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Identification of Molds with Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry: Performance of the Newly Developed MSI-2 Application in Comparison with the Bruker Filamentous Fungi Database and MSI-1

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ABSTRACT Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) represents a promising tool for the rapid and efficient identification of molds, but improvements are still necessary to achieve satisfactory results when identifying cryptic species. Here, we aimed to validate a new web application, MSI-2, which replaces MSI-1, an application that was built and deployed online in 2017. For the evaluation, we gathered 633 challenging isolates obtained from daily hospital practice that were first identified with DNA-based methods, and we submitted their corresponding mass spectra to three identification programs (Bruker, MSI-1, and MSI-2). The MSI-2 application had a better identification performance at the species level than MSI-1 and Bruker, reaching 83.25% correct identifications, compared with 63.19% (MSI-1), 38.07% (Bruker with a 1.7 threshold), and 21.8% (Bruker with a 2.0 threshold). The MSI-2 application performed especially well for *Aspergillus* and *Fusarium* species, including for many cryptic species, reaching 90% correct identifications for *Aspergillus* species and 78% for *Fusarium* species compared to 69% and 43% with MSI-1. Such an improvement may have a positive impact on patient management by facilitating the identification of cryptic species potentially associated with a specific antifungal resistance profile.

KEYWORDS mold, online identification, MSI, database, MALDI-TOF mass spectrometry, molds

Molds are saprophytic environmental fungi that are widely used in biotechnologies, but they can also be responsible for infections in plants, animals, and humans. They also cause food spoilage and indoor damage. Phytopathogens can destroy hectares of agricultural crops, and fungal infections in livestock herds can result in the loss of many animals. Mold infections in humans are diverse, ranging from skin and nail infections to invasive and disseminated forms, mostly seen in immunocompromised patients. Fungal species do not all have the same antifungal susceptibility or the same pathogenicity in humans and animals. Consequently, their precise identification is essential to diagnose and manage the infections they provoke. The identification of fungi is usually based on complementary approaches: morphological identification and molecular biology. For a long time, molecular biology approaches were mainly based on the comparison of DNA sequences to reference sequences in Internet databases or on sequence alignments with reference data on phylogenetic trees. This technique is still considered the gold standard for identification, but it is expensive and time-consuming. Since the beginning of 2000,

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matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (MS) has provided a faster and less expensive method to identify bacteria, yeasts, and molds by analyzing the protein profiles of microorganisms and comparing them to an available database. Hence, MALDI-TOF MS has been used for a decade to identify filamentous fungi, mainly those implicated in clinical human or veterinary pathology (1–6). MALDI-TOF MS suppliers propose fungal spectra databases that include the species implicated in the most frequent fungal infections encountered in medical practice. However, these databases cover only a portion of the hundreds of already recognized human, animal and plant pathogens. In 2017, the first Web application was built and deployed online (MSI, for mass-spectral identification) to allow mycologists around the world to identify fungal MALDI-TOF mass spectra against a larger database (7). However, this application presented problems regarding the curation of the references that could hardly be deleted or modified due to iterative updates in the Java language that was used. Since 2019, a new application has been developed and coded in Python, a less sensitive language. The MSI-2 application is currently available at https://msi.happy-dev.fr. The performance of this application has not yet been formally evaluated in the identification of molds of medical interest. In this study, we assessed the identification results obtained with a panel of 633 mold isolates that had been identified via DNA-based methods with the new MSI application (MSI-2) and compared them to those obtained with the current version of the Bruker database and with the first MSI application (MSI-1), which was available until 2019.

MATERIALS AND METHODS

Isolates. All the mold isolates available at the Mycology Laboratory of La Pitié-Salpêtrière Hospital (Paris, France) for which DNA sequence-based identification was available were included in the study.

