Usefulness of matrix-assisted laser desorption ionisation–time-of-flight mass spectrometry for identifying clinical *Trichosporon* isolates

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Abstract

Trichosporon spp. have recently emerged as significant human pathogens. Identification of these species is important, both for epidemiological purposes and for therapeutic management, but conventional identification based on biochemical traits is hindered by the lack of updates to the species databases provided by the different commercial systems. In this study, 93 strains, or isolates, belonging to 16 *Trichosporon* species were subjected to both molecular identification using IGS1 gene sequencing and matrix-assisted laser desorption ionisation–time-of-flight (MALDI–TOF) analysis. Our results confirmed the limits of biochemical systems for identifying *Trichosporon* species, because only 27 (36%) of the isolates were correctly identified using them. Different protein extraction procedures were evaluated, revealing that incubation for 30 min with 70% formic acid yields the spectra with the highest scores. Among the six different reference spectra databases that were tested, a specific one composed of 18 reference strains plus seven clinical isolates allowed the correct identification of 67 of the 68 clinical isolates (98.5%). Although until recently it has been less widely applied to the basidiomycetous fungi, MALDI–TOF appears to be a valuable tool for identifying clinical *Trichosporon* isolates at the species level.

Keywords: Identification, main spectrum database, MALDI–TOF mass spectrometry, protein extraction procedures, *Trichosporon*
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Introduction

Trichosporon spp. are well recognized as causative agents of white piedra, a benign cutaneous infection characterized by the presence of irregular nodules in hairy regions [1]. However, more recently, the *Trichosporon* genus has emerged as a life-threatening opportunistic pathogen responsible for invasive infections in immunosuppressed patients [2–5]. Disseminated infection is their most common clinical picture [6,7]. Their reported mortality rates range between 50% and 80%, eventually reaching 100% in onco-haematological patients with persistent neutropenia [2,3,8].

To date, at least 17 different species have been considered as aetiological agents of *Trichosporon* infections [8–10]. Species identification is important for epidemiological purposes and to better define species-specific clinical associations [11,12]. In addition, certain *Trichosporon* species may be more resistant to antifungal drugs [8]. However, identifying *Trichosporon* species may be difficult because the databases based on commercial biochemical systems lack some of the newly described species.

Even the target to use for molecular identification is the subject of debate. Indeed, analysis of the ITS1 region and the D1/D2 domain of the large subunit of rDNA can fail in the identification of *Trichosporon* species, because these sequences may only represent 1% of the nucleotide divergence of closely related species [13–15]. However, Sugita *et al.* [14] have shown that IGS1 sequencing allows closely related species to be distinguished and should be considered as the method of choice for accurately identifying *Trichosporon* species.

Matrix-assisted laser desorption ionisation–time-of-flight (MALDI–TOF) mass spectrometry (MS) has recently emerged as a powerful tool for identifying yeast species and some of the species of filamentous fungi from the Ascomycota phylum [16–18]. To the best of our knowledge, a single study has focused on *Trichosporon* identification using MS [19]. This study, however, included IGS1 sequencing analysis for only a limited number of strains [19].

In the present study, we used a large panel of well-characterized strains and clinical isolates belonging to the main pathogenic *Trichosporon* species to evaluate the performance of MALDI–TOF for identifying clinical *Trichosporon* isolates.

Materials and Methods

Strains

Ninety-three *Trichosporon* strains were included (Table 1). Nineteen of these were reference strains from the CBS collection (http://www.cbs.knaw.nl/collections/Biolomics.aspx?Table=Yeasts%202011). Seventy-four were clinical isolates collected between 2004 and 2012, which were recovered from different body sites (blood, urine, stools, skin and respiratory tract) and maintained as frozen stocks at −80°C in yeast-extract peptone dextrose medium. They belong to the collections of large university hospitals in São Paulo, Brazil, and Paris, Tours, Reims, Bordeaux and Nantes in France. Their initial identification was based on their phenotypic traits (arthrospores and positive urea test) and biochemical profile (obtained using either the 20AUX/ID32C panels or the VITEK2 automated system) (Biomérieux, Marcy l'Etoile, France) and/or the sequences of the internal transcribed spacer 1 (ITS1) region or intergenic spacer 1 region of their rDNA (IGS1) [14,20].

