ABSTRACT  In the present study, an innovative top-down liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the identification of clinically relevant fungi is tested using a model set of dermatophyte strains. The methodology characterizes intact proteins derived from *Trichophyton* species, which are used as parameters of differentiation. To test its resolving power compared to that of traditional Sanger sequencing and matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF), 24 strains of closely related dermatophytes, *Trichophyton rubrum*, *T. violaceum*, *T. tonsurans*, *T. equinum*, and *T. interdigitale*, were subjected to this new approach. Using MS/MS and different deconvolution algorithms, we identified hundreds of individual proteins, with a subpopulation of these used as strain- or species-specific markers. Three species, i.e., *T. rubrum*, *T. violaceum*, and *T. interdigitale*, were identified correctly down to the species level. Moreover, all isolates associated with these three species were identified correctly down to the strain level. In the *T. tonsurans-equinum* complex, eight out of 12 strains showed nearly identical proteomes, indicating an unresolved taxonomic conflict already apparent from previous phylogenetic data. In this case, it was determined with high probability that only a single species can be present. Our study successfully demonstrates applicability of the mass spectrometric approach to identify clinically relevant filamentous fungi. Here, we present the first proof-of-principle study employing the mentioned technology to differentiate microbial pathogens. The ability to differentiate fungi at the strain level sets the stage to improve patient outcomes, such as early detection of strains that carry resistance to antifungals.

KEYWORDS  clinical mycology, dermatophytes, identification, LC-MS/MS, *Trichophyton*
matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF) mass spectrometry has been applied to a broad range of species (4–9) or species groups of dermatophytes (10, 11). Even with these recent advances in identification and characterization using MALDI-TOF, accuracy rates remain at the 50 to 60% range with very high no-call rates (12), which is partly due to inadequate taxonomy of dermatophytes at the DNA level (3). In other fungal groups, identification of clinically relevant filamentous fungi has been shown to be possible with the addition of custom acquired data supplementing IVD databases (6, 7).

As an alternative strategy to the MALDI-TOF fingerprint approach, proteome-based strategies involve identification of proteins derived from microbial extracts. Two fundamentally different mass spectrometric strategies are available for protein identification: bottom-up and top-down. In bottom-up proteomics, purified proteins or complex protein mixtures are subjected to proteolytic cleavage prior to MS analysis. In top-down proteomics, intact protein ions or large protein fragments are injected directly into the mass spectrometer, where they are further fragmented. The main advantage of top-down analysis is the ability to reveal intact protein masses, structural amino acid sequence variants, and (combinations of) posttranslational modifications.

In the present study, we utilize liquid chromatography-tandem mass spectrometry (LC-MS/MS) to separate proteins from dermatophyte extracts and analyze them sequentially in an Orbitrap tandem mass spectrometer. Amino acid sequence information obtained from tandem mass spectrometry is used to identify the observed proteins, which in turn leads to the correct classification of clinically relevant dermatophytes. This MS/MS process, termed collision-induced dissociation (CID), imparts excess energy to the intact protein ions, resulting in smaller-mass amino acid sequence-specific protein fragments which are used to directly identify any given protein undergoing this process. Several thousand highly informative MS/MS spectra from the fragmented proteins or peptides are obtainable this way in a single run. The key difference of this approach compared to fingerprinting/pattern recognition by MALDI-TOF is the accurate assignment of intact protein and fragment masses that allows for statistically relevant high-confidence protein identification (13). In turn, these identified proteins, either singly or in combination, can be used as diagnostic markers of clinically relevant microorganisms.

The goal of the present study was to provide a proof-of-principle experiment employing Orbitrap LC-MS/MS for discrimination of filamentous fungi and to establish a proteomic approach for detailed characterization of strain diversity of the investigated taxa. As a model, two closely related but different pairs of species were compared. The members of one species pair are known to belong to unambiguously different species, whereas the separation of the other pair or lineages is doubtful, possibly comprising only a single species (Fig. 1). The former set concerns the *Trichophyton rubrum* group, comprising two species: *T. rubrum*, with a global prevalence and mainly causing tinea corporis and tinea pedis, and *T. violaceum*, which mostly causes tinea capitis and is endemic to northern Africa and the Middle East. *Trichophyton soudanense* belongs to the latter group but is generally judged a synonym of *T. violaceum* (14). This set is compared to the *Trichophyton tonsurans* complex, which comprises two lineages that are often regarded as synonymous (15): *T. tonsurans* and *T. equinum*. The former is an anthropophilic entity causing tinea capitis in humans, while its zoophilic counterpart, *T. equinum*, causes ringworm in horses but is also found in humans (3).

Our study successfully demonstrates a solution to a long-existing technical challenge, i.e., the possibility of employing liquid chromatography coupled with ultra-high-resolution Orbitrap mass spectrometry for microbial species identification. Massive quantities of fully resolved individual microbial proteins render Orbitrap mass spectrometry several orders of magnitude higher in sensitivity and specificity than currently existing proteomic technologies. Subsequently this will set the stage to improve patient care, significantly enabling microbial identification down to the strain level.
MATERIALS AND METHODS

Strains and growth conditions. Strains studied were acquired from the reference collection of Centraalbureau voor Schimmelcultures at the Westerdijk Fungal Biodiversity Institute (Table 1). Strains were part of a taxonomic study applying multilocus sequencing (3) and included (neo)type strains of synonymized species *Trichophyton raubitschekii*, *T. rubrum* var. *nigricans*, *T. fischeri*, *T. soudanense*, and *T. violaceum* in the *T. rubrum* group and *Trichophyton areolatum*, *T. floriforme*, *T. equinum*, and *T. equinum* var. *autotrophicum* in the *T. tonsurans* group. Two strains of *T. interdigitale* were included as closest relatives of *T. tonsurans*, serving as a marker of nonidentity. Nine out of 12 strains in this group had variously been classified as either *T. tonsurans* or *T. equinum* (Table 1). Lyophilized or cryopreserved

![Image: Two species groups, *T. rubrum* with *T. violaceum* and *T. tonsurans* with *T. equinum*. The members of the *T. rubrum* group are considered to be different, whereas the separation of the other pair is doubtful, possibly involving only a single species.]

