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–Time of Flight (MALDI-TOF) Mass  
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# Matrix-Assisted Laser Desorption Ionization–Time of Flight (MALDI-TOF) Mass Spectrometry Using the Vitek MS System for Rapid and Accurate Identification of Dermatophytes on Solid Cultures

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The objective of this research was to extend the Vitek MS fungal knowledge base version 2.0.0 to allow the robust identification of clinically relevant dermatophytes, using a variety of strains, incubation times, and growth conditions. First, we established a quick and reliable method for sample preparation to obtain a reliable and reproducible identification independently of the growth conditions. The Vitek MS V2.0.0 fungal knowledge base was then expanded using 134 well-characterized strains belonging to 17 species in the genera *Epidermophyton*, *Microsporum*, and *Trichophyton*. Cluster analysis based on mass spectrum similarity indicated good species discrimination independently of the culture conditions. We achieved a good separation of the subpopulations of the *Trichophyton* anamorph of *Arthroderma benhamiae* and of anthropophilic and zoophilic strains of *Trichophyton interdigitale*. Overall, the 1,130 mass spectra obtained for dermatophytes gave an estimated identification performance of 98.4%. The expanded fungal knowledge base was then validated using 131 clinical isolates of dermatophytes belonging to 13 taxa. For 8 taxa all strains were correctly identified, and for 3 the rate of successful identification was >90%; 75% (6/8) of the *M. gypseum* strains were correctly identified, whereas only 47% (18/38) of the African *T. rubrum* population (also called *T. soudanense*) were recognized accurately, with a large quantity of strains misidentified as *T. violaceum*, demonstrating the close relationship of these two taxa. The method of sample preparation was fast and efficient and the expanded Vitek MS fungal knowledge base reliable and robust, allowing reproducible dermatophyte identifications in the routine laboratory.

Dermatophytes in the genera *Epidermophyton*, *Microsporum*, and *Trichophyton* are usually characterized and identified by cultural and morphological characters and physiological tests or, more recently, by sequencing (1). Morphological identification is time-consuming and complex, usually requiring expert mycological knowledge, while sequencing is comparatively expensive and at least 2 to 3 days elapse before sequencing results are available.

Matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (MS) is a reliable technique for the identification and typing of microbial pathogens such as bacteria (2–7), yeasts (8–10), and filamentous fungi, including dermatophytes (7, 11–17). Recent studies confirm that this technique may be very attractive for dermatophyte identification (18–22). In one study, the rate of correct identification of isolates belonging to the *T. mentagrophytes* complex was 89% (19), and in others, the overall rates of successful identification of dermatophyte species reached 95.8% (18), 97.8% (21), and 99.3% (20), demonstrating the potential of MALDI-TOF MS to replace classical identification methods. The technique has now also been used for the direct identification of dermatophytes in clinical samples (23).

Technical challenges to its routine use include the simultaneous presence in a sample of different growth forms (mycelium or conidia), as well as the potential influence of duration of incubation, culture medium, preparation methods (solid or liquid cultures), the amount of biomass, and the type of instrument used (24, 25).

The objective of this work was to establish a simple and rapid sample preparation method suitable for routine laboratory work and to extend the Vitek MS fungal knowledge base version 2.0.0 to allow the robust identification of clinically relevant dermato-

phytes, using a variety of strains, incubation times, and growth conditions. The sample preparation method, based on previously described protocols for yeasts (9) and filamentous fungi (19, 21), combines organism inactivation and protein extraction and allows working with material taken directly from agar plates, thus avoiding any environmental or instrument contamination. We first established a spectrum database using 134 reference strains belonging to 17 species in the three anamorphic genera *Epidermophyton*, *Microsporum*, and *Trichophyton*. In the second step, 131 clinical strains not used to build the knowledge base and belonging to 13 taxa were identified using the new knowledge base to validate its performance. Results were compared with sequencing and/or previous MALDI-TOF MS analyses carried out using the SARAMIS system (18).