Matrix-assisted laser desorption ionisation–time-of-flight mass spectrometry identification

Mass spectra were acquired using a Microflex LT MALDI–TOF mass spectrometer (Bruker, Bremen, Germany). The post-analytical procedures were conducted using Biotyper software and its associated database version 3.0 (Bruker). Identification

TABLE 1. Strains utilized in this study according to species, phenotypic and genotypic identification, country and strain name.

Strain name	Country	Phenotypic identification	Molecular identification	MS identification
DLC4	Brazil	*Trichosporon asahii*[a]	*T. asahii*[f]	*T. asahii*
DLC7[g]	Brazil	*T. asahii*[a]	*T. asahii*[f]	*T. asahii*
DLC9	Brazil	*T. asahii*[a]	*T. asahii*[f]	*T. asahii*
DLC12	Brazil	*T. asahii*[a]	*T. asahii*[f]	*T. asahii*
DLC14	Brazil	*T. asahii*[a]	*T. asahii*[f]	*T. asahii*
IAL2	Brazil	*T. asahii*[a]	*T. asahii*[f]	*T. asahii*
IAL5	Brazil	*T. asahii*[a]	*T. asahii*[f]	*T. asahii*
IAL6	Brazil	*T. asahii*[a]	*T. asahii*[f]	*T. asahii*
IAL7	Brazil	*T. asahii*[a]	*T. asahii*[f]	*T. asahii*
IAL11	Brazil	*T. asahii*[a]	*T. asahii*[f]	*T. asahii*
IAL12	Brazil	*T. asahii*[a]	*T. asahii*[f]	*T. asahii*
F2201	France	*T. asahii*[a]	*T. asahii*[f]	*T. asahii*
FT01	France	*T. asahii*[b]	ND	*T. asahii*
FT02	France	*T. asahii*[b]	ND	*T. asahii*
FHD01	France	*T. asahii*[c]	ND	*T. asahii*
FKBCT01	France	*T. asahii*[c]	ND	*T. asahii*
FSA02	France	*T. asahii*[c]	ND	*T. asahii*
FNA01	France	*T. asahii*[a]	ND	*T. asahii*
FNA02	France	*T. asahii*[a]	ND	*T. asahii*
FNA03	France	*T. asahii*[a]	ND	*T. asahii*
FNA04	France	*T. asahii*[a]	ND	*T. asahii*
FNA05	France	*T. asahii*[d]	ND	*T. asahii*
CBS8972[g]	Israel	NA	*T. asahii*[f]	*T. asahii*
CBS7631[g]	France	NA	*T. asahii*[f]	*T. asahii*
CBS2479	Japan	NA	*T. asahii*[f]	*T. asahii*
DLC2	Brazil	*T. asahii*[a]	*Trichosporon inkin*[f]	*T. inkin*
DLC3	Brazil	*T. asahii*[a]	*T. inkin*[f]	*T. inkin*
DLC6	Brazil	*T. asahii*[a]	*T. inkin*[f]	*T. inkin*
DLC11[g]	Brazil	*T. asahii*[a]	*T. inkin*[f]	*T. inkin*
DLC15	Brazil	*T. asahii*[a]	*T. inkin*[f]	*T. inkin*
DLC16	Brazil	*T. asahii*[a]	*T. inkin*[f]	*T. inkin*
F0302	France	*T. asahii*[a]	*T. inkin*[f]	*T. inkin*
F2501	France	*T. inkin*[b]	*T. inkin*[f]	*T. inkin*
F1001	France	*T. inkin*[b]	*T. inkin*[f]	*T. inkin*