**FIG 1** Two species groups, *T. rubrum* with *T. violaceum* and *T. tonsurans* with *T. equinum*. The members of the *T. rubrum* group are considered to be different, whereas the separation of the other pair is doubtful, possibly involving only a single species.

**TABLE 1** *Trichophyton* strains analyzed in this study

| *Trichophyton* strain<sup>a</sup> | Taxonomy change<sup>b</sup> | Source | Clinical picture | Country | GenBank accession no. |
|---------------------------------|----------------------------|--------|------------------|---------|----------------------|
| *T. rubrum* CBS 115314          |                           | Human  | Onychomycosis    | Greece  | KT155714             |
| *T. rubrum* CBS 100084 (T)     | *T. raubitschekii*        | Human  | Skin             | Canada  | KT155667             |
| *T. rubrum* CBS 1000238        | *T. rubrum* var. *nigicans* | Human  |                  |         | KT155669             |
| *T. rubrum* CBS 288.86         | *T. fischeri*             | Contaminant |                |         | AJ270793             |
| *T. rubrum* CBS 202.88         | *T. raubitschekii*        | Human  | Tinea pedis      | Canada  | AJ270804             |
| *T. rubrum* CBS 118892         |                           | Human  | Tinea pedis      | Germany | KT155731             |
| *T. violaceum* CBS 120320      |                           | Human  | Tinea capitis    | Switzerland | KT155740       |
| *T. violaceum* CBS 120316      |                           | Human  | Tinea capitis    | Switzerland | KT155737       |
| *T. violaceum* CBS 201.88      | *T. soudanense*           | Human  | Tinea faciei     | Canada  | KT310173             |
| *T. violaceum* CBS 452.61      | *T. soudanense*           | Human  | Endotheix variants and tinea capitis | Zaire | AJ270809 |
| *T. tonsurans* CBS 285.30 (T)  | *T. areolatum*            | Human  | Endotheix        | Argentina | KT155645         |
| *T. tonsurans* CBS 318.31 (T)  | *T. floriforme*           | Human  |                  |         | KT310170             |
| *T. tonsurans* CBS 856.71      | *T. equinum* var. *equinum* | Horse | Hair             | The Netherlands | KT310172      |
| *T. tonsurans* CBS 127.97      | *T. equinum*, *T. equinum* var. *equinum* | Human | Onychomycosis and tinea manuum | Finland | KT310169             |
| *T. tonsurans* CBS 109033      | *T. equinum*              | Horse  | Skin             | Canada  | KT155681             |
| *T. tonsurans* CBS 112186      | *T. tonsurans*             | Human  |                  | England  | KT155688            |
| *T. equinum* CBS 270.66 (NT)   | *T. equinum* var. *equinum*, *T. tonsurans* | Horse |                  | USA      | KT155643             |
| *T. equinum* CBS 634.82        | *T. equinum* var. *autotrophicum*, *T. tonsurans* | Horse | Tinea            | New Zealand | KT310171         |
| *T. equinum* CBS 100080 (T)   | *T. equinum* var. *autotrophicum*, *T. tonsurans* | Horse |                  | New Zealand | KT155665         |
| *T. equinum* CBS 112188        | *T. tonsurans*             | Horse  |                  | England  | EF043277             |
| *T. equinum* CBS 112193        | *T. tonsurans*             | Horse  |                  | England  | KT155693             |
| *T. equinum* CBS 112198        | *T. tonsurans*             | Human  |                  | England  | EF043275             |
| *T. interdigitale* CBS 119447  |                           | Human  | Tinea capitis    | Gabon    | KT155733             |
| *T. interdigitale* CBS 120318  |                           | Human  | Tinea capitis    | Switzerland | KT155738       |

<sup>a</sup>T, type; NT, neotype.

<sup>b</sup>Data for taxonomy changes are all name changes recorded in the CBS database (with previous nomenclatural changes for a particular strain).
Material was activated on Sabouraud’s glucose agar plates (SGA; Oxoid, Thermo Scientific) and incubated at 24°C for 3 weeks due to slow growth of *T. violaceum*.

**DNA extraction, PCR, and sequencing.** Genomic DNA was extracted using Illumina’s MasterPure DNA purification kit (Illumina) according to the manufacturer’s protocol. Ribosomal DNA (rDNA) ITS was sequenced using ITS5 and ITS4 primers under standard conditions (16). PCR products were purified with FastAP thermo-sensitive alkaline phosphatase and shrimp alkaline phosphatase (Fermentas, Thermo Scientific). Sequencing reactions were done in 10-μl volumes using Thermo Scientific BigDye Terminator v.3.1 on a 3730XL instrument (Thermo Scientific). Sequences were deposited at NCBI GenBank (Table 1).