Gold standard identification of the selected isolates. As fungal taxonomy is always evolving, the DNA sequences of the selected isolates were searched against updated DNA databases to confirm their identities in October 2020 (NCBI-BLAST [https://blast.ncbi.nlm.nih.gov/Blast.cgi] and MycoBank-BLAST [https://www.mycobank.org/page/Pairwise_alignment]). For each of the selected isolates, with the exception of the *Aspergillus* and *Fusarium* isolates, identification relied on the sequencing of the internal transcribed spacer (ITS) sequence (8, 9). Isolates were included in this study only if the sequences obtained were sufficiently discriminant to support identifications at the species level. As large international databases such as NCBI-BLAST and MycoBank-BLAST are difficult to keep updated with the constant evolution of *Aspergillus* and *Fusarium* taxonomy, we considered identifications through the usual BLAST programs not accurate enough to discriminate between the cryptic species inside species complexes. Therefore, we performed DNA identifications by submitting the sequences to locally built phylogenetic trees that included reference strain sequences obtained using their published accession numbers (10–14). For most *Aspergillus* isolates, the sequence of beta-tubulin was required to ensure the correct identification of cryptic species. However, in some cases, where paralogous genes exist, calmodulin sequences were also used for identification (10). For *Fusarium* isolates, the ITS sequences were not sufficiently discriminant. Hence, the transcription elongation factor 1α sequence was used to perform the identification at the species level (9).

Mass spectrum preparation. For all isolates, protein extracts were obtained following the previously published protocol for culture on solid media. Briefly, approximately 1 mm^3 of the fungal culture was gently scraped with a scalpel blade, with extra care taken not to collect any agar. The samples were suspended in 70% high-performance liquid chromatography (HPLC) ethanol for inactivation. After a 2- to 5-min centrifugation step at 13,000 $\times$ *g* (depending on the sporulation of the fungal colony, a longer centrifugation might be required to fix the pellet on the microtube), the hydroalcoholic solution was removed, and the pellets were suspended in at least 10 μl of 70% formic acid (or enough volume to cover the pellet). The fungal samples were homogenized in formic acid by pipetting up and down. After a 5-min incubation step that allowed the cell walls to be destroyed by contact with the formic acid, an equal volume of HPLC acetonitrile was added, and the two reagents were mixed by pipetting up and down. After 5 min of incubation at ambient temperature for neutralization of the acid and precipitation of the proteins, the sample was centrifuged for 2 min at 13,000 $\times$ *g*, and 1-μl drops of the supernatant were deposited onto the polished steel targets in two to four replicates. Each deposit was covered with 1 μl of HCCA matrix (α-cyano-4-hydroxycinnamic acid) and dried at room temperature before processing on a Microflex mass spectrometer (Bruker Daltonics).

MSI-2 application. MSI-2 is an online mass spectrum identification application developed by Sorbonne University (Paris, France) and available at https://msi.happy-dev.fr. Original algorithms, different from those utilized in MSI-1, have been applied to optimize identification performances and to shorten the duration of calculations. After cleaning the spectrum of noise and applying a baseline subtraction, the most intense peaks are selected and compared to a set of reference spectra. The most similar spectrum is identified, and a resemblance score is calculated. The new application is coded in Python and framed in a Django web environment that allows an easier management of modifications among the reference spectra (addition of references and correction or deletion of incorrect references). New functions for diagnostic and epidemiological purposes have been developed. The application contains