FR03	France	*T. inkin*[c]	ND	*T. inkin*
FR05	France	*T. inkin*[c]	*T. inkin*[f]	*T. inkin*
FR13	France	*Trichosporon* sp.[d]	*T. inkin*[f]	*T. inkin*
FNA06	France	*T. inkin*[c]	*T. inkin*[e]	*T. inkin*
FNA07	France	*T. inkin*[c]	*T. inkin*[f]	*T. inkin*
CBS7630[g]	United Kingdom	NA	*T. inkin*[f]	*T. inkin*
DLC10	Brazil	*Trichosporon mucoides*[a]	*Trichosporon dermatis*[f]	*T. dermatis*
F2101	France	*T. mucoides*[a]	*T. dermatis*[f]	*T. dermatis*
F1601	France	*T. mucoides*[a]	*T. dermatis*[f]	*T. dermatis*
F2401	France	*T. mucoides*[a]	*T. dermatis*[f]	*T. dermatis*
F2301	France	*T. mucoides*[a]	*T. dermatis*[f]	*T. dermatis*
F1801	France	*T. mucoides*[a]	*T. dermatis*[f]	*T. dermatis*
F1301	France	*T. mucoides*[a]	*T. dermatis*[f]	*T. dermatis*
F0801[g]	France	*T. mucoides*[a]	*T. dermatis*[f]	*T. dermatis*
F1401	France	*T. mucoides*[a]	*T. dermatis*[f]	*T. dermatis*
F2801	France	*T. mucoides*[a]	*T. dermatis*[f]	*T. dermatis*
FR02	France	*T. mucoides*[c]	*T. dermatis*[f]	*T. dermatis*
FR04	France	*T. mucoides*[c]	*T. dermatis*[f]	*T. dermatis*
FR06	France	*T. mucoides*[c]	*T. dermatis*[f]	*T. dermatis*
FR11	France	*T. mucoides*[c]	*T. dermatis*[f]	*T. dermatis*
FR14	France	*T. mucoides*[c]	*T. dermatis*[f]	*T. dermatis*
FR15	France	*Trichosporon* sp.[d]	*T. dermatis*[f]	*T. dermatis*
FR16	France	*T. mucoides*[c]	*T. dermatis*[f]	*T. dermatis*
FR21	France	*T. mucoides*[c]	*T. dermatis*[f]	*T. dermatis*
FR23	France	*T. mucoides*[c]	*T. dermatis*[f]	*T. dermatis*
FR25	France	*T. mucoides*[c]	*T. dermatis*[f]	*T. dermatis*
FR26	France	*T. mucoides*[c]	*T. dermatis*[f]	*T. dermatis*
FT03	France	*T. mucoides*[b]	*T. dermatis*[f]	*T. dermatis*
CBS10495[g]	Argentine	NA	*T. dermatis*[f]	*T. dermatis*
CBS2043[g]	Germany	NA	*T. dermatis*[f]	*T. dermatis*
F1901	France	*T. asahii*[a]	*Trichosporon faecale*[f]	*T. faecale*
FSA03	France	*T. asahii*[b]	*T. faecale*[f]	*T. faecale*
IAL03[g]	Brazil	*T. asahii*[a]	*T. faecale*[f]	*T. faecale*
IAL04	Brazil	*T. inkin*[a]	*T. faecale*[f]	*T. faecale*
CBS4828[g]	Brazil	NA	*T. faecale*[f]	*T. faecale*
F2701[g]	France	*Trichosporon* sp.[d]	*Trichosporon jirovecii*[f]	*T. jirovecii*
F2702	France	*Trichosporon* sp.[d]	*T. jirovecii*[f]	*T. jirovecii*

Table 1 (*Continued*)