Obtained sequences were manually edited, and consensus sequences were aligned with MAFFT v. 6.850b with default settings (17). Identification was performed by querying sequences against NCBI GenBank and the Westerdijk Institute website (www.westerdijkinstitute.nl).

**Protein extraction and purification.** Protein extractions were performed on three biological replicates per strain. Briefly, approximately 5 mg biomass was harvested with a scalpel from a culture plate and transferred to a microvial (Eppendorf) with lysis buffer containing formic acid and acetonitrile (proprietary ratios). Cells were disrupted and then centrifuged for 1 min at 14,000 rpm. The supernatant was then transferred to a new vial. Extracts were diluted to 10% acetonitrile and desalted using Lab_in_a_Plate plates (Glygen Corp., USA). Equilibration, loading, and washing steps were done according to the manufacturer’s protocol, with minor changes. Samples were eluted in 40% acetonitrile with 0.1% formic acid.

**LC-MS/MS analysis, data processing, and identification.** Chromatographic separation was done by injecting 8 μl of the protein extracts on a Thermo Scientific EASY-Spray Accucore C4 column (15 cm, 75-μm inner diameter, 2.6-μm particles, and 150-Å pore size). Protein separation was achieved with a 1-h gradient starting with buffer A (0% acetonitrile, 0.1% formic acid) to 60% buffer B (60% acetonitrile and 0.1% formic acid) in 50 min at a column temperature of 60°C and a flow rate of 200 μl/min. The LC system was coupled with a Thermo Scientific Q Exactive Plus hybrid quadrupole mass spectrometer. Mass analysis was done with the top-down method of 5 microscans, a scan range of 350 to 2,000 Da, and loop counts for data-dependent (dd) analysis being 15.

Algorithms used for further analysis of the acquired data are given in Fig. 2. Deconvolution of the mass spectra was performed via two algorithms. The first employed Thermo Fisher Scientific proprietary software (algorithm A1) to deconvolute raw spectra in m/z space into monoisotopic protein masses. An alternative approach was conducted via Thermo Scientific ProSightPC 3.0 for deconvolution of intact protein mass spectra and analysis of MS/MS fragment spectra (Thermo Scientific Xtract build-in). Subsequently, MS/MS fragment spectra were queried against a custom database obtained from UniProt.
(http://www.uniprot.org/) using ProSightPC 3.0 and containing amino acid sequences of the genera *Trichophyton*, *Microsporum*, *Epidermophyton*, and *Arthroderma* (883,412 predicted proteoforms in total). The queries were performed as absolute mass search (considering disulfide linkages and Δm applied) in a specified intact mass window of 1,000 Da; selected parameters were defined as 15-ppm fragment mass tolerance, with acetylation and posttranslational modifications (PTM) applied as criteria and a cutoff expectation value (E value) of \( < 0.0001 \). Identified protein sequences with a confidence (E value) score higher than \( 1.0 \times \) \( 10^{-4} \) were further analyzed in the versatile custom sequence database and analysis software ProteinCenter (Thermo Fisher Scientific). Using ProteinCenter, the identified proteins were subjected to homology search using an 80% similarity cutoff in an attempt to find identical or similar sequences in other dermatophytes. Subsequent searches were constrained to 100% homology level to clear redundancies.

In addition to inferring species affiliations with identified protein sequences from MS/MS fragment spectra, we ran two additional analyses to classify the strains and to predict the species where the analyzed strains belong. An unreported Thermo Fisher proprietary classification algorithm (algorithm A2) inferred the strain classification analysis. The prediction was repeated four times in order to establish variance between replicates. The resulting classification accuracy has values from 0 to 1, with 1 being all three replicates of a strain that were correctly identified in all four predictions.

Species prediction was performed with algorithm A4. The same data were used to establish statistical independence over the current taxonomic species affiliation but constrained to a chosen reference strain to guide the species prediction. The analysis was conducted twice with different reference strains, each analysis with three iterations for the individual taxa. Two analyses were conducted with one or two reference strains; in the first analysis, one or two (neo)type strains or randomly chosen strains were used as reference strains to infer accuracy. Prior to the second analysis, we performed clustering of the 500 most consistently measured monoisotopic masses using a Thermo Scientific proprietary clustering algorithm, A3 (data not shown). Clustering of strains with these monoisotopic masses revealed which strains have the highest number of shared masses. Based on this criterion, one or two of these strains were chosen as references. The latter step was required to avoid atypical selections.

**Accession number(s).** Sequences determined in this work were deposited at NCBI GenBank and are listed in Table 1.

### RESULTS

**DNA-based identification.** All strains had been identified prior to protein analysis using rDNA ITS as a barcode. Nucleotide sequence differences were established by separately aligning the two complexes. In the *T. rubrum* complex, *T. rubrum* strains differed from *T. violaceum* (including *T. soudanense*) with 4 single-nucleotide polymorphisms (SNPs) at positions 167 (ITS1), 525, 543, and 544 (ITS2). Differences in the number of AT repeats at the end of ITS2 reported in the literature were not found to differ consistently in this alignment and therefore were not taken into account. The six ITS sequences of *T. rubrum* were identical. In *T. violaceum*, CBS 452.61, denominated as *T. soudanense*, was identical to the remaining two *T. violaceum* strains, while the second *T. soudanense* strain, CBS 201.88, had a deletion of 36 bp, as reported in the literature. In the *T. tonsurans/T. equinum* group, the known C/T SNP was not distributed, as expected, between *T. tonsurans* and *T. equinum* strains; only CBS 318.31 had a C SNP, while all remaining 11 strains, including the neotype of *T. equinum* CBS 270.66, had a T SNP. The sequence of CBS 318.31 was identical to that of the neotype of *T. tonsurans* CBS 496.48. *Trichophyton interdigitale* differed from the *T. tonsurans/T. equinum* group in 9 nucleotides.