## MATERIALS AND METHODS

### Isolates studied. (i) Isolates used to build up the fungal knowledge base.

A total of 134 reference strains belonging to 17 species of the genera *Epidermophyton*, *Microsporum*, and *Trichophyton* and characterized by

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TABLE 1 Performance of the expanded Vitek MS database for the identification of reference dermatophyte spectra

Taxon	No. of strains	No. of spectra	% (no.) of correctly identified spectra (no.)	Discrimination			% (no.) of unidentified spectra	% (no.) of spectra with discordant results <sup>d</sup>	Erroneous taxon <sup>d</sup>
				% (no.) with single-choice result <sup>a</sup>	% (no.) with low-discrimination result <sup>b</sup>	Second match <sup>c</sup>			
<i>E. floccosum</i>	11	107	100 (107)	100 (107)					
<i>M. audouinii</i>	8	74	98.6 (73)	94.6 (70)	4 (3)	<i>M. canis</i>		1.4 (1)	<i>M. canis</i>
<i>M. canis</i>	10	98	100 (98)	100 (98)					
<i>M. fulvum</i>	2	16	100 (16)	100 (16)					
<i>M. gypseum</i>	9	66	98.5 (65)	98.5 (65)			1.5 (1)		
<i>M. persicolor</i>	5	38	97.4 (37)	97.4 (37)			2.6 (1)		
<i>M. praecox</i>	1	11	100 (11)	100 (11)					
<i>Trichophyton</i> sp. ( <i>A. benhamiae</i> )	14	81	95.1 (77)	93.8 (76)	1.2 (1)	<i>T. erinacei</i>	3.7 (3)	1.2 (1)	<i>T. rubrum</i>
<i>T. erinacei</i>	7	53	100 (53)	92.4 (49)	7.6 (4)	<i>A. benhamiae</i>			
<i>T. equinum</i>	1	16	100 (16)	100 (16)					
<i>T. interdigitale</i> (anthropophilic and zoophilic)	19	185	99.5 (184)	98.4 (182)	1.1 (2)	<i>T. tonsurans</i>	0.6 (1)		
<i>T. mentagrophytes</i>	2	25	88 (22)	64 (16)	24 (6)	<i>T. equinum</i> (3), <i>T. interdigitale</i> (3)	8 (2)	4 (1)	<i>T. interdigitale</i>
<i>T. rubrum</i> (African and <i>sensu stricto</i> )	16	152	98.7 (150)	93.4 (142)	5.3 (8)	<i>T. violaceum</i>	0.7 (1)	0.7 (1)	<i>T. violaceum</i>
<i>T. terrestre</i> ( <i>A. quadrifidum</i> )	3	24	100 (24)	100 (24)					
<i>T. tonsurans</i>	10	82	97.6 (80)	91.5 (75)	6.1 (5)	<i>T. interdigitale</i>	2.4 (2)		
<i>T. verrucosum</i>	3	24	95.8 (23)	87.5 (21)	8.3 (2)	<i>T. erinacei</i>	4.2 (1)		
<i>T. violaceum</i>	13	78	97.4 (76)	80.8 (63)	16.7 (13)	<i>T. rubrum</i>	1.3 (1)	1.3 (1)	<i>T. rubrum</i>
Total	134	1,130	98.4 (1,112)	94.5 (1,068)	3.9 (44)		1.1 (13)	0.5 (5)	

<sup>a</sup> The mass spectrum matched the right species (high discrimination).

<sup>b</sup> The mass spectrum matched the right species.

<sup>c</sup> The mass spectrum also matched another, closely related species.

<sup>d</sup> The mass spectrum matched another, unexpected species.

genetic (internal transcribed spacer [ITS] sequencing) and morphological (18) analyses were used for the Vitek MS knowledge base creation (Table 1). They originated from culture collections (CBS, Utrecht, The Netherlands; Micoteca da Universidade do Minho, Braga, Portugal), external quality control programs, and individual collections (Department of Dermatology, University Hospital of Zurich, Zurich, Switzerland; Laboratory of Applied Microbiology, University of Applied Sciences and Arts of Southern Switzerland, Bellinzona, Switzerland). Strains were inoculated on Sabouraud dextrose (SDA; bioMérieux, La Balme Les Grottes, France) and potato dextrose (PDA; Becton Dickinson, Franklin Lakes, NJ) agar plates and incubated at 30°C for 5 and 10 days to capture two distinct growing stages. Because of their low growth rates, *Microsporum audouinii* and *Trichophyton violaceum* were incubated for 10, 15, and 20 days to obtain enough biomass.