Strain name	Country	Phenotypic identification	Molecular identification	MS identification
F2601	France	*Trichosporon* sp.[d]	*T. jirovecii*[f]	*T. jirovecii*
FSA01	France	*Trichosporon* sp.[d]	*T. jirovecii*[f]	*T. jirovecii*
CBS6864[g]	Czech Republic	NA	*T. jirovecii*[f]	*T. jirovecii*
F0501[g]	France	*T. inkin*[a]	*Trichosporon montevideense*[f]	*T. montevideense*
F0701	France	*T. inkin*[a]	*T. montevideense*[f]	*T. montevideense*
FR01	France	*T. asahii*[c]	ND	*T. montevideense*
CBS8605[g]	Netherlands	NA	*T. montevideense*[f]	*T. montevideense*
FR29[g]	France	*T. asahii*[c]	*Trichosporon coremiiforme*[f]	*T. coremiiforme*
FR31	France	*Trichosporon* sp.[d]	ND	*T. coremiiforme*
CBS2482[g]	Costa Rica	NA	*T. coremiiforme*[f]	*T. coremiiforme*
FR12	France	*T. mucoides*[c]	*T. mucoides*[f]	*T. mucoides*
CBS7625[g]	Belgium	NA	*T. mucoides*[f]	*T. mucoides*
FR07	France	*T. inkin*[c]	ND	*Trichosporon lactis*
CBS9051[g]	Austria	NA	*T. lactis*[f]	*T. lactis*
FBOR01	France	*Trichosporon* sp.[d]	*Trichosporon loubieri*[e]	*T. loubieri*
CBS7719[g]	Australia	NA	*T. loubieri*[f]	*T. loubieri*
CBS7556[g]	Italy	NA	*Trichosporon ovoides*[f]	*T. ovoides*
CBS2466[g]	France	NA	*Trichosporon cutaneum*[f]	*T. cutaneum*
CBS8280[g]	Japan	NA	*Trichosporon domesticum*[f]	*T. domesticum*
CBS8641[g]	Japan	NA	*Trichosporon japonicum*[f]	*T. japonicum*
CBS9756[g]	Germany	NA	*Trichosporon mycotoxinovorans*[f]	*T. mycotoxinovorans*
CBS2481[g]	Switzerland	NA	*Trichosporon asteroides*[f]	*T. asteroides*

[a]Identification using the VITEK2 system (Biomérieux, Marnes-la-Coquette, France).
[b]Identification using the API 20 AUX system (Biomérieux).
[c]Identification using the API 32 ID system (Biomérieux).
[d]Identification based on arthrospores and positive urease test.
[e]Identification using ITS1 rDNA sequencing.
[f]Identification using IGS1 rDNA sequencing.
[g]Strains included in main spectrum databases.

compatibility relies upon comparing a given spectrum against a main spectra (MSP) database, and its value is expressed as a logscore (LS). LS values vary between 0 and 3, with 0 indicating no relationship and 3 indicating identical spectrum profiles. According to the manufacturer's recommendations, LS values between 1.7 and 2 are acceptable for genus identification, and an LS value ≥2.0 is required for accurate species identification.

Protein extraction protocols

Because the spectra that were generated using intact cells exhibited only a limited number of peaks (data not shown), we first compared three different protein extraction protocols to determine which led to spectra with the highest logscores. The isolates were slant-subcultured on Sabouraud dextrose agar containing chloramphenicol and gentamicin (BioRad, Marnes-la-Coquette, France) for 48 h at 30°C. All three of the tested protocols relied on the extraction efficacy of ethanol 70% and formic acid 70% (FA) plus acetonitrile 100% (ACN) for the colonies harvested from the slant cultures. The first protocol followed the manufacturer's recommendations for yeast identification. The second and third protocols were recently published by Cassagne *et al.* [16] and Sendid *et al.* [21], respectively. They differed in the time of the initial centrifugation, the time of incubation, the volumes of FA and ACN that were used and the volume of the final deposit on the slide (Table S1).