**Deconvolution of raw mass spectra.** Results from deconvoluting MS1 spectra employing a proprietary algorithm (A1) and ProSightPC 3.0 (Xtract), followed by processing MS/MS fragment spectra, are summarized in Table 2. Total numbers of identified monoisotopic protein masses are given per replicate, with shared masses per strain, average numbers, standard deviations (SD), and coefficients of variation (CV) representing biological variation. With algorithm A1, the lowest number of monoisotopic masses (52) was observed in *T. rubrum* CBS 115314 and the highest (259) in *T. interdigitale* CBS 119447. Standard deviations ranged between 3 and 53 in *T. rubrum* CBS 202.88 and *T. violaceum* CBS 120316, respectively. The CV was above 20% for five strains, being the lowest (1%) in *T. rubrum* CBS 118892 and the highest (27%) in *T. equinum* CBS 112198.

Deconvolution employing ProSightPC 3.0 (Xtract) rendered the lowest number of protein masses (126) in *T. tonsurans* CBS 856.71 and the highest (523) in *T. rubrum* CBS 118892. Standard deviations ranged between 8 in *T. tonsurans* CBS 127.97 and 38 in *T. rubrum* CBS 115314. The CV was 3% in *T. violaceum* CBS 201.88 and was highest, at 35%,
in *T. rubrum* CBS 115314. ProteinCenter analysis of protein masses obtained by ProSightPC 3.0 resulted in a total of 413 proteins present in at least one replicate of one of the 24 strains at 100% sequence homology. The number of identified proteins at the 100% homology level is given in Table 2 after postprocessing of initial ProSight results. The list of these proteins is given in Table S1 in the supplemental material. In all subsequent analyses, only deconvoluted masses (algorithm A1) and/or identified proteins (ProSightPC 3.0) present in all three biological replicates were used.

### Strain classification and species prediction.

Monoisotopic masses obtained by A1 were further analyzed in order to select unique masses per strain, to classify the strains, and to predict species affiliations. Strain classification performed on all replicates with four prediction runs is given in Table S2. The highest classification accuracy (Table 3) is achieved with a score of one, while a no call (i.e., no classification) is defined as zero. The results indicate that all strains affiliated with *Trichophyton rubrum*, *T. violaceum*, and *T. interdigitale* were correctly classified with a classification accuracy of 1. In the *Trichophyton tonsurans/T. equinum* group, four strains, CBS 318.31, CBS 285.30, CBS 100080, and CBS 865.71, were correctly identified with the maximum classification accuracy. The remaining eight strains in this group were identified with a classification accuracy ranging from 0.42 to 0.92. Unique masses per strain were those masses present in all three replicates of a given strain and absent from all replicates from the remaining 24 strains.

Species prediction was performed using two independent analyses, each applying one or two reference strains (types or randomly chosen strains) for each cluster (Table 4). While selection of a single reference resulted in inconsistent species calls for both species complexes, adding another strain to the classifier improved classification accuracy by assessing proteome variability. With minor ambiguities, all strains in the *T. rubrum* complex were correctly identified. In the *T. tonsurans* complex, one reference-based prediction yielded a random spread of *T. tonsurans* and *T. equinum* calls. Addition

