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Identification of dermatophytes by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

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In this study we evaluated the suitability of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) for the identification of dermatophytes in diagnostic laboratories. First, a spectral database was built with 108 reference strains belonging to 18 species of the anamorphic genera *Epidermophyton*, *Microsporum* and *Trichophyton*. All strains were well characterized by morphological criteria and ITS sequencing (gold standard). The dendrogram resulting from MALDI-TOF mass spectra was almost identical with the phylogenetic tree based on ITS sequencing. Subsequently, MALDI-TOF MS SuperSpectra were created for the identification of *Epidermophyton floccosum*, *Microsporium audouinii*, *M. canis*, *M. gypseum* (teleomorph: *Arthroderma gypseum*), *M. gypseum* (teleomorph: *A. incurvatum*), *M. persicolor*, *A. benhamiae* (Tax. Entity 3 and Am-Eur. race), *T. erinacei*, *T. interdigitale* (anthropophilic and zoophilic populations), *T. rubrum*/*T. violaceum*, *T. tonsurans* and *T. terrestre*. Because *T. rubrum* and *T. violaceum* did not present enough mismatches, a SuperSpectrum covering both species was created, and differentiation between them was done by comparison of eight specific peptide masses. In the second part of this study, MALDI-TOF MS with the newly created SuperSpectra was tested using 141 clinical isolates representing nine species. Analyses were done with 3-day-old cultures. Results were compared to morphological identification and ITS sequencing; 135/141 (95.8%) strains were correctly identified by MALDI-TOF MS compared to 128/141 (90.8%) by morphology. Therefore, MALDI-TOF MS has proven to be a useful and rapid identification method for dermatophytes.

Keywords dermatophytes, morphology, phylogeny, taxonomy, ITS sequencing, MALDI-TOF MS

Introduction

Classical identification of dermatophytes relies on culture characteristics, microscopic morphology, physiological tests and clinical data [1]. Their overlapping phenotypic characteristics, however, may be confusing [2] and the identification requires growth of the organisms in culture for at least one week, which delays the diagnosis.

Molecular techniques allow a fast and reliable identification of dermatophytes [3–5]. ITS sequencing is presently considered the gold standard for molecular identification and phylogenetic analyses of dermatophytes. However, DNA sequencing is expensive and at least 2–3 days are required before a sequence is obtained from a culture.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is now used routinely in clinical diagnostic laboratories as it is faster than PCR and requires little sample handling. It is a cost-effective and reliable technique for the identification and typing of microbial pathogens including bacteria [6–11], yeasts [12–14], and filamentous fungi [11,15–18]. MALDI-TOF

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MS has already been used for the identification of dermatophytes, either after their isolation and cultivation [19–21] or directly from clinical material [22]. However, the number of dermatophyte species analyzed was limited in two studies [19,20], and in one of them [20] only 59.6% of 171 strains were identified at the species level, which is insufficient for the routine application. A recent study [21] analyzed 12 dermatophyte species, but some important ones (*Microsporum audouinii*, *M. gypseum*, *Arthroderma benhamiae*, *Trichophyton verrucosum*) were not included. Moreover, the time of incubation was 3 weeks, making the MALDI-TOF MS system not attractive for diagnostics.

Our study aimed to test MALDI-TOF MS with most of the important dermatophyte species, analyzing them after only 3 days of incubation. First, a database was built with 108 reference strains and SuperSpectra were created. In a second step, 141 clinical isolates were tested and the results compared with those obtained from morphological and ITS sequencing studies.

Materials and methods

Fungal strains

A total of 108 reference strains belonging to 18 species of *Arthroderma*, *Epidermophyton*, *Microsporum* and *Trichophyton* were included to construct the reference database (Supplementary Table 1 to be found online at http://informahealthcare.com/doi/abs/10.3109/13693786.2012.746476). They were obtained from culture collections (CBS, Utrecht, The Netherlands; Micoteca da Universidade do Minho (*MUM*), Braga, Portugal), external quality control programs or laboratories own collections (*Z*, Zürich; *bM*, Bellinzona). For the MALDI-TOF MS identification study, 141 clinical isolates belonging to nine species were used (Supplementary Table 2 to be found online at http://informahealthcare.com/doi/abs/10.3109/13693786.2012.746476), all of them originated from Zurich (*z*) and Bellinzona (*M*).