To compare the performances of the protocols, three MSP libraries (one for each extraction procedure) were built. Each library contained five MSPs, one for each of the following strains: *Trichosporon dermatis* F0801, *Trichosporon montevideense* CBS8605, *Trichosporon inkin* CBS7630, *T. dermatis* CBS2043 and *Trichosporon asahii* CBS8972. Each MSP was created from 20 spectra that were obtained from four slant cultures (five extractions per culture) using Biotyper software 3.0 with the MSP standard method parameters. Each of the 20 raw spectra composing an MSP was submitted for MS identification and the best-matched LS values were calculated, generating 100 LS values per extraction protocol, which were then compared.

Comparison of different databases for identifying *Trichosporon* isolates

The BioTyper vs. 3.0 database (database 1) contains 11 MSPs, which represent six different species. To enlarge this spectrum of species, we created MSPs for 18 CBS reference strains and seven clinical isolates of 16 species that had been identified through the sequences of the IGS1 region (Table S2). Five additional databases encompassing the MSPs of the reference strains (database 2), combinations of databases 1 and 2, and the MSPs of the clinical isolates were evaluated for the identification of the remaining 68 strains.

Protein extracts of the 68 strains that were not included in the MSP database were tested in quadruplicate. For qualitative analysis (i.e. correct or incorrect identification at the genus and species levels) only the spot returning the highest LS was considered. For the 51 strains for which IGS1 rDNA region was sequenced, the spectrometric identification was considered correct if the results of both methods were congruent. For the remaining 17 strains, the spectrometric identification was considered correct if the BioTyper analysis returned an LS of greater than 2 for only one species.

Statistical analysis

The statistical analyses were performed using Prism software, version 6.0 (GraphPad Software, La Jolla, CA, USA). The LSs obtained using each extraction protocol were compared using the non-parametric rank sum Kruskal–Wallis test. In the case of a significant difference, a *post-hoc* analysis was conducted using pairwise matched Dunn's tests with adjustment for multiple comparisons. In every case, two-sided tests were utilized and a p value of <0.05 was considered significant.

Results

Performance of biochemical-based identification

Phenotypic identification led to a high number of mis- or non-identifications (Table 1). All isolates of *T. dermatis* were misidentified as *T. mucoides*. In addition, half (seven of 14) of the *T. inkin* isolates were misidentified as *T. asahii*. Misidentification as *T. asahii* or *T. inkin* was also noted for *Trichosporon faecale* (n = 4), *T. montevideense* (n = 3), *Trichosporon coremiiforme* (n = 1) and *Trichosporon lactis* (n = 1). Finally, all of the *Trichosporon jirovecii* isolates (n = 4) were not identified at the species level but had only a genus characterization.

Protein extraction protocol

Three different protein extraction protocols were compared for their ability to generate high LSs (Fig. 1). A significant difference in the performance of these protocols (p 0.014) was observed. A *post hoc* analysis revealed that protocol 3 performed better than protocol 1 (p 0.012) but not protocol 2 (p 0.86) and that protocol 1 and protocol 2 performed similarly (p 0.2). Because protocol 3 provided more homogeneous results than the other methods, it was selected for further experiments.

Comparison of the main spectra databases for species identification

The performances of the six databases that were tested for *Trichosporon* species identification are summarized in Fig. 2. The Biotyper 3.0 database (database 1) returned the lowest level of correct identification at the genus (85.1%) and the species (31.3%) levels. In contrast, 100% identification at the genus level and 98.5% identification at species level were achieved using database 5, which contained 25 MSPs of clinical and CBS strains. The rate of identification with an LS of >2.0 ranged from 28.36% (database 1) to 91.04% (database 5).