| Strain           | Algorithm A1 | ProSightPC 3.0 |
|------------------|--------------|----------------|
|                 | Total no. of identified proteins | Statistics | Shared mass | Total no. of identified proteins | Statistics | Shared mass |
|                 | rep 1 | rep 2 | rep 3 | Avg | SD | CV | rep 1 | rep 2 | rep 3 | Avg | SD | CV |
| *T. rubrum* CBS 115314 | 140 | 197 | 130 | 156 | 36 | 23 | 52 | 306 | 552 | 320 | 393 | 138 | 35 | 153 | 16 |
| *T. rubrum* CBS 100084 | 182 | 214 | 162 | 186 | 26 | 14 | 88 | 392 | 373 | 320 | 362 | 37 | 10 | 195 | 21 |
| *T. rubrum* CBS 288.86 | 417 | 433 | 340 | 397 | 50 | 13 | 205 | 785 | 818 | 749 | 784 | 35 | 4 | 486 | 51 |
| *T. rubrum* CBS 202.88 | 228 | 233 | 227 | 229 | 3 | 1 | 130 | 464 | 644 | 551 | 553 | 90 | 16 | 432 | 45 |
| *T. rubrum* CBS 118892 | 346 | 311 | 367 | 341 | 28 | 8 | 195 | 675 | 655 | 810 | 553 | 90 | 16 | 432 | 45 |
| *T. violaceum* CBS 120320 | 287 | 190 | 249 | 242 | 49 | 20 | 95 | 395 | 385 | 451 | 410 | 36 | 9 | 304 | 30 |
| *T. violaceum* CBS 120316 | 218 | 323 | 277 | 273 | 53 | 19 | 147 | 383 | 591 | 536 | 503 | 108 | 21 | 309 | 39 |
| *T. violaceum* CBS 201.88 | 264 | 257 | 286 | 269 | 15 | 6 | 160 | 697 | 711 | 664 | 663 | 121 | 18 | 313 | 32 |
| *T. violaceum* CBS 452.61 | 394 | 380 | 338 | 371 | 29 | 8 | 218 | 535 | 775 | 680 | 661 | 121 | 18 | 313 | 32 |
| *T. tonsurans* CBS 285.30 | 336 | 282 | 305 | 308 | 27 | 18 | 197 | 535 | 547 | 419 | 500 | 71 | 14 | 277 | 43 |
| *T. tonsurans* CBS 318.31 | 153 | 170 | 126 | 150 | 22 | 15 | 81 | 290 | 313 | 227 | 277 | 46 | 15 | 202 | 28 |
| *T. tonsurans* CBS 856.71 | 124 | 116 | 80 | 107 | 23 | 22 | 57 | 137 | 193 | 159 | 163 | 27 | 12 | 126 | 22 |
| *T. tonsurans* CBS 127.97 | 345 | 327 | 337 | 336 | 9 | 3 | 193 | 456 | 441 | 454 | 450 | 8 | 2 | 340 | 50 |
| *T. tonsurans* CBS 109033 | 322 | 350 | 308 | 327 | 21 | 7 | 148 | 459 | 340 | 607 | 469 | 134 | 29 | 241 | 43 |
| *T. tonsurans* CBS 112186 | 313 | 251 | 225 | 263 | 45 | 17 | 119 | 494 | 473 | 353 | 440 | 76 | 17 | 262 | 42 |
| *T. equinum* CBS 270.66 | 134 | 185 | 205 | 175 | 37 | 21 | 86 | 333 | 448 | 385 | 389 | 58 | 15 | 279 | 46 |
| *T. equinum* CBS 634.82 | 274 | 313 | 262 | 283 | 27 | 9 | 162 | 297 | 399 | 290 | 329 | 61 | 19 | 231 | 32 |
| *T. equinum* CBS 100080 | 382 | 396 | 365 | 381 | 16 | 4 | 222 | 479 | 550 | 648 | 559 | 85 | 15 | 329 | 62 |
| *T. equinum* CBS 112188 | 204 | 256 | 265 | 242 | 33 | 14 | 116 | 486 | 483 | 523 | 497 | 22 | 4 | 325 | 48 |
| *T. equinum* CBS 112193 | 184 | 225 | 177 | 195 | 26 | 13 | 103 | 368 | 434 | 350 | 384 | 44 | 12 | 266 | 73 |
| *T. equinum* CBS 112198 | 242 | 145 | 243 | 210 | 56 | 27 | 91 | 420 | 387 | 421 | 409 | 19 | 5 | 300 | 29 |
| *T. interdigitale* CBS 119447 | 395 | 387 | 450 | 411 | 34 | 8 | 259 | 683 | 612 | 697 | 664 | 46 | 7 | 474 | 71 |
| *T. interdigitale* CBS 120318 | 305 | 304 | 292 | 300 | 7 | 2 | 165 | 605 | 568 | 497 | 557 | 55 | 10 | 369 | 54 |

*Variation between shared masses between all replicates for ProSight/ProteinCenter data processing, with results from different E value/homology level cutoff criteria (see Materials and Methods).*
of a second reference strain assigned only CBS 318.31 and CBS 285.30 to *T. tonsurans*, while the other 10 strains were assigned to *T. equinum* (exception in one call for CBS 109033; Table S2).

A second analysis was performed with strains in each species with the highest number of shared masses. The references for *T. rubrum*, CBS 202.88 and CBS 118892, identified correctly the remaining *T. rubrum* strains, while in *T. violaceum* addition of a second reference strain, CBS 452.61, to CBS 120316 did not improve the outcome. Species identifications for all strains primarily identified as *T. equinum* resulted consistently in *T. equinum* but never *T. tonsurans*. Strains CBS 318.31 and CBS 285.30 were consistently assigned as *T. tonsurans*. The *T. interdigitale* strains matched each other in the second analysis.

Selection of unique protein markers. Unique masses selected by one of the two or both algorithms, A1 and ProSightPC 3.0, are given in Table 5. Homologous proteins in other dermatophytes were matched using 80% for global and 100% for stringent sequence similarity filtering. In the *T. rubrum* group, both algorithms identified two out of six unique monoisotopic masses. Mass 6,391.358 was identified as hypothetical protein H100_08464 from *Trichophyton rubrum* MR850 (UniProt entry A0A022T914). Homology search in ProteinCenter revealed this protein in *T. equinum*, *T. interdigitale*, and *Microsporum gypseum* (*Nannizzia gypsea*), with a monoisotopic mass of 6,419.364 and one amino acid substitution. A second mass of 13,490.093 was identified as V-type ATPase G subunit from *Trichophyton rubrum* CBS 100081 (UniProt entry A0A022VE64). Homologs were found in *T. interdigitale* and *T. tonsurans* with monoisotopic masses of 13,476.077 and 13,480.072, respectively. Four other protein masses identified with ProSightPC 3.0 only were 7,553.051, identified as 40S ribosomal protein S28 from *Trichophyton rubrum* CBS 100081 (UniProt entry A0A022UXP6), 7,869.165, identified as hypothetical protein H106_04186 from *Trichophyton rubrum* CBS 735.88 (UniProt entry A0A028JNY6), 10,476.424, identified as hypothetical protein H102_06602 from *Tricho-