Morphological identifications

All 249 isolates were grown on potato dextrose agar (Becton Dickinson, France) plates at 25–30°C in the dark until sporulation. Physiological tests (Christensen's urea agar, BCP milk glucose agar, *in vitro* hair perforation test, and temperature enhancement analysis) were conducted when morphology alone did not allow reliable identification. Identifications were made using standard keys in the literature [23–25], those isolates that did not demonstrate all typical features were reported as species-like (e.g., *T. interdigitale*-like).

ITS sequencing

All 249 isolates were inoculated on Sabouraud gentamycin chloramphenicol 2 agar plates (bioMérieux, France) and incubated at 25–30°C for 3–7 days. DNA was extracted by phenol-chloroform or using the Nexttec Genomic DNA Kit (Nexttec, Germany). The ITS region was amplified using primers ITS1 and ITS4 [26]. Sequences were generated with the ITS4 primer and compared to the publicly available database (http://www.cbs.knaw.nl/dermatophytes/BioloMICSID.aspx) using the pairwise sequence alignment tool (Supplementary Tables 1 and 2 to be found online at http://informahealthcare.com/doi/abs/10.3109/13693786.2012.746476). A phylogenetic tree was constructed using Mega 4.0 (Neighbor-Joining method, Kimura two-parameter distances, bootstrap with 1,000 replicates).

MALDI-TOF MS

All strains were grown on Sabouraud gentamycin chloramphenicol 2 agar plates (bioMérieux SA, Marcy l'Etoile, France) and incubated at 30°C for 3 days. Young, growing mycelium was extracted using 25% formic acid, followed by a step of lipid elimination with methanol and chloroform to avoid any competition between proteins and lipids during the phase of protonization (Guido Vogel, Mabritec AG, personal communication). The supernatant obtained after centrifugation was mixed with a matrix containing 30–40 mg of α-cyano-4-hydroxy-cinnamic acid (CHCA) in acetonitrile/ethanol/water (1:1:1) supplemented with 3% trifluoroacetic acid (Sigma-Aldrich, Switzerland). For each isolate, 1 μl was spotted in quadruplicate onto wells of a 48-position stainless steel FLEXImass target plate (Shimadzu Biotech, Kyoto, Japan). MS analyses were performed in positive linear mode in the range of 2,000–20,000 mass-to-charge ratio (*m/z)* with delayed, positive ion extraction (delay time: 104 ns with a scale factor of 800) and an acceleration voltage of 20 kV on an AXIMA Confidence (Shimadzu Biotech, Kyoto, Japan) mass spectrometer equipped with a 50 Hz nitrogen laser (pulse width: 3 nS). Averaged profile spectra fulfilling the quality criteria were collected from 5 laser shot cycles. For every sample, 50 averaged profile spectra were used for analysis. All spectra were processed using the MALDI-TOF MS Launchpad 2.8 software (Shimadzu Biotech, Kyoto, Japan) with baseline correction, peak filtering and smoothing.

The resulting peak lists were imported into the BioNumerics software package (Version 6.0, Applied Maths NV, Sint-Martens-Latem, Belgium). Distances were computed using the Pearson's correlation (0.5% tolerance) and the UPGMA agglomeration algorithm to produce a dendrogram (one mass spectrum per isolate, mass range from 3,000–20,000 *m/z*).

MALDI-TOF MS identification strategy

Reference spectra were imported into the SARAMIS software package (bioMérieux, La Balme, France). SuperSpectra™ containing the most representative peptide masses were created for the taxonomic clusters and integrated in the SARAMIS database for future rapid identification of clinical samples.