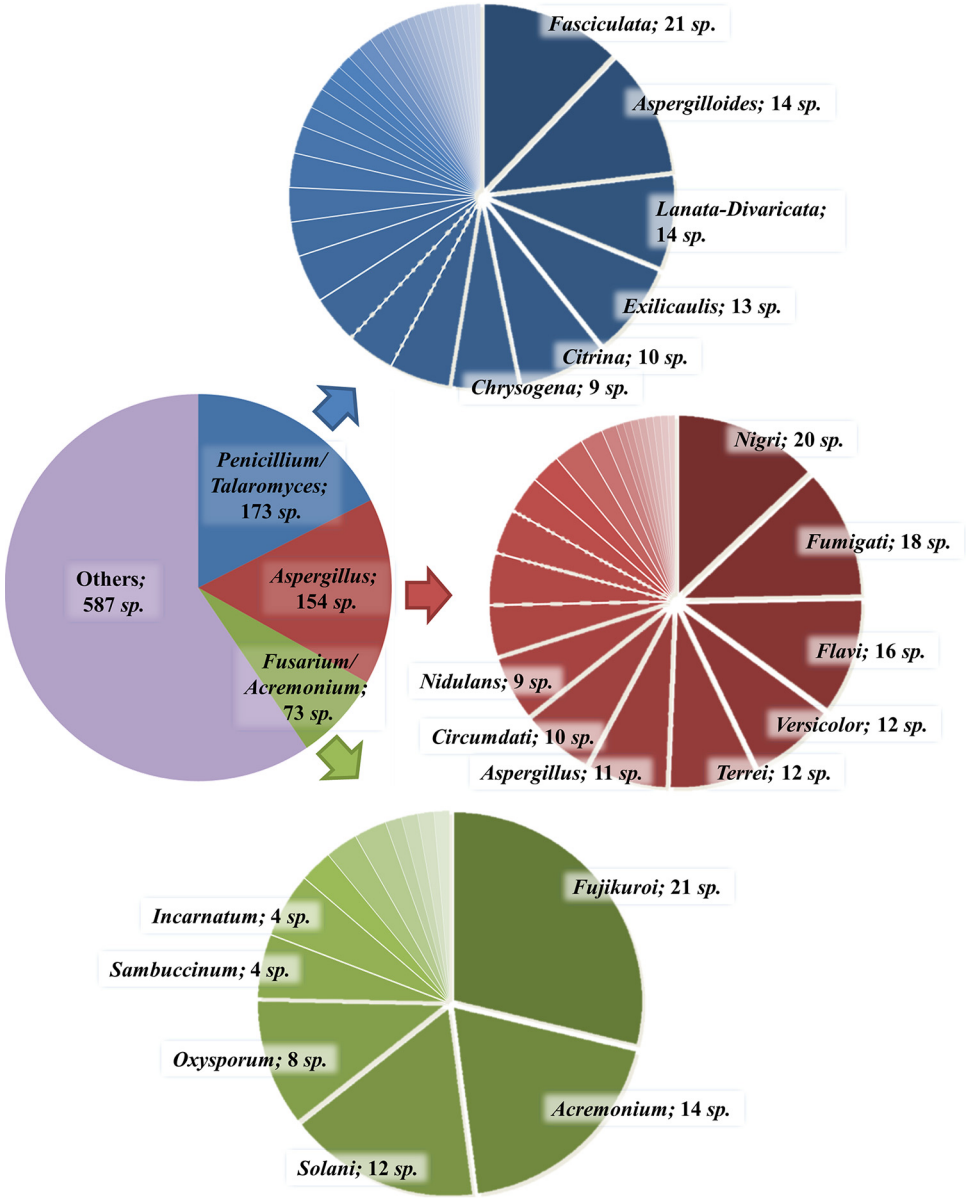

FIG 1 Graphical representation of the MSI-2 mold database; inside the *Penicillium/Talaromyces*, *Aspergillus*, and *Fusarium/Acremonium* taxa, representation of the most represented complexes and the number of species in each one. The "others" category groups 258 genera, represented by 1 (as is the case for 153 genera) to 28 (genus *Trichoderma*) species.

several spectral databases, and the main database, which is used for the identification of fungal species, was jointly developed by Sorbonne University and Sciensano (Brussels, Belgium). The list of the 1,301 fungal species and the 9,969 references included in the corresponding reference database is available at https://msi.happy-dev.fr/identification/bankspecieslist/10/. The strains whose spectra are used for the reference library have been extensively identified and stored in the BCCM/IHEM collection at Sciensano. This collection has successfully undergone audits for ISO 17025 accreditation and ISO 9001 certification.

Mass spectrum identification. After confirmation of the identification of the isolates, spectra were compared to the local MSI-1 version (7) (no longer available online; the list of reference species is available in Table S1), to the newly developed MSI-2 application, and to the Bruker research-use-only (RUO) database (BDAL revision 9) coupled with the filamentous fungus database (revision 3), containing a total of 1,450 fungal references (yeasts, molds [535 references], and dermatophytes [101 references] included). A graphical description of the content of the MSI-2 mold database can be found in Fig. 1. For the MSI-2 application identifications, as some of the isolates of the panel had been used previously to build up the library of reference spectra, we discarded the results corresponding to self-recognition of isolates present in both the panel and the library. The three databases differed in several parameters, both in the identification algorithms and in the reference composition. References parameters are compared in Table 1.