| Trichophyton strain | CA (A2) | Unique masses per strain (A1) |
|---------------------|---------|------------------------------|
| *T. rubrum* CBS 115314 | 1 | (1) 22,241.054 |
| *T. rubrum* CBS 100084 (T) | 1 | (4) 18,812.048, 7,306.868, 7,384.616, 7,983.656 |
| *T. rubrum* CBS 100238 | 1 | (6) 7,480.315, 7,975.984, 22,046.173, 6,201.287, 21,707.120, 7,309.791 |
| *T. rubrum* CBS 288.86 | 1 | (8) 16,934.943, 9,368.722, 7,881.010, 11,873.910, 18,185.271, 7,779.975, 19,733.724, 11,861.896 |
| *T. rubrum* CBS 202.88 | 1 | (3) 5,437.895, 8,037.319, 5,227.340 |
| *T. rubrum* CBS 118892 | 1 | (3) 5,209.149, 12,397.609, 5,338.210 |
| *T. violaceum* CBS 120320 | 1 | (8) 9,852.837, 13,331.557, 5,301.844, 9,865.257, 5,073.543, 7,874.989, 10,851.584, 7,281.114 |
| *T. violaceum* CBS 120316 | 1 | (7) 6,067.292, 6,198.456, 7,139.672, 19,264.358, 5,146.600, 5,251.738, 12,669.770 |
| *T. violaceum* CBS 201.88 | 1 | (6) 6,660.335, 8,404.081, 9,832.479, 7,433.682, 9,363.639, 12,924.041 |
| *T. violaceum* CBS 452.61 | 1 | (7) 5,522.724, 12,042.842, 6,582.882, 6,500.904, 7,781.364, 20794.42548, 12339.29146 |
| *T. tonsurans* CBS 285.30 (T) | 1 | (11) 11,388.283, 10,124.535, 23,933.015, 19,316.943, 9,897.097, 13,636.961, 5,183.505, 10,010.170, 15,833.425, 8,114.057, 20915.14524 |
| *T. tonsurans* CBS 318.31 (T) | 1 | (5) 6,868.244, 8,272.557, 5,271.958, 7,306.539, 5,723.449 |
| *T. tonsurans* CBS 856.71 | 1 | (17) 5,169.601, 5,250.649, 5,918.855, 5,934.868, 6,686.769, 6,702.530, 7,005.085, 7,808.024, 8,007.802, 8,148.177, 8,199.929, 8,560.452, 9,659.657, 9,717.965, 13,008.799, 17,112.085 |
| *T. tonsurans* CBS 127.97 | 0.42 | (1) 9,945.362 |
| *T. tonsurans* CBS 109033 | 0.42 | |
| *T. tonsurans* CBS 112186 | 0.92 | |
| *T. equinum* CBS 270.66 (NT) | 0.42 | |
| *T. equinum* CBS 634.82 | 0.83 | |
| *T. equinum* CBS 100080 (T) | 1 | (11) 11,196.118, 6,496.162, 14,082.605, 5,679.384, 7,750.926, 14,327.736, 6,955.962, 13,134.323, 7,118.017, 6,398.192, 6,105.138 |
| *T. equinum* CBS 112188 | 0.67 | |
| *T. equinum* CBS 112193 | 0.75 | |
| *T. equinum* CBS 112198 | 0.67 | |
| *T. interdigitale* CBS 119447 | 1 | (16) 9,770.501, 9,659.784, 9,282.621, 5,373.858, 17,020.294, 11,145.854, 28,820.684, 10,022.215, 21,168.266, 21,271.700, 11,174.914, 19,542.778, 19,529.736, 21,354.18, 9,908.134, 7,323.754 |
| *T. interdigitale* CBS 120318 | 1 | (7) 5,065.410, 5,091.356, 5,316.956, 6,488.034, 9,175.903, 13,247.418, 19,206.537 |

*a*, type; *NT*, neotype.

CA, coefficient of accuracy.

The values in parentheses are the numbers of unique masses per strain (in Da).
phyton rubrum CBS 100081 (UniProt entry A0A022UY55), and 10,974.876, identified as hypothetical protein H107_03773 from Trichophyton rubrum CBS 202.88 (UniProt entry A0A023AI34).

In the *T. tonsurans* group (with *T. interdigitale* as species parameter), two unique masses were found and identified. One was a hypothetical protein, TEQG_02912, from *Trichophyton equinum* CBS 127.97 (UniProt entry F2PPR0) with a mass of 7,883.18. This protein differs from its counterpart in the *T. rubrum* group (7,869.165) by one amino acid. The second one was hypothetical protein TEQG_00161, with a mass of 10,450.372.