All 141 clinical isolates were submitted to identification by SuperSpectra but if identification with SuperSpectra was not possible ($<70\%$), spectra were compared directly with the reference spectra of the database (COMPARE function of the SARAMIS software). Identifications through the COMPARE function were considered valid when the similarity was $\geq 40\%$. As *T. rubrum* and *T. violaceum* presented too few mismatches to be distinguishable, a strategy of comparison of specific masses was created to discriminate them.

Results

Reference strains

The morphological identification of 91/108 (84.3%) of the isolates was in accord with ITS sequencing, in 4/108 (3.7%) identification was not possible due to the lack of spore formation in culture, and in 13/108 (12.0%) morphological results were discrepant with ITS sequencing (Table 1, Supplementary Table 1 to be found online at http://informahealthcare.com/doi/abs/10.3109/13693786.2012.746476).

The ITS sequences of the 108 reference strains were 535–686 nucleotides long. The best and second best database matches are presented in Supplementary Table 1 to be found online at http://informahealthcare.com/doi/abs/10.3109/13693786.2012.746476. More than 94% of the strains were identified with a score $\geq 99.7\%$. In one case (*Arthroderma* sp. bM 128), the homology to the best-fitting database sequence was $<98\%$, leading to identification at the genus level [27]. All 18 species included in the study were well separated based on the ITS analysis (Fig. 1A), including species that are morphologically indistinguishable (e.g., *A. incurvatum* and *A. gypseum* or *T. mentagrophytes* and *T. interdigitale*).

MALDI-TOF MS (Fig. 1B) essentially confirmed the phylogenetic results (Fig 1A). Exceptions were the *T. terrestre* complex that formed a distinct group and *M. praecox*, which did not cluster with other species of *Microsporum*. At the species level, a clear separation that was in agreement with the ITS results was generally seen with MALDI-TOF MS. Some of the closely related species or strains (*A. gypseum* and *A. incurvatum*, *T. interdigitale* and *T. mentagrophytes*, the two *A. benhamiae* subgroups, and *M. audouinii* and *M. canis*) formed distinct clusters. For *T. interdigitale*, two clusters could be observed, i.e., one containing only anthropophilic strains and the other anthropophilic and zoophilic strains (Fig. 1B). *T. rubrum* was heterogeneous with one group clustering together with *T. violaceum* and containing most 'classical' *T. rubrum* strains and one of the African population. Two smaller and more heterogeneous subgroups included most strains of the African population.

Creation of MALDI-TOF MS SuperSpectra

Species-specific biomarkers (SuperSpectra) identifying *E. floccosum*, *M. audouinii*, *M. canis*, *M. gypseum* (*A. gypseum*), *M. gypseum* (*A. incurvatum*), *M. persicolor*, *A. benhamiae* (covering Tax. Entity 3 and Am-Eur. race), *T. erinacei*, *T. interdigitale* (covering both anthropophilic and zoophilic populations), *T. tonsurans* and *T. terrestre* were created.

No species-specific SuperSpecta could be created for *T. rubrum* and *T. violaceum* and as a result, a combined SuperSpectum for both species was generated. For further discrimination, the reference spectra of *T. rubrum* and *T. violaceum* were compared mass by mass and eight specific peptides (*m/z* 5,052, 5,436, 5,575, 5,706, 6,647, 7,550, 8,607, 11,409) were selected. *m/z* 7,550 was prevalent in *T. violaceum* (96.8%) and the others were prevalent in 68.4–95.7% of all *T. rubrum* reference spectra. Subsequently, when a clinical strain was identified as *T. rubrum/T. violaceum* the presence/absence of the eight masses described above was checked, giving to each other a value proportional to the frequencies found in the reference spectra. After multiplying all the values, the frequencies were normalized by dividing each by their sum and multiplying by 100 to give the *T. rubrum* and *T. violaceum* identification percentages as previously described [28,29].