TABLE 1 Comparison of the references parameters between the Bruker RUO, MSI-1, and MSI-2 databases

Database	Type of references	No. of:			Species	Genera	Origins of the strains
		Subcultures per strain	Spectra per strain	References per strain			
Bruker RUO	Meta-spectra	1	24	1	411	111	Collections
MSI-1	Spectra	1–4	10–40 (10 per subculture)	1–4 (one per subculture)	818	209	Collections and individual spectra from various hospitals
MSI-2	Spectra	1–4	2–40 (2 to 10 per subculture)	1–4 (1 per subculture)	1,301	358	Collections

Performance of the MS databases. For each isolate, 2 to 4 extraction replicates were deposited on the steel target, but only the identification corresponding to the highest score was considered. As previous publications proposed lowering the Bruker identification threshold from 2.0 to 1.7 (15–18), we considered the performance of the Bruker database with both thresholds. For each isolate, the retained identification was compared to the DNA-based identification, and 5 categories of identification accuracy were established: (i) correct at the species level, when the MS identification was identical to the DNA-based identification; (ii) correct at the complex level, when the MS identification was different from the DNA-based identification at the species level but belonged to the same complex of species; (iii) correct at the genus level, when both species and complex of species were incorrect but the identification belonged to the same genus as the DNA-based identification; (iv) incorrect at the genus level, when different genera were identified by the MS- and DNA-based identifications (with the exception of closely related genera such as *Paecilomyces* and *Byssochlamys*, for which the taxonomy is still doubtful and for which we considered an identification correct at the genus level when it occurred); and (v) under the defined identification threshold, when the score was lower than the 1.7 or 2.0 thresholds for Bruker and lower than 20 for the two MSI applications.

Statistical analyses. The identification performances using each of the databases were compared based on the proportions and 95% exact binomial confidence intervals (http://statpages.info/confint.html) of the categorical identifications for each database. Significances of the differences between the contingency tables were calculated using Pearson's chi-squared test (http://biostatgv.sentiweb.fr/?module=tests/chideux).

RESULTS

Fungal diversity of the study panel. Analyses were performed on 633 isolates corresponding to 124 species and 26 genera of nondermatophytic filamentous fungi. A description of the species, complexes of species, and genera of the isolates included in this study is shown in Table 2.

Comparison of the identification performances of the mass spectrometry databases. The spectra obtained for the 633 isolates were subjected to three different MALDI-TOF MS identifications. Of the 124 species included in our panel, only 51 were represented in the three corresponding databases. The overall results are presented in Table 3.

The identification performance at the species level of the MSI-2 application was significantly better than that of either the MSI-1 application ($P < 0.001$) or the Bruker software ($P < 0.001$). The Bruker software showed better identification performances for the 51 species represented in the three databases than for the whole panel (66% for the 51 species versus 38% for all species, at a 1.7 threshold). Both of the MSI applications allowed the identification of more than 80% of the isolates corresponding to these 51 species, marking large improvements compared to the Bruker software. Improvements made in the latest MSI-2 application regarding these 51 species were significant compared to the previous MSI-1 application, with approximately 92% correct identifications at the species level, compared to approximately 84% with MSI-1. The percentages of identification at the species level per database and per submitted species for the 51 species represented in the three databases are shown in Table S2.

DISCUSSION

Here, we performed a comparative study of three MALDI-TOF mass-spectral identification systems. We selected 633 isolates for which we had already obtained DNA identifications and mass spectrum acquisitions. In our laboratory, we do not perform systematic molecular identifications for every mold that we obtain in culture. Instead, we

TABLE 2 DNA-based identification of the 633 isolates included in the selected panel for the comparison of the mass spectrometry databases