Analysis using the BioTyper 3.0 database revealed misidentifications (LS >1.7) for isolates of six species that are not represented in the database (Table 2). This was particularly the case for *T. dermatis* isolates (n = 21). In addition, ten *T. asahii* isolates were identified as *T. cutaneum*. To dissect these discrepancies, cross-identification of the MSPs in the Biotyper 3.0 database with those derived for the CBS strains and the clinical isolates showed that *T. cutaneum* BioTyper MSP(s) were identified as that/those of *T. asahii,* whereas the *Trichosporon ovoides* BioTyper MSP was recognized as that of

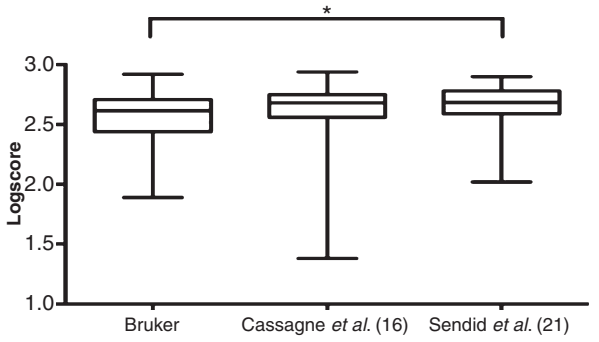

FIG. 1. Box-and-whiskers plot of the logscore values obtained using the three extraction procedures tested in this study. Protocol 1: Bruker's recommended protocol for yeast identification. Protocol 2: described by Cassagne *et al.* [16]. Protocol 3: described by Sendid *et al.* [21]. * Statistically significant difference (p 0.012).

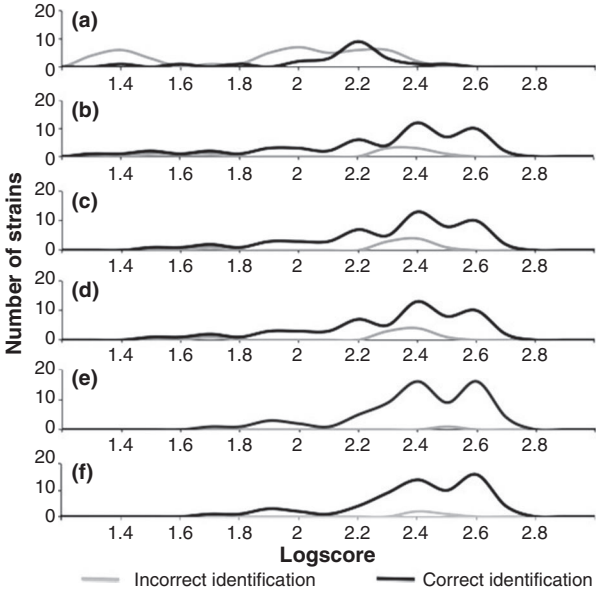

FIG. 2. Distribution of the logscore (LS) values for 68 strains obtained using matrix-assisted laser desorption ionisation–time-of-flight (MALDI–TOF), according to the reference main spectra (MSP) databases that were searched. The dark line represents the best-matched LS values of the correct identification results, whereas the grey line shows the best-matched LS values of the misidentification results. (a) Biotyper 3.0, database 1: several incorrect identifications with LS values below 1.6 (mainly related to the absence of the species in the main spectra (MSP) database) and with LS values above 2.0 (MSPs with incorrect identification); (b) CBS strains, database 2; (c) Biotyper 3.0 and CBS strains, database 3; (d) Biotyper 3.0 and clinical strains, database 4; (e) CBS and clinical strains, database 5; (f) Biotyper 3.0, CBS and clinical strains, database 6. Databases 5 and 6 yielded the best results, but incorrect identifications of *Trichosporon dermatis* as *Trichosporon mucoides* with LS values above 2.0 were noted for one and three isolates, respectively.