Validation of the MALDI-TOF MS identification method on clinical strains

A total of 141 clinical isolates belonging to nine species were identified by MALDI-TOF MS, morphology and ITS sequencing (Supplementary Table 2 to be found online at http://informahealthcare.com/doi/abs/10.3109/13693786.2012.746476). Identification by MALDI-TOF MS allowed an overall correct identification of 95.8% of all clinical isolates (Table 2) which included the correct identification of all strains belonging to *M. audouinii* (five), *M. canis* (16), *M. gypseum* (*A. gypseum*) (two), *T. erinacei* (one), and *T. tonsurans* (four) (Table 2, Supplementary Table 2 to be found online at http://informahealthcare.com/doi/abs/10.3109/13693786.2012.746476). Four *T. interdigitale* and two *T. rubrum* isolates required the use of the COMPARE function. In two of 12 *A. benhamiae*, three of

Table 1 Reference strains with discordant results between morphology and ITS/MALDI-TOF MS.

Strain	ITS sequencing identification			MALDI-TOF MS cluster	Morphological identification
	Genus, species	% ID	Mismatches		
CBS 100.64	*Microsporum gypseum*	100	*0/585*	*Microsporum gypseum* complex	*Trichophyton rubrum* (african pop.): sterile, colony orange-yellow, corresponding to former *Trichophyton soudanense*
bM 134	*Microsporum canis*	100	0/656	*Arthroderma otae* complex	*Chrysosporium inops*: thick walled aleurospores present, no macroconidia
CBS 318.56	*Trichophyton mentagrophytes*	100	0/604	*Arthroderma vanbreuseghemii* complex	*Trichophyton erinacei*: no spiral hyphae seen
CBS 101546	*Trichophyton mentagrophytes*	100	0/604	*Arthroderma vanbreuseghemii* complex	Atypical *Trichophyton tonsurans*: microconidia variable, tear-drop-shaped to clavate, colony flat, white with some brown colour, hair perforation test negative.
bM 126	*Trichophyton erinacei*	100	0/598	*Arthroderma benhamiae* complex	No identification: many diverse microconidia, colony white, cottony
CBS 623.66	*Arthroderma benhamiae* (Am-Eur race)	99.8	1/598	*Arthroderma benhamiae* complex	Atypical *Trichophyton erinacei*: microconidia slender, clavate, colony flat, cottony, reverse yellow.
CBS 624.66	*Arthroderma benhamiae* (Am-Eur race)	99.8	1/598	*Arthroderma benhamiae* complex	*Trichophyton interdigitale*: micro- and macroconidia typical for *Trichophyton interdigitale*, colony reverse yellowish-brown.
bM 123	*Arthroderma benhamiae* (tax. entity 3)	100	0/599	*Arthroderma benhamiae* complex	Atypical *Trichophyton tonsurans:* microconidia abundant and of variable size, cylindrical to clavate or balloon-shaped. Colony white, flat.
bM 132	*Trichophyton verrucosum*	99.8	1/597	*Arthroderma benhamiae* complex	*Trichophyton concentricum*: no chlamydospores as illustrated for *Trichophyton verrucosum*. Seems to have better growth than typical *Trichophyton verrucosum*.
MUM 09.15	*Trichophyton rubrum* (African pop.)	100	0/589	*Trichophyton rubrum* complex	No identification: microconidia variable, slender to short-clavate, macroconidia cylindrical, 2–7-celled, colony slow-growing, reverse yellow.
CBS 517.63	*Trichophyton rubrum* (african pop.)	99.5	3/609	*Trichophyton rubrum* complex	Atypical *Trichophyton erinacei*: microconidia slender, clavate, terminally abundant. Colony cottony, white, reverse yellowish.
CBS 518.63	*Trichophyton rubrum* (african pop.)	99.5	3/609	*Trichophyton rubrum* complex	No identification: sterile, colony white, fluffy
Neqas 9649	*Trichophyton rubrum* (african pop.)	100	0/541	*Trichophyton rubrum* complex	*Trichophyton tonsurans*: microconidia abundant and of variable size, cylindrical to clavate or balloon-shaped. Colony yellowish with red-brown reverse