(ii) **Clinical isolates used for testing.** The test of the expanded Vitek MS knowledge base was carried out on 131 isolates not used to build the knowledge base and including the *Trichophyton* anamorph of *Arthroderma benhamiae* ( $n = 3$ ), *Epidermophyton floccosum* ( $n = 3$ ), *M. audouinii* ( $n = 3$ ), *M. canis* ( $n = 6$ ), *M. gypseum* ( $n = 4$ ), *M. persicolor* ( $n = 2$ ), *T. erinacei* ( $n = 1$ ), *T. interdigitale* ( $n = 38$ ), *T. rubrum sensu stricto* ( $n = 34$ ), African *T. rubrum* (*T. soudanense*) ( $n = 19$ ), *T. terrestre* ( $n = 1$ ), *T. tonsurans* ( $n = 5$ ), and *T. violaceum* ( $n = 12$ ) (Table 2). All of these originated from clinical material received from the Microbiology and Virology Unit, Papa Giovanni XXIII Hospital (Bergamo, Italy); the Department of Dermatology, University Hospital of Zurich (Zurich, Switzerland); and the Laboratory of Applied Microbiology, University of Applied Sciences and Arts of Southern Switzerland (Bellinzona, Switzerland). They had previously been characterized by morphology, ITS sequencing, and/or MALDI-TOF MS (SARAMIS system).

Strains were grown on SDA plates and on Sabouraud gentamicin chloramphenicol 2 agar plates (SGC2; bioMérieux) to test the system's performance with cultures grown on a medium routinely used in clinical diagnostics laboratories. All strains were incubated at 30°C for 5 days, except for *M. audouinii* and *T. violaceum* samples, which were grown at 30°C for 9 to 10 days.

**Sample preparation.** Sample preparation consisted of a combination of organism inactivation and protein extraction. It was based on a previously published method (9, 19, 21), with minor modifications. Fungal material containing all growth phases of the colony was scraped from a surface of approximately 1 cm<sup>2</sup> using a wet swab and suspended in 300  $\mu$ l of sterile deionized water. Nine hundred microliters of >99.8% (vol/vol) ethanol was added. After vortexing and centrifugation at 10,600  $\times$  g for 2 min, the supernatant was discarded, 40  $\mu$ l of 70% formic acid was added to the resulting pellet, and 40  $\mu$ l of acetonitrile was added after vortexing again. After a final vortexing and centrifugation step at 10,600  $\times$  g for 2 min, 1  $\mu$ l of the supernatant was spotted in duplicate directly on a MALDI-TOF MS target plate (bioMérieux) and air dried. One microliter of  $\alpha$ -cyano-4-hydroxy-cinnamic acid matrix (CHCA; bioMérieux) was added to each spot and air dried before MALDI-TOF MS analysis.

**MALDI-TOF MS.** The bioMérieux Vitek MS Plus system used in this study is composed of an AXIMA (Shimadzu) mass spectrometer combined with both a closed database (Vitek MS V2.0.0) containing 755 species and an open database (SARAMIS version 4.09) comprising 1,202 species. Spectrum acquisition for knowledge base expansion was performed using the software LaunchPad version 2.9.3. Acquisition parameters such as laser power, detector voltage, etc., were set according to bioMérieux requirements for the Vitek MS system. Peak lists ( $m/z$  2,000 to 20,000) were imported into the SARAMIS software (bioMérieux) for fur-

TABLE 2 Identification of clinical strains using the expanded Vitek MS database<sup>a</sup>