**TABLE 4** Species prediction of individual *Trichophyton* strains and the references employed

| Trichophyton strains | Analysis 1 | Analysis 2 |
|----------------------|------------|------------|
|                      | Reference 1 | Reference 1 |
| **T. rubrum** CBS 115314 | Tr Tr Tr | Tr Tr Tr |
| **T. rubrum** CBS 100081 (T) | Reference | Reference |
| **T. rubrum** CBS 1000238 | Tr Tv Tr | Tr Tr Tr |
| **T. rubrum** CBS 288.86 | Tr Tv Tr | Tr Tv Tr |
| **T. rubrum** CBS 202.88 | Tr Tv Tr | Tr Tr Tr |
| **T. rubrum** CBS 118892 | Tr Tv Tr | Tr Tr Tr |
| **T. violaceum** CBS 120320 | Reference | Reference |
| **T. violaceum** CBS 120316 | Tr Tr Tr | Tr Tr Tr |
| **T. violaceum** CBS 201 | Tr Tr | X Tv Tr |
| **T. violaceum** CBS 452.61 | Tr Tr Tr | Tr Tr Tr |
| **T. tonsurans** CBS 285.30 (T) | Reference | Reference |
| **T. tonsurans** CBS 318.31 (T) | Tt Tt | Reference |
| **T. onsurans** CBS 856.71 | Te Te Te | Te Te Te |
| **T. tonsurans** CBS 127.97 | Te Te Te | Te Te Te |
| **T. tonsurans** CBS 109033 | Te Tt Tt | Te Tt Tt |
| **T. tonsurans** CBS 112186 | Tt Tt Te | Te Te Te |
| **T. equinum** CBS 270.66 (NT) | Reference | Reference |
| **T. equinum** CBS 634.82 | Te Te Te | Te Te Te |
| **T. equinum** CBS 100080 (T) | Reference | Reference |
| **T. equinum** CBS 112188 | Tt Tt Te | Te Te Te |
| **T. equinum** CBS 112193 | Tt Tt Tt | Te Te Te |
| **T. interdigitale** CBS 119447 | Te Te Te | Reference |
| **T. interdigitale** CBS 120318 | Tt Tt Tt | Ti Ti Ti |

*References are individual mass spectra used relative to the taxon name identified by ITS sequencing. For analysis 1, types, neotypes, and/or randomly chosen strains were used as reference strains. For analysis 2, central strains with the highest number of shared masses (clustering analysis) were used as reference strains. T, type; NT, neotype; Tr, *T. rubrum*; Tv, *T. violaceum*; Tt, *T. tonsurans*; Te, *T. equinum*; Ti, *T. interdigitale*; X, no call. Colors visualize taxa.*

**TABLE 5** Unique masses per group and per species

| Group/species | Unique masses* |
|---------------|---------------|
| *T. rubrum*/*T. violaceum* | (6) 6,391.358*, 13,490.093*, 7,553.051, 7,869.165, 10,476.424, 10,974.876 |
| *T. tonsurans*/*T. equinum*/*T. interdigitale* | (2) 7,883.18*, 10,450.732* |
| *T. tonsurans*/*T. equinum* | (3) 7,902.81*, 8,787.278, 11,464.894 |
| *T. equinum*/*T. interdigitale* | (3) 12,993.799, 15,859.410, 15,888.449 |
| *T. interdigitale* | (5) 6,961.324, 9,014.567, 9,254.624, 9,481.539, 15,829.423 |
| *T. tonsurans* | (2) 13,152.935, 17,474.951* |

*Unique masses were identified by proprietary algorithm 1 and/or ProSightPC 3.0. Proteins marked with an asterisk were identified by both algorithms. The values in parentheses are the numbers of unique masses per strain (in Da).
found in *T. equinum* CBS 127.97 (UniProt entry F2PGT9), differing from the *T. rubrum* protein (mass of 10,476.424) by having one extra amino acid.

The *T. tonsurans* group (without *T. interdigitale*) had three unique masses, of which both algorithms identified mass 7,906.81 as hypothetical protein TEQG_01010 from *Trichophyton equinum* CBS 127.97 (UniProt entry F2PJA2). The two other monoisotopic masses, 8,787.278 and 11,464.894, could not be identified by ProSightPC 3.0. *Trichophyton equinum* and *T. interdigitale* share three masses, 12,993.799, 15,859.411, and 15,888.449. There were no entries for these masses in ProSightPC 3.0. In contrast, *T. tonsurans* did not have masses in common with *T. interdigitale*, implying that *T. interdigitale* likely is closer phylogenetically to *T. equinum* than to *T. tonsurans*.

At the species level, no unique masses were found defining *T. rubrum*, *T. violaceum*, *T. equinum*, or *T. tonsurans*. In contrast, *T. interdigitale* had five unique masses (6,961.324, 9,014.567, 9,254.624, 9,481.539, and 15,829.423), none of which could be matched to any protein sequence predicted from the corresponding genomes. For *T. tonsurans*, as derived from the second species prediction approach, the two strains CBS 318.31 and CBS 285.30 affiliated with *T. tonsurans* share two unique masses: 13,152.935, found by algorithm A1, and 17,474.951, found by both algorithms, identified as hypothetical protein TESG_03051 from *T. tonsurans* CBS 112818 (UniProt entry F2RWA2).

**DISCUSSION**

In this study, we evaluated the resolution power of LC-MS/MS as a novel method to delimit clinically relevant filamentous fungi with two groups of dermatophytes, each containing two very closely related species as a model set. Nucleic acid-based approaches, like rDNA ITS sequence data, are used as a gold standard, as this gene is judged to be optimal for dermatophyte diagnostics (3).

Separation of species within both groups is problematic and highly controversial. On the basis of molecular data, *Trichophyton tonsurans* (on humans) and *T. equinum* (on horses) had been regarded as synonyms (15). Matruchot and Dassonville (18) already reported transmission from horse to human in their original description of *T. equinum*. In the present study, two of the analyzed strains were used that had been transmitted from horse to human: CBS 127.97 (19) and CBS 270.66 (20). Woodgyer (21) distinguished the species by a T/C SNP in ITS1 (C nucleotide in *T. tonsurans*, T nucleotide in *T. equinum*), and Chollet et al. (22) listed some phenotypic differences. We were unable to find correspondence between these criteria among our strains using the intact protein-based approach described here. All but one strain (CBS 318.31) listed as *T. tonsurans* had the *T. equinum*-associated T nucleotide, which was also present in all six *T. equinum* strains. To verify the validity of this SNP in a larger data set, we randomly selected 64 strains from the CBS collection (data not shown). All *T. equinum* strains from horse had a T nucleotide, but 35% of the *T. tonsurans* strains from humans had the same T nucleotide (data not shown). De Hoog et al. (3) were also unable to distinguish the two species using additional genes. More detailed patient and phenotypic information is necessary to establish whether *T. equinum* is a separate species at all. Using MALDI-TOF, Nenoff et al. (8) and De Respinis et al. (4) distinguished *T. tonsurans* and *T. equinum*, but the authors did not present the grounds on which they denominated the strains as *T. tonsurans* or *T. equinum*. In our study, discrimination at strain level resulted in identification of 4 out of 12 strains of the *T. tonsurans* lineage. The remaining eight strains formed two clusters with overlapping proteomes: cluster 1 with CBS 642.82, CBS 127.97, and CBS 109033, and cluster 2 with two subclusters, CBS 270.66 (overlapping with 112188), CBS 112198 (overlapping with 112186), and CBS 112193 (see Table S2 in the supplemental material). In Fig. 1, these two clusters would be placed in the intersection of the two species clouds. Note that both clusters have strains isolated from both humans and horses, which contradicts the hypothesis of host-based distinction in the two species. In the species prediction analysis (Table 4), only CBS 318.31 and CBS 285.30 were affiliated with *T. tonsurans*, which is not in concordance with a T/C SNP for CBS 285.30. So far, typing strains within the *T. tonsurans/T. equinum* species complex...
appears to be challenging (pending method improvements) due to insufficiently resolved taxonomic definitions of known reference strains, which is likely due to conspecificity.

In MALDI-TOF analyses of De Respinis et al. (4), some T. tonsurans spectra were misidentified as T. interdigitale. Calderaro et al. (23) noted the same misidentification before the supplementation to Bruker’s BioTyper database. With nine nucleotides of difference in the ITS region, T. interdigitale should be easily distinguishable from the T. tonsurans/T. equinum complex. Separation was confirmed in all our analyses, with strain classification accuracies of 1, five unique species masses (Table 5), and their clustering as a distinct group in species prediction analysis (Table 4).

Analyses of the T. rubrum group were in concordance with previous findings. According to Gräser et al. (14), the T. rubrum group comprises only two anthropophilic species, T. rubrum and T. violaceum, the latter species with T. soudanense as a probable mutant and prevalently causing tinea capitis. Trichophyton violaceum is endemic to Africa (14, 24), while T. rubrum is cosmopolitan. Microsatellite analysis has revealed that T. violaceum is more variable than T. rubrum, with some strains being closer to T. rubrum than the others (25). Trichophyton rubrum and T. violaceum are morphologically very different but are similar in their DNA profiles. Our analyzed strains differed in four positions in ITS (data not shown). MALDI-TOF analyses frequently proved to be unable to separate the two species (4, 7, 8, 11). Summarized misidentifications and/or unreliable identifications of T. tonsurans (misidentified with T. rubrum and vice versa), T. violaceum, and T. soudanense were recently reported by Sanguinetti and Posteraro (26).

In the newest evaluation study of the Vitek v3.0 system for the identification of filamentous fungi (27), Trichophyton species were regarded as particularly problematic, with T. interdigitale, T. tonsurans, and T. violaceum having success percentages of 97%, 91%, and 41%, respectively, in the first attempt.

With LC-MS/MS, discrimination at the strain level was achieved with all six T. rubrum and four T. violaceum strains classified with a classification analysis of 1. In this analysis, optimal species association was achieved with CBS 118892 as the reference for T. rubrum and CBS 120316 as the reference for T. violaceum. Notably, taxonomic types may be located eccentrically in the species cloud and thus provide less optimal results. Our analysis showed that T. violaceum strain CBS 120320 shares some features with T. rubrum (with one call as T. rubrum) (Table 4). Both strains denominated in the CBS collection as T. soudanense, CBS 452.61 and CBS 201.88, were affiliated with T. violaceum, fitting the ITS data. Interestingly, the only strain with a 36-bp deletion, CBS 201.88, had one nonsense and one T. violaceum call in the first species prediction analysis (Table 4).

Conclusions. Whole-protein top-down LC-MS/MS analysis has significant diagnostic potential because of its analytical performance level being higher than that of MALDI-TOF, particularly below the species level, i.e., at the lineage or strain level. The accurate detection of protein masses, separation of high numbers of individual proteins, and detection of single-amino-acid exchanges are responsible for the high performance. The proprietary Thermo Scientific algorithms A2, A3, and A4 showed a potential to recognize individual strains that can be applied in epidemics or outbreak scenarios. However, detailed studies are required, since the choice of reference strains is crucial for appropriate species affiliation, as routine selection of taxonomic types may not provide optimal results. Species limits and species variability in dermatophytes, which were classically distinguished on the basis of clinical and phenotypic criteria, have to be newly defined in order to develop reliable and predictive taxonomy and meaningful diagnostics tools.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JCM.00102-18.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.
SUPPLEMENTAL FILE 2, PDF file, 0.1 MB.
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