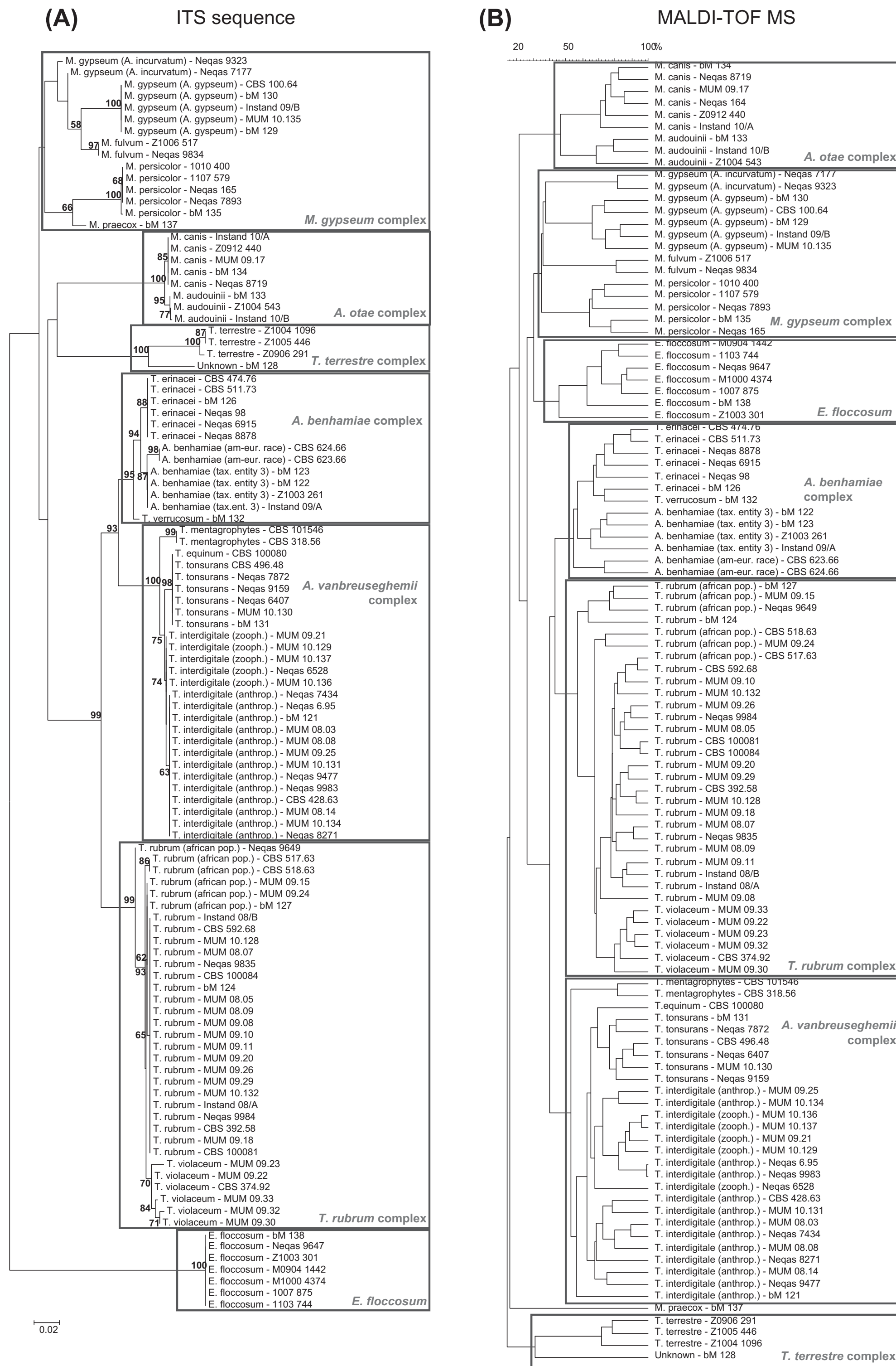

Fig. 1 (A) Phylogenetic tree based on ITS sequencing. Neighbor-Joining method, Kimura 2-parameter, bootstrap from 1,000 replicates. (B) Dendrogram based on MALDI-TOF mass spectra. Mass range (*m/z*: 3,000–20,000, Pearson's correlation, UPGMA. Indications of complex memberships according to [2].

Table 2 Comparison of identifications between ITS sequencing, morphology and MALDI-TOF MS for the 141 clinical strains.

Genus, species	No. of isolates investigated[a]	Morphology (no.)			MALDI-TOF MS (no.)		
		Correct identification	No identification	Misidentification	SuperSpectra™ (% ID)[b]	[c]COMP	No identification
Microsporum audouinii	5	4	1	0	5 (78.0–99.9)	0	0
Microsporum canis	16	12	4	0	16 (87.0–99.9)	0	0
Microsporum gypseum (*Arthroderma gypseum*)	2	1	1	0	2 (99.9)	0	0
Arthroderma benhamiae	12	10	0	2	10 (75.0–99.9)	0	2
Trichophyton erinacei	1	1	0	0	1 (97.0)	0	0
Trichophyton interdigitale	46	42	2	2	39 (78.2–99.9)	4	3
Trichophyton rubrum	48	47	1	0	45 (82.0–99.9)	2	1
Trichophyton tonsurans	4	4	0	0	4 (95.5–99.9)	0	0
Trichophyton violaceum	7	7	0	0	7 (93.0–99.9)	0	0
			9 (6.4%)	4 (2.8%)	129 (91.6%)	6 (4.2%)	
Total (141)	141 (100%)	128 (90.8%)	13 (9.2%)		135 (95.8%)		6 (4.2%)

[a]As identified by ITS sequencing; [b]% ID, percentage given by identification with the SuperSpectra™; [c]COMP, COMPARE function from the SARAMIS™ system.

46 *T. interdigitale*, and one of 48 *T. rubrum* isolates, identification by MALDI-TOF MS was not possible. The other *T. rubrum* (47) and all *T. violaceum* (seven) isolates were submitted to the *T. rubrum* versus *T. violaceum* differentiation system using the eight discriminating peptide masses. All were correctly assigned to their respective species, with percentages of reliability between 99.9 and 100% for *T. rubrum*, and 95.6–99.9% for *T. violaceum* (Table 2, Supplementary Table 2 to be found online at http://informahealthcare.com/doi/abs/10.3109/13693786.2012.746476).

Some 128 of the 141 (90.8%) isolates were correctly identified by morphology at the species level, whereas nine (6.4%) could not be identified due primarily to the lack of conidial formation and four (2.8%) were discrepant compared to sequencing and mass spectrometry (Supplementary Table 2 to be found online at http://informahealthcare.com/doi/abs/10.3109/13693786.2012.746476, Table 2).

Specificity, sensitivity, positive predictive values and negative predictive values were computed for the outcomes of identifications by morphology and MALDI-TOF MS for each of the nine species, using ITS sequencing as the gold standard. MALDI-TOF MS was always comparable to morphology in terms of the parameters computed. i.e., its sensitivity was significantly better for four species (*M. gypseum* [*A. gypseum*], *M. audouinii*, *M. canis*, *T. interdigitale*) and the specificity was superior for three (*T. interdigitale*, *T. tonsurans*, *T. erinacei*). Its efficiency was also better for five (*M. gypseum* [*A. gypseum*], *M. audouinii*, *M. canis*, *T. interdigitale*, *T. tonsurans*), the positive predictive values for three (*T. interdigitale*, *T. tonsurans*, *T. erinacei*), and the negative predictive values for four species (*M. gypseum* [*A. gypseum*], *M. audouinii*, *M. canis*, *T. interdigitale*) (Supplementary Table 3 to be found online at http://informahealthcare.com/doi/abs/10.3109/13693786.2012.746476).

Discussion

This study has shown that MALDI-TOF MS is a reliable, rapid and effective method for the routine identification of dermatophytes. Overall, 95.8% of the clinical isolates were correctly identified by MALDI-TOF MS. In addition, it was very powerful in discriminating closely related and morphologically indistinguishable species, as it allowed for the differentiation of *A. gypseum* and *A. incurvatum*, *T. interdigitale* and *T. mentagrophytes* and the two *A. benhamiae* groups.

Morphological identification is straightforward for those isolates forming typical macro- and/or microconidia or other characteristic features. However, it relies on the experience of the staff and in daily practice the mycology laboratory is often confronted with strains that do not produce

typical characteristics, e.g., atypical colony morphology, physiological tests, conidia, or sterile growth. Molecular/proteomic methods are faster and less dependent on strain conditions and on the expertise of the staff.

Molecular analyses, and in particular DNA sequencing, are expensive and rather time-consuming. MALDI-TOF MS, on the other hand, has been shown to be very cost-effective with limited expenses for material and reagents and there is a considerable reduction in turnaround time compared to routine cultural work or even molecular identifications [30]. This applies to dermatophytes as well as consumable costs amount to less than €5 for each identification and results can be provided within 3–4 days after culture.

Currently three studies have used MALDI-TOF MS for the identification of dermatophytes [19–21]. Erhard and co-workers [19] used the SARAMIS system with a limited number of isolates. They used a different method for extraction and a different matrix and the SuperSpectra they created (already available in our database) could therefore not be used in our study. Theel and collaborators [20] analyzed dermatophytes using the Bruker Biotyper MALDI-TOF MS system. The number of species was limited to seven and only 59.6% of 171 isolates were identified at the species level which limited the application of their method. Recently, Alshawa and co-workers [21] used MALDI-TOF MS for the diagnosis of dermatophytes with the Andromas system. They obtained a correct identification at the species level for 331/360 (91.9%) of the isolates, which is comparable to the values obtained in this study. However, they used 3-week-old cultures (as compared to 3 days in our study), which obviously eliminates one of the main advantages of MALDI-TOF MS, i.e., speed of analysis.

MALDI-TOF MS of fungi is not as straightforward as described for bacteria. A careful standardization that takes into account culture media, time of incubation, quantity and type of colony material employed for the analysis is needed to obtain reproducible results [11,18]. Sometimes bead beating and trifluoroacetic acid, acetonitrile or formic acid treatments are required to disrupt the fungal cell walls, increase the protein extraction rate and provide high quality mass spectra [31].

In this study, an open database was used, to which new reference spectra and SuperSpectra can be easily added, which allows the identification at two levels, i.e., (i) directly and rapidly with SuperSpectra or (ii) with the less rapid (yet still quite quick and robust) COMPARE function that allows comparison of an unknown spectrum with reference spectra in the database. In difficult cases, the use of both steps allowed an identification of 95.8%, which is higher compared to a one step identification system [20]. The use of an open database has the additional advantage of customisation by each laboratory [10,17].

Although *T. rubrum* and *T. violaceum* are quite easily distinguishable by morphology, MALDI-TOF MS, similar to ITS sequencing, showed a high homology between the two species (Fig. 1). Therefore, to be able to identify *T. rubrum* and *T. violaceum* correctly, we had to resort to a third level of identification, based on the presence of eight specific peptide masses in the reference spectra. This allowed the correct identification of all 47 *T. rubrum* and seven *T. violaceum* isolates (Table 2).

Our study has some limitations. The 141 clinical dermatophytes only included nine species and were dominated by *T. rubrum*, *T. interdigitale*, *M. canis* and by the American-European strains of *A. benhamiae*. *M. gypseum* (*A. gypseum*), *T. erinacei* and *T. tonsurans* were under-represented with only 2, 1 and 4 isolates, respectively. SuperSpectra™ were created for *E. floccosum*, *M. gypseum* (*A. incurvatum*), *M. persicolor*, and *T. terrestre* but could not be tested, because no clinical strains were obtained during the study period.

Conclusions

This study has shown that MALDI-TOF MS is a fast, robust and reliable method for the identification of the most frequent dermatophyte species isolated in the clinic. Under-represented and uncommon taxa, however, must be included in further studies to confirm the present results and to cover the whole dermatophyte species spectrum.

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Supplementary material available online

Supplementary Tables 1–3.