Taxon	Discrimination of strains grown on SDA					Discrimination of strains grown on SGC2			
	Total no.	No. (%) positive	No. with single-choice result	No. with low-discrimination result	No. (%) with discordant result	No. (%) positive	No. with single-choice result	No. with low-discrimination result	No. (%) with discordant result
<i>Trichophyton</i> sp. ( <i>A. benhamiae</i> )	3	3 (100)	3			3 (100)	3		
<i>E. floccosum</i>	3	3 (100)	3			3 (100)	3		
<i>M. audouinii</i>	3	3 (100)	3			3 (100)	3		
<i>M. canis</i>	6	6 (100)	6			6 (100)	6		
<i>M. gypseum</i>	4	3 (75)	3		1 (25)	3 (75)	3		1 (25)
<i>M. persicolor</i>	2	2 (100)	2			2 (100)	2		
<i>T. erinacei</i>	1	1 (100)	1			1 (100)	1		
<i>T. interdigitale</i>	38	38 (100)	37	1		37 (97.4)	35	2	1 (2.6)
<i>T. rubrum sensu stricto</i>	34	34 (100)	31	3		29 (85.3)	25	4	5 (14.7)
African <i>T. rubrum</i>	19	15 (78.9)	7	8	4 (21.1)	3 (15.8)	1	2	16 (84.2)
<i>T. terrestre</i>	1	1 (100)	1			1 (100)	1		
<i>T. tonsurans</i>	5	5 (100)	5			5 (100)	5		
<i>T. violaceum</i>	12	11 (91.7)	11		1 (8.3)	11 (91.7)	8	3	1 (8.3)
Total	131	125 (95.4)	113	12	6 (4.6)	107 (81.7)	96	11	24 (18.3)

<sup>a</sup> For abbreviations and definitions, see the text.

ther taxonomic analyses. To be considered, the quality of the strains and spectra had to have a relative intraspecific similarity of >65%, a relative similarity between duplicates of >70%, and an absolute intraspecific similarity of >50 peaks. Spectra fulfilling these quality criteria were included in the Vitek MS dermatophyte spectral base.

**Expanded Vitek MS knowledge base performance estimation by cross validation.** A 5-fold cross validation method (26) was used to estimate the performance of the expanded knowledge base. The whole Vitek MS spectrum base (including the new dermatophyte spectra) was divided into five subsets, four of which were used as references and the remaining one as a test subset (to predict identification). Dermatophyte spectra from the test subset were submitted to identification by comparing them with spectra from the four reference subsets. This process was repeated five times with permutations of the five subsets. The identification results of the five rounds were used to estimate the identification performance. A correct identification included (i) a single-choice result (high discrimination), meaning that the spectrum matched only the right species, and (ii) a low-discrimination result, when the spectrum matched the right species and another, closely related species.

## RESULTS

**Vitek MS fungal knowledge base extension.** A total of 1,130 dermatophyte mass spectra were retained and used for the Vitek MS fungal knowledge base extension (Table 1). The average number of strains and mass spectra per species were 7.9 and 66.5, respectively. Separation of all strains was achieved at least at the species level. In general, the mass spectra were very homogeneous when generated from material from SDA plates and less homogeneous for samples originating from PDA plates.

**Microsporium sp. and Trichophyton anamorph of Arthroderma benhamiae.** All *Microsporium* strains were well separated at the species level by MALDI-TOF MS (Fig. 1A). For *M. audouinii*, *M. canis*, *M. persicolor*, and *M. praecox*, MALDI-TOF MS results were similar to results obtained by ITS sequencing (Fig. 1B), with *M. audouinii* clustering together with *M. canis* and *M. persicolor* with *M. praecox*. *Microsporium audouinii* was separated clearly from *M. canis* by MALDI-TOF MS, although the ITS sequence

similarity of the two species is >99.6% (18). The anamorphic *M. gypseum* strains used in this study belong to two teleomorphic species, *Arthroderma gypseum* and *A. incurvatum*, both well distinguished by MALDI-TOF MS (Fig. 1A).

Both ITS sequencing and MALDI-TOF MS were able to separate reliably the American-European race and taxonomic entity 3 of the *Trichophyton* anamorph of *A. benhamiae* (Fig. 1).

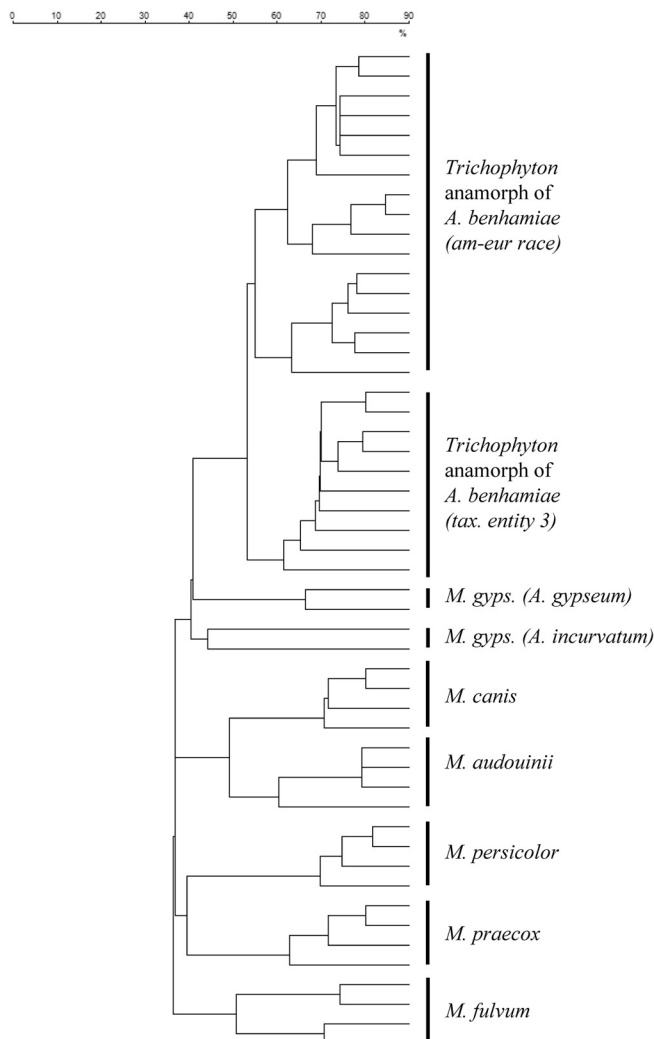
**Trichophyton sp.** Previous ITS sequencing showed that *T. violaceum* differed from *T. rubrum* by only 7/538 nucleotides (Fig. 2B) (18); MALDI-TOF MS was able to distinguish the two species (Fig. 2A). *T. rubrum sensu stricto* and its African population are genetically very similar (sequence similarity of >99.5%) (18), and MALDI-TOF MS could not separate them.

Both *T. interdigitale* and *T. mentagrophytes* species could be identified unambiguously by MALDI-TOF MS. In addition, *T. interdigitale* spectra obtained from samples grown on SDA allowed separation of anthropophilic from zoophilic populations (Fig. 2A).

**Performance of the expanded Vitek MS knowledge base.** Overall, the 1,130 dermatophyte mass spectra generated with the 134 reference strains gave a cross-validated performance of 98.4% (1,112 correctly identified spectra): 94.5% (1,068) of them provided a single-choice result (high discrimination) and 3.9% (44) a correct identification with low discrimination (Table 1). Only 1.1% (13) of the spectra could not be identified, and 0.5% (5) provided discordant identifications. One hundred percent of the spectra were correctly identified for 7, >95% for 9, and 88% for 1 (*T. mentagrophytes*) of the 17 species. For species with low-discrimination values or a discordant result, the second match was in general a taxon closely related to the correct one.

**Evaluation of the expanded knowledge base with clinical samples.** The overall rate of correct identification of the 131 clinical isolates tested to estimate the knowledge base performance was 88.5% (95.4% for samples on SDA and 81.7% on SGC2) (Table 2). For 8 taxa, all strains were correctly identified, and for 3 the rate of successful identification was >90%; 75% of the *M. gypseum*

## A. MALDI-TOF MS



## B. ITS sequencing

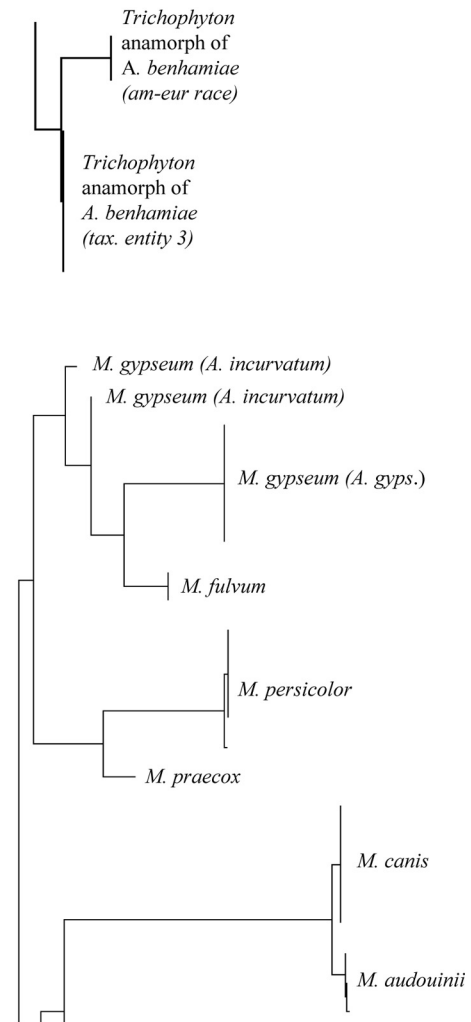


FIG 1 *Microsporium* and *Trichophyton* anamorph of *A. benhamiae*. (A) Dendrogram (Dice coefficient, single-linkage agglomerative clustering) based on the MALDI-TOF MS analysis of some of the reference strains used for the Vitek MS knowledge base creation (SDA and PDA plates; molecular mass range, 3 to 20 kDa). (B) Phylogenetic tree from ITS sequencing (reproduced from reference 18 with permission of Informa Healthcare).

strains were correctly identified, whereas only 47% (18/38) of the African *T. rubrum* population (*T. soudanense*) were recognized accurately, with a large rate of strains misidentified as *T. violaceum*, demonstrating the close relationship of these two taxa. Increasing the incubation time of cultures to 10 days and/or repeating the analyses did not improve results (data not shown).

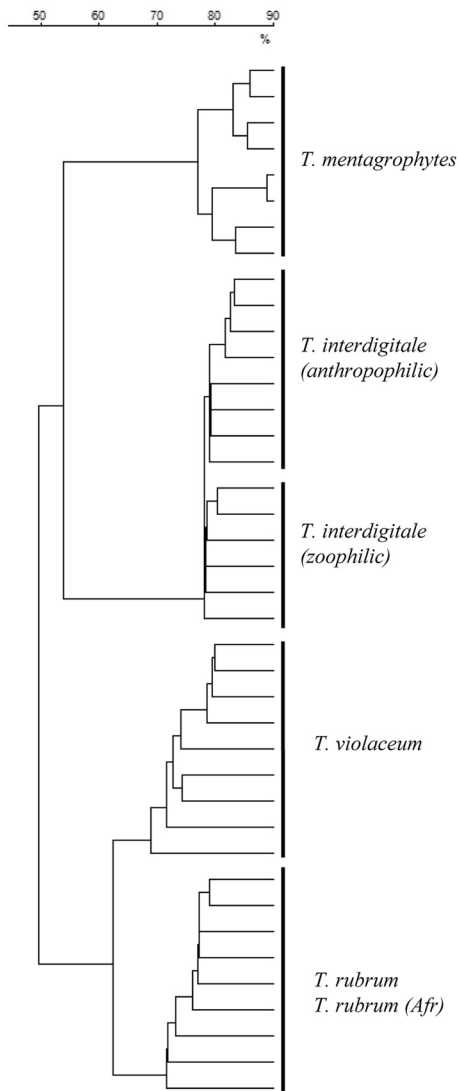
## DISCUSSION

**Sample preparation for dermatophyte identification by Vitek MS.** The Vitek MS fungal knowledge base constructed using sample spectra obtained with the method described here proved to be reliable and robust, allowing reproducible dermatophyte identifications in the routine laboratory.

Obtaining high-quality MALDI-TOF MS spectra of fungi is more challenging than spectrum acquisition from most bacteria. Direct spotting does not provide reproducible results, and a careful standardization of the extraction and preparation method is necessary. This can be done only by taking into account culture

media, incubation time, and amount and type of material (7, 25). Published extraction protocols include bead beating and the use of trifluoroacetic acid, acetonitrile, or formic acid treatments to disrupt the fungal cell walls, to increase protein extraction, and to provide high-quality mass spectra (27). In this study, we have used a newly developed protocol, combining microorganism inactivation and protein extraction, to obtain reliable, high-quality, and reproducible spectra from material grown on solid media. This protocol is rapid and safe, as it uses only nontoxic solvents and ensures inactivation of the molds, thus avoiding health hazards and the contamination of the instrument during measurements. It is also most convenient in the routine laboratory, where solid culture media are mainly used; for instance, nails and hairs suspected to contain dermatophytes are generally incubated on agar plates. Identification by MALDI-TOF MS can, therefore, be carried out immediately from material derived from the original culture, with no additional time-consuming steps described for other

## A. MALDI-TOF MS



## B. ITS sequencing

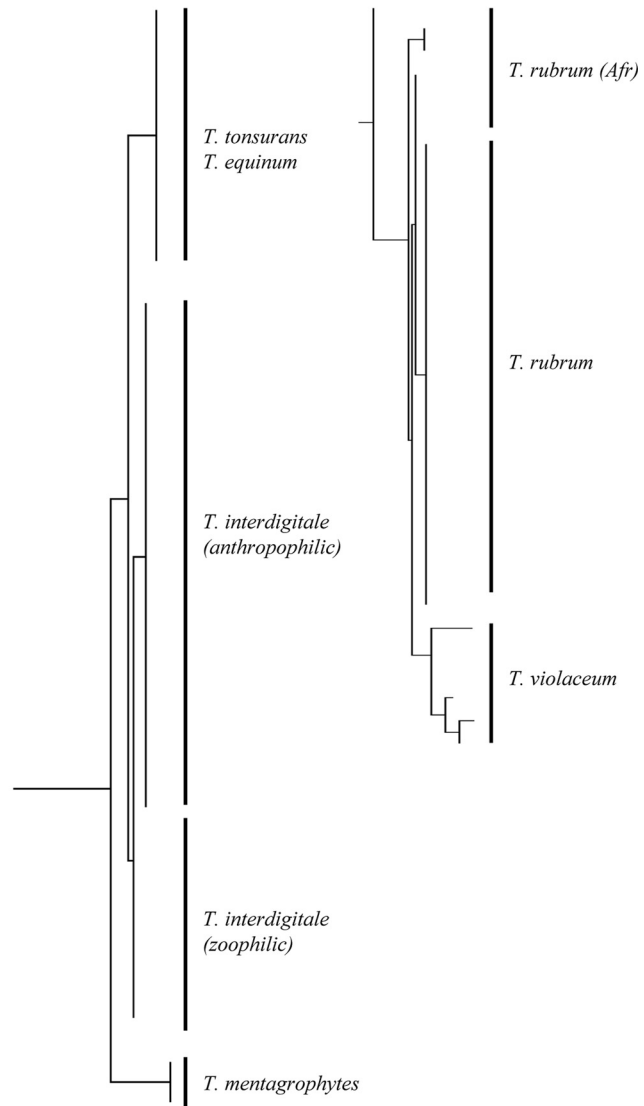


FIG 2 *Trichophyton*. (A) Dendrogram (Dice coefficient, single-linkage agglomerative clustering) based on the MALDI-TOF MS analysis of some of the reference strains used for the Vitek MS knowledge base creation (SDA plates; molecular mass range, 3 to 20 kDa). (B) Phylogenetic tree from ITS sequencing (reproduced from reference 18 with permission of Informa Healthcare).

protocols (28). The use of this protocol allows also the user to visually identify distinct types of molds growing on the same agar plate, thus avoiding the potential pitfall of liquid cultures that could mask the presence of several organisms.

**Extension of the Vitek MS knowledge base.** In this study, we used several strains per species (7.9 on average) and several culture conditions (different media and times of incubation) to capture the variability of each species and to create a robust knowledge base taking into account possible peak pattern variations in response to different cultivation conditions.

Spectral profiles obtained after incubation on SDA were in general very homogeneous. The presence of different growth stages on PDA with stage-specific spectral profiles resulted in more heterogeneous MALDI-TOF mass spectra than for organisms grown on SDA, on which sporulation generally is delayed.

This made a separation of species grown on PDA possible, but often it did not allow distinguishing intraspecific populations or cryptic species.

To our knowledge, this is the first study to achieve a good separation of intraspecific taxa in some dermatophyte species. We obtained an excellent separation of the subpopulations of the *Trichophyton* anamorph of *A. benhamiae* grown on SDA or PDA (Fig. 1A) and of the anthropophilic and zoophilic strains of *T. interdigitale* grown on SDA (Fig. 2A). This confirms the high discriminatory power of the method used.

**Identification of clinical strains.** Clinical strains were grown on SDA and on SGC2 agar plates, the latter being a medium frequently used in clinical diagnostic laboratories. This was done intentionally to ensure that the extraction and identification method would also work on material taken from a medium not

used to construct the database. The identification of strains grown on SDA plates was very reliable, with the exception of some strains of the African population of *T. rubrum* (*T. soudanense*). Identification using spectra of material grown on SGC2, however, was less reliable for *T. rubrum sensu stricto* and unsatisfactory for the African population of *T. rubrum*. Identification of *T. violaceum* was less problematic, with only 1 out of 12 clinical strains wrongly identified as *T. rubrum*.

Gräser et al. (1) describe the *T. rubrum* complex to include only the two anthropophilic species *T. rubrum sensu lato* and *T. violaceum*, other taxa previously recognized being reclassified or synonymized as *T. rubrum* or *T. violaceum* (29, 30). Both *T. violaceum* and the African *T. rubrum* (*T. soudanense*) are endemic in Africa, whereas *T. rubrum sensu stricto* strains are cosmopolitan. *T. rubrum sensu lato* and *T. violaceum* are phylogenetically very close, even if morphologically quite distinct, and their taxonomic position has been under discussion for a long time (1, 29, 31–34). ITS sequence data would support *T. soudanense* as a synonym of *T. violaceum* (29, 35, 36); the two taxa share identical ITS2 sequences different from those of *T. rubrum sensu stricto* (36). In contrast, studies using microsatellite markers, known to evolve at a high mutation rate, are in disagreement with ITS data and suggest *T. soudanense* to be closely related to *T. rubrum sensu stricto* (33, 34). In our study, we followed the proposal of Gräser et al. to consider *T. soudanense* as belonging to *T. rubrum sensu lato* (1, 29, 33).

Six reference strains belonging to the African population of *T. rubrum* (*T. soudanense*) were used for the knowledge base creation and, as expected, were all closely related to *T. rubrum sensu stricto*. Identification of 19 clinical African *T. rubrum* strains was clearly less reliable than that of *T. rubrum sensu stricto* (78.9% for material grown on SDA and 15.8% on SGC2) (Table 2). Several strains were also misidentified as *T. violaceum* (4/19 strains grown on SDA and 16/19 on SGC2). De Respinis et al. (18) already showed a high degree of homology between *T. violaceum* and *T. rubrum sensu lato* by MALDI TOF MS. They could not achieve a direct identification and had to use the direct comparison of eight specific masses to reliably distinguish these two taxa. Other authors described similar problems in differentiating taxa from the *T. rubrum* complex. Nenoff et al. (20) reported a very high percentage (99.3%) of correct dermatophyte identifications, the only two strains that could not be identified belonging to *T. violaceum*. L'Ollivier et al. (21) reported that half of the *T. violaceum* strains they studied were misidentified as *T. soudanense*. The close relationship of taxa within the *T. rubrum* complex is confirmed by our results, and the high similarity between *T. violaceum* and African *T. rubrum* observed with MALDI-TOF MS is in accordance with the ITS sequence data (29, 35, 36).

**Conclusions.** Our study has demonstrated that the inactivation-extraction method is rapid and reliable and that the newly built Vitek MS knowledge base provides a robust tool for the reproducible identification to the species (and in selected cases to the intraspecific) level of dermatophytes grown on common routine media such as SDA, SGC2, and PDA.

The quality of the spectra obtained is high and the method suitable for research and diagnostic work with dermatophytes and with filamentous fungi in general.

The overall identification performance of clinical strains was high, but strains identified as *T. violaceum* by MALDI-TOF MS should undergo additional morphological and/or molecular anal-

ysis to exclude any confusion with the African population of *T. rubrum*.

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