TABLE 2. Discordant results with logscore values above 1.7 that were obtained using databases 1 and 2

Database	Spectrometric identification (number)	Logscore range	Molecular identification[a]	MPS present in the database (number)
Database 1 (Biotyper 3.0)	*Trichosporon mucoides* (n = 19)	1.709–2.477	*Trichosporon dermatis*	No
	Trichosporon ovoides (n = 2)	2.04–2.014	*T. dermatis*	No
	Trichosporon cutaneum (n = 10)	2.146–2.338	*Trichosporon asahii*	Yes (2)
	T. mucoides (n = 1)	1.864	*Trichosporon jirovecii*	No
Database 2 (CBS strains)	*T. mucoides* (n = 6)[b]	2.36–2.467	*T. dermatis*	Yes (2)
	T. ovoides (n = 1)	1.754	*Trichosporon inkin*	Yes (1)

[a]As identified using IGS1 sequencing.
[b]One of these strains (FR16) was later identified as *Trichosporon mucoides* using a multilocus approach (Congress of the French Society of Medical Mycology, Dijon, 16–17 May 2013, abstract 15).

T. dermatis. Similarly, the database composed using CBS strains only (database 2) misidentified seven isolates of *T. dermatis* as *T. mucoides* and one *T. inkin* isolate as *T. ovoides*.

Interestingly, misidentifications resulting from using database 2 led us to sequence the IGS1 region of the strain CBS10495, originally described as *T. mucoides* but found to be *T. dermatis* after final identification (GenBank accession number AB066412.1). Finally, one isolate (FR16) that was identified as *T. mucoides* using the MALDI-TOF results to search database 5 (LS = 2.549) had 99% identity with *T. dermatis* according to its IGS1 rDNA sequences (GenBank accession number JF302985.1). However, using a multilocus phylogenetic approach, the strain was found to be closely related to *T. mucoides* (Congress of the French Society of Medical Mycology, Dijon, 16–17 May 2013, abstract 15).

Discussion

Trichosporon spp. have recently emerged as life-threatening opportunistic pathogens [8]. Species identification in this genus that would allow the description of possible new epidemiological traits and would facilitate the difficult decision-making regarding an antifungal strategy has become a challenging issue. Biochemical profiling methods appear clearly insufficient for the reliable identification of *Trichosporon* species, even if, due to the heterogeneous phenotypic methods utilized for the initial characterization, a one to one comparison with the molecular or proteomic identification was not possible. In contrast, MALDI–TOF MS has emerged as a powerful and reliable tool for the identification of fungi [22,23]. Some of these studies included a small number of *Trichosporon* isolates [21,22,24–27]. For example, Stevenson *et al.* [24] included nine species for an in-house database construction, but did not include *T. dermatis*, for which the distinction from *T. mucoides* is the most challenging. More recently, Kolecka *et al.* [19] studied a large collection of arthroconidial yeasts, including *Trichosporon* spp., using MALDI–TOF MS. The overall rate of accurate species identification ranged from 95% (CBS strain collection) to 97% (clinical isolates). As in our study, discrepancies were demonstrated for some of the clinically relevant, closely related species, such as *T. inkin*/*T. ovoides* and *T. mucoides*/*T. dermatis*. The frequency of these discrepancies may have been underestimated in that study because (i) only *T. asahii* strains were subjected to IGS1 sequencing and (ii) only a limited number of *T. mucoides*/*T. dermatis* strains were tested; this distinction appeared in our experience to be the most challenging issue [19]. Thus, a robust database including well-identified *Trichosporon* isolates (IGS1 rDNA sequenced) representing the majority of species that are related to human infections is required to identify the entire spectrum of *Trichosporon* infections.

Although direct deposit of intact cells on the target plate is the simplest method of sample processing, several authors have stated that protein extraction provided better results [21,26,27]. Similarly, the 'smear procedure' described by the manufacturer was not achievable, because some of the isolates produced colonies that were difficult to homogeneously spot onto the plate. In contrast, the extraction protocols previously described [16,21] were shown to be powerful adjuncts for MALDI-TOF analysis of *Trichosporon* species, allowing reliable and reproducible results to be achieved. We believe that the longer exposure to formic acid (30 min) was responsible for the better results obtained using protocol 3, compared with protocol 1. However, in routine practice, the additional incubation period is time consuming, and protocol 2 may be preferred.