Genus	Complex	Species	No. of isolates
Acrophialophora		*Acrophialophora levis*	1
Alternaria		*Alternaria abundans*	1
		Alternaria alternata	4
Aphanocladium		*Aphanocladium album*	1
Artdrinium		*Artdrinium arundinis*	3
Artdrographis		*Artdrographis curvata*	1
Aspergillus	*Aspergillus*	*Aspergillus montevidensis*	2
		Aspergillus pseudoglaucus	1
	Circumdati	*Aspergillus affinis*	1
		Aspergillus insulicola	3
		Aspergillus ochraceopetaliformis	4
		Aspergillus ochraceus	6
		Aspergillus persii	5
		Aspergillus sclerotiorum	16
		Aspergillus subramanianii	1
		Aspergillus westerdijkiae	14
	Flavi	*Aspergillus alliaceus*	1
		Aspergillus flavus	38
		Aspergillus parasiticus	1
		Aspergillus tamarii	1
	Fumigati	*Aspergillus felis*	3
		Aspergillus fischeri	3
		Aspergillus fumigatus	26
		Aspergillus hiratsukae	13
		Aspergillus lentulus	9
		Aspergillus tdermomutatus	18
		Aspergillus tsurutae	1
		Aspergillus udagawae	3
	Nigri	*Aspergillus niger*	13
		Aspergillus tubingensis	5
	Terrei	*Aspergillus floccosus*	1
		Aspergillus terreus	18
	Unguis	*Aspergillus unguis*	2
	Usti	*Aspergillus calidoustus*	5
	Nidulantes	*Aspergillus nidulans*	5
		Aspergillus spinulosporus	4
		Aspergillus sublatus	18
		Aspergillus amoenus	1
		Aspergillus creber	9
		Aspergillus hongkongensis	1
		Aspergillus jensenii	10
		Aspergillus protuberus	3
		Aspergillus puulaauensis	3
		Aspergillus sydowii	5
Bipolaris		*Bipolaris hawaiiensis*	1
Ceriporia		*Ceriporia lacerata*	3
Cladosporium		*Cladosporium cladosporioides*	4
Engyodontium		*Engyodontium album*	1
Eutypella		*Eutypella scoparia*	2
Fomes		*Fomes fomentarius*	3
Fomitopsis		*Fomitopsis pinicola*	1
Fusarium/Acremonium	Acremonium	*Acremonium sclerotigenum*	3
	Dimerum	*Bisifusarium dimerum*	5
	Fujikuroi	*Fusarium acutatum*	1
		Fusarium andiyazi	2
		Fusarium lactis	1
		Fusarium proliferatum	48
		Fusarium sacchari	3
		Fusarium verticillioides	8
	Incarnatum	*Fusarium bubalinum*	1
		Fusarium equiseti	2
		Fusarium flagelliforme	1

(Continued on next page)

TABLE 2 (Continued)

Genus	Complex	Species	No. of isolates
		Fusarium incarnatum	2
		Fusarium tanahbumbuense	1
	Oxysporum	*Fusarium carminascens*	1
		Fusarium contaminatum	1
		Fusarium cugenangense	1
		Fusarium curvatum	7
		Fusarium elaeidis	1
		Fusarium gossypinum	1
		Fusarium languescens	1
		Fusarium nirenbergiae	13
		Fusarium oxysporum	3
		Fusarium triseptatum	1
		Fusarium veterinarium	53
	Redolens	*Fusarium redolens*	1
	Sambucinum	*Fusarium culmorum*	1
	Solani	*Fusarium breviconum*	1
		Fusarium falciforme	8
		Fusarium keratoplasticum	11
		Fusarium lichenicola	1
		Fusarium metavorans	1
		Fusarium petroliphilum	14
		Fusarium solani	2
		Fusarium solani.FSSC5	8
		Fusarium solani.FSSC9	2
		Fusarium solani.new.sp.1	8
		Fusarium solani.new.sp.2	2
		Fusarium solani f. sp. robiniae	2
Geosmithia		*Rasamsonia argillacea*	1
Geotrichum	Candidum	*Gallactomyces candidum*	2
		Gallactomyces geotrichum	5
	Capitatum	*Geotrichum capitatum*	6
Mucor		*Mucor circinelloides*	1
Paecilomyces	Lilacinum	*Paecilomyces lilacinus*	2
	Variotii	*Paecilomyces formosus*	1
		Paecilomyces variotii	2
Penicillium/Talaromyces	Aspergilloides	*Penicillium glabrum*	19
		Penicillium palmense	1
	Canescentia	*Penicillium canescens*	1
	Chrysogena	*Penicillium chrysogenum*	20
	Citrina	*Penicillium citrinum*	3
	Exilicaulis	*Penicillium corylophilum*	1
	Fasciculata	*Penicillium crustosum*	1
	Islandici	*Talaromyces rugulosus*	1
	Lanata-divaricata	*Penicillium oxalicum*	1
	Penicillium	*Penicillium expansum*	1
	Talaromyces	*Penicillium crