterization, while providing similar reliability [12].

The aim of the present study was to evaluate the suitability of MALDI-TOF MS as a rapid and reliable method for the identification of *Exophiala* species previously identified using ITS sequencing. We also compared the relative efficacies of two media, in other words, Sabouraud glucose agar (SGA) and Columbia agar (CA). To our knowledge, this study is the first to use this technology to identify black yeasts and to generate a library for peptide masses of *Exophiala* environmental isolates representing potentially pathogenic (*E. dermatitidis*, *E. heteromorpha*, and *E. phaeomuriformis*) and saprobic (*E. crusticola*) species.

Materials and methods

Fungal Strains

In this study, the following 110 *Exophiala* isolates were investigated, including 15 CBS reference strains; 61 *E. dermatitidis* (genotypes A, B, and C [45:11:5]), 36 *E. phaeomuriformis* (genotypes 1 and 2 [8:28]), 9 *E. crusticola*, and 4 *E. heteromorpha*. They had previously been recovered from railway sleepers and household dishwashers and had been identified by ITS sequencing (Refgen Biotechnology, Ankara, Turkey), genotyped, and examined for their physiological characteristics as described in recent studies conducted by our group [3,4,6]. The study strains were maintained at the laboratory collection facility at the Division of Mycology, Department of Microbiology, Medical Faculty of Çukurova University, Adana, Turkey. Table 1 presents the list of the reference strains that were examined in the present study; these strains have already been deposited in the reference collection of the CBS-KNAW Fungal Biodiversity Centre, Utrecht, the Netherlands, and in the Macit Ilkit working collection, Adana, Turkey.

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Table 1. *Exophiala* species that were isolated from dishwashers and railway sleepers in Turkey and added to the Bruker Standard Reference Library.

Species	Genotype	Origin	City	CBS No.	MI No.
E. dermatitidis	A	Dishwasher	Mersin	CBS 132752	19680
E. dermatitidis	A	Dishwasher	Mersin	CBS 132751	19681
E. dermatitidis	B	Dishwasher	Mersin	CBS 132753	19682
E. dermatitidis	B	Dishwasher	Mersin	CBS 132754	19683
E. dermatitidis	C	Dishwasher	Mersin	CBS 132755	19684
E. phaeomuriformis	1	Dishwasher	Mersin	CBS 132756	19685
E. phaeomuriformis	1	Dishwasher	Mersin	CBS 132757	19686
E. phaeomuriformis	2	Dishwasher	Mersin	CBS 132758	19687
E. phaeomuriformis	2	Oak-wood[a]	Adana	CBS 134012	19688
E. phaeomuriformis	2	Oak-wood	Adana	CBS 134013	19689
E. crusticola	-	Concrete[b]	Adana	CBS 134043	20093
E. crusticola	-	Oak-wood	Adana	CBS 134427	20094
E. crusticola	-	Concrete	Niğde	CBS 134428	20095
E. heteromorpha	-	Oak-wood	Kayseri	CBS 134041	20096
E. heteromorpha	-	Concrete	Niğde	CBS 134042	20097

[a,b]Railway sleeper type; CBS, Centraalbureau voor Schimmelcultures; MI, Macit Ilkit's working collection.

MALDI-TOF MS sample and target plate preparation

MALDI-TOF MS was conducted at the Department of Microbiology, Medical Faculty of Akdeniz University, Antalya, Turkey. All isolates were subcultured on SGA (Merck, Darmstadt, Germany) and CA with 5% sheep's blood (Becton Dickinson, Heidelberg, Germany) and incubated at 28°C for 48 hours. Physiologically active cells were selected from fresh cultures for MALDI analysis. Material was harvested when the colony diameter reached 1–2 mm. A single colony (5–10 mg) was detached with an inoculation loop and transferred to an Eppendorf Safe-Loc tube containing 300 μl deionized water, and formic acid-ethanol protein extraction was performed according to the recommendations of Bruker Daltonik GmbH (Bremen, Germany). The supernatant (1 μl) was transferred to a MALDI target plate (MSP 96 BC ground steel target; Bruker Daltonik) and allowed to dry at room temperature. In the final step, the sample was covered with 1 μl of saturated α-cyano-4-hydroxy cinnamic acid solution (HCCA matrix; Bruker) as a matrix for the co-crystallization. The matrix and sample were allowed to dry at room temperature. The Bacterial Test Standard (BTS; Bruker) was used in the mass calibration procedure and showed a typical *Escherichia coli* DH5 alpha peptide and protein profile plus additional protein peaks. Bruker BTS solution (1 μl) was applied to the appropriate spot on the MALDI target plate covered with 1 μl HCCA matrix and allowed to dry at room temperature.

MS was performed using the Microflex LT model (Bruker Daltonik GmbH) MALDI-TOF instrument. The linear positive-ion mode was used within a mass range of 2,000–20,000 Da for microbial identification. The instrument was equipped with a 60-Hz nitrogen laser and for each spectrum, 240 laser shots from different positions of the sample spot were accumulated and analyzed.

Construction of the in-house spectrum database

Currently, the MALDI Biotyper database includes 5,627 bacterial, mycobacterial, and fungal species of which only one belongs to the genus *Exophiala*, in other words, *E. dermatitidis* CBS 207.35 (= ATCC 28869). Prior to the MALDI-TOF analysis, the related spectra of 15 CBS reference strains were added to the MALDI Biotyper database to create an in-house database with the help of a trained MALDI specialist. As a subsequent step, the spectra of the remaining 95 *Exophiala* strains were compared and identified using the Bruker Standard Reference Library and the in-house spectrum database.

To create the in-house database of 15 CBS reference *Exophiala* strains, the following steps were performed. Formic acid-ethanol protein extraction was applied for each CBS strain. The supernatant from each strain was transferred to eight different spots on the MALDI target plate, and the spots were covered with 1 μl HCCA matrix and allowed to dry at room temperature. The laser settings (offset and range) were checked prior to the analysis, which was performed in triplicate for each site and involved $8 \times 3 = 24$ measurements for each strain. The Bruker BTS solution was applied to one spot for internal calibration and as the positive control. Each strain produced 24 spectra, which were analyzed and subjected to smoothing and baseline subtraction for spectrum quality control by using the FlexAnalysis software. Each of the spectra ($n = 24$) was

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examined, and spectra with mass-deviations ≥500 ppm were removed from the list. For each strain, at least 20 spectra were selected for further analysis. These selected spectra for each strain were simultaneously analyzed via Biotyper 3.0 (Bruker Daltonik) and added to the database constructed in-house.

MALDI-TOF MS-based Identification

Mass spectra of the remaining 95 *Exophiala* strains were measured and identified with MALDI-TOF MS by using the in-house database. Each strain was analyzed twice. The identification data generated by MALDI-TOF MS were classified following the manufacturer's instructions: a log-score value ≥2.0 indicated species identification, a score of 1.7–1.99 indicated identification at the genus level, and a score <1.7 indicated non-reliable identification (NRI).

Results

In general, MALDI-TOF MS results were consistent with those obtained using ITS sequence analysis. Notably, 15 CBS reference strains had log-score values ≥2.0 by using SGA and CA. By using MALDI-TOF MS-based identification, the results of the remaining 95 strains with log-score values ≥2.0 and 1.7–1.99 fully corresponded to the identification at the genus level determined by ITS. In the present study, SGA and CA provided similar identification rates of 96.8% and 90.5% for *Exophiala* strains at the genus (n = 92) and species (n = 86) levels, respectively. We obtained a log-score value ≥ 2.0 by using SGA and CA in 58.9% and 26.3% of the 95 strains, respectively. Using SGA, 100% of *E. dermatitidis* and *E. heteromorpha* strains, 80.6% of *E. phaeomuriformis*, and 50% of *E. crusticola* strains were correctly identified. Most notably, six *E. phaeomuriformis* strains were misidentified as *E. dermatitidis*; three generated log-score values ≥2.0, and three generated log-score values in the range of 1.7–1.99. Three *E. crusticola* strains had log score values < 1.7 and were considered to show NRI.

Using CA, 25 (26.3%) *Exophiala* strains had log-score values ≥2.0, 67 (70.5%) strains had log-score values of 1.7–1.99, and three (3.2%) strains had log-score values < 1.7 and were considered to show NRI. Specifically, using CA, 100% of *E. crusticola* (n = 6) and *E. heteromorpha* (n = 2) strains, and 92.9% of *E. dermatitidis* strains (52 out of 56) were correctly identified at the species level. One of the four misidentified *E. dermatitidis* strains was determined as *E. phaeomuriformis*, and three strains had log-score values of < 1.7 and were considered to show NRI. We also correctly identified 83.9% of *E. phaeomuriformis* (26 out of 31) strains at the species level; however, five strains were misidentified as *E. dermatitidis* (16.1%). One *E. dermatitidis* and three *E. phaeomuriformis* misidentified strains generated log-score values of ≥2.0; two *E. phaeomuriformis* strains misidentified as *E. dermatitidis* generated log-score values of 1.7–1.99 (Table 2). In this study, only the 15 CBS reference strains were included in the Bruker Standard Reference Library and are listed in Table 1.

Discussion

In the present study, we investigated the applicability of MALDI-TOF MS analysis in the identification of 95 environmental *Exophiala* strains, which were previously recognized by ITS sequencing. In addition, we obtained the same correct identification rates (96.8% and 90.5%) for *Exophiala* strains at the genus and species levels, respectively, through the use of SGA or CA. Most notably, we obtained superior results in species recognition, a log-score value ≥ 2.0, using SGA (58.9%) than when using CA (26.3%). Therefore, we suggest that SGA might be more suitable than CA for providing higher species-level identification scores. In contrast, we obtained less reliable results with SGA (50%) than with CA (100%) in identifying *E. crusticola* strains. Conclusively, seven *Exophiala* strains that had log-score values ≥ 2.0 were misidentified by using SGA (n = 3) or CA (n = 4).

Most of the *E. crusticola* and *E. heteromorpha* strains were identified correctly, which can be explained by the fact that these species are phylogenetically distant from each other, as well as from the *E. dermatitidis* and *E. phaeomuriformis* species groups [16]. In addition, it is sometimes difficult to distinguish between thermophilic *E. dermatitidis* and *E. phaeomuriformis* because of their close phylogenetic position as members of the *dermatitidis* clade [17]. Therefore, the ITS sequencing data in this case need to be checked again to ensure that there were no errors in labeling of strains or data. Overall, the discrimination rate of opportunistic black yeasts (*E. dermatitidis*, *E. heteromorpha*, and *E. phaeomuriformis*) by MALDI-TOF was at least 80.6%, which, in our experience, is superior to the results obtained by conventional methods. Although we evaluated the performances of both SGA and CA, we constructed a database dependent on SGA, which is used for primary culture of human samples and will be helpful to the fungal research community.

The current CD-ROM version of the Atlas of Clinical Fungi describes over 500 fungal species recovered from humans with mycotic diseases, of which many are rare, drug-resistant, or poorly sporulating species [1]. MALDI-TOF MS has provided a major breakthrough in medical mycology diagnostics and will improve the identification rate of fungal pathogens, including rare and nonsporulating fungi,

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Table 2. Log-score values of four *Exophiala* species obtained by the MALDI-TOF MS analysis using SGA and CA.

	No. of isolates with Bruker score of:						
	≥2.0		1.7–1.99		<1.7		
ITS-based identification (no. of isolates)	SGA	CA	SGA	CA	SGA	CA	Discordant species-level identification
E. dermatitidis (56)	29	7	27	46	–	3	*E. phaeomuriformis* (1[b])
E. phaeomuriformis (31)	25	18	6	13	–	–	*E. dermatitidis* (6[a] and 5[b])
E. crusticola (6)	–	–	3	6	3	–	–
E. heteromorpha (2)	2	–	–	2	–	–	–

[a]SGA, Sabouraud glucose agar;
[b]CA, Columbia agar.

in regular clinical laboratories [12,14,15,18,19]. Currently, however, no MALDI-TOF MS-generated data are available for any black yeast species. For example, *E. dermatitidis* has capsules; therefore, the effect of the thick cell walls containing melanin on the results obtained with this technique needs to be clarified.

Using MALDI-TOF MS, Firacative et al. [14] successfully identified subspecies and major molecular types within the *Cryptococcus neoformans*/*C. gattii* complex. Recently, Ranque et al. [19] reported that MS dramatically improved the identification of the non-*Aspergillus* filamentous fungi with a 31–61% increase in accuracy, and showed superior performance to the laborious, highly subjective, and costly conventional methods. The present study confirms that MALDI-TOF MS is capable of identifying organisms such as black yeasts, which are relatively more difficult to identify, down to the species level. However, the relatively low identification rates (80.6–83.9%) observed for *E. phaeomuriformis* suggest that expanding the number of species included in the reference library is critical to improve the identification of *Exophiala* species in a timely manner, such as by using MALDI-TOF MS. MS technology is, in theory, capable of identifying a given species that is already included in the commercial database.

Both the ITS-based and MALDI-TOF MS-based identification modalities need accurate, quality-controlled reference databases. It is important to understand the limitations of each approach. Thus, a very small percentage of fungi have been sequenced in the ITS region, and the taxonomic reliability of fungal ITS sequences available in public repositories is inadequate [20,21]. Some fungal isolates have poor-quality mass spectra and insufficient database entries, which hinders MALDI-TOF MS-based identification [12], as demonstrated in this study. For the current investigation, the main limitation was a relatively small number of *Exophiala* strains analyzed.

Rapid and reliable identification of clinical *Exophiala* isolates will further our current knowledge of these emerging pathogens. We have demonstrated that MALDI-TOF MS is an easy-to-use, reliable, highly practical, and cost-effective method for the identification of *Exophiala* species, which can be an alternative to the current "gold-standard" ITS-based approach.

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Transparency Declaration:

Neither Bruker Daltonik GmbH (Bremen, Germany) nor any other organization influenced the design of the study, data collection and analysis, decision to publish, or preparation of the manuscript.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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