Moreover, the reliability of the identification was obviously dependent on the MSP library that was used. The results presented here showed that the manufacturer's MSP library is insufficient to identify several of the *Trichosporon* species of medical importance. This finding reflects this database's lack of MSPs for some of the common species, such as *T. dermatis*, and the inclusion of MSPs for strains with probable erroneous identification. The former case has already been described because some unusual species could be identified only after the original MSP library was upgraded [28,29]. The latter situation led to the misidentification of 52.4% (11 out of 21) of the *T. asahii* strains as *T. cutaneum* when the Bruker

database was used. We are not aware of any molecular data concerning the two strains (120PSB and 95PSB) that were used to generate the Biotyper MSP that is representative of *T. cutaneum*. Stevenson *et al.* [24] also described the erroneous identification of several of the *Trichosporon* reference strains. This was also the case for the CBS 10495 strain, described as *T. mucoides* based on ITS and D1/D2 regions sequencing but identified in this study as *T. dermatis* based on IGS1 sequencing, in accordance with the MS identification.

Considering these different features, we were able to build a specific database that allowed a 98.5% correct identification rate at the species level. The library contained 25 MSPs from either the CBS strains or the clinical isolates that had a definitive molecular-based identification (IGS1 sequencing), representing 16 different species. The recommendation that each species should be represented by several isolates was followed [16,24]. Interestingly, whereas misidentifications of *T. dermatis* as *T. mucoides* using phenotypical or molecular methods have been reported [11,12,15], our optimized database unambiguously identified 21 isolates from both of these species. Moreover, the single discrepancy found in this study between IGS1 sequencing-based analysis, which provided a 99% identity of the isolate as *T. dermatis*, and MALDI-TOF MS-based analysis, which identified it as *T. mucoides*, referred to an isolate that was related to *T. mucoides* using a multilocus analysis. More strains of *T. mucoides* need to be studied to confirm this observation.

Using this database, we found that, consistent with previous studies, *T. asahii* was the species that was most frequently isolated from clinical specimens [12,30]. There are increasing data supporting the existence of *T. asahii* isolates with high minimal inhibitory concentrations for azole derivatives [8], which are frequently used as the first-line therapy, reinforcing the need for methods that provide rapid and accurate identification of *Trichosporon* isolates. Thus, in addition to the epidemiological importance of correct species identification, rapid and reliable identification of *T. asahii* certainly will facilitate the choice of an optimal antifungal therapy. Identification of fungal pathogens is essential, and our results illustrate its importance in epidemiology and the prescription of the appropriate antifungal therapy and possibly in taxonomic studies. Revisiting the fungal epidemiological findings using reliable identification techniques is crucial to reveal the pathogenic potential of species not currently considered problematic and, conversely, to rule out some of the species that were formerly considered pathogenic. MALDI–TOF MS appears to be particularly suitable for investigating the *Trichosporon* genus, but the Biotyper 3.0 database requires improvement.

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Author Contributions

All authors have seen and approved the manuscript and they contributed significantly to the work. Study design: DSYF, GMBDN, JG, DM, GB and CH. Acquisition of data: JNAJ, DSYF, DT, AL, FM, EB, AA and CH. Analysis and interpretation of data: JNAJ, GMBDN, DM and CH. Article construction: JNAJ, DSYF, DT, GMBDN, AL, FM, EB, AA, DM and CH. Final review of the version to be published: JNAJ, GB and CH.

Transparency Declaration

The authors declare that there are no conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Summary of the different protein extraction protocols used in this study

Table S2. Composition (number of representative species and code of the strains) of the reference spectra databases used in this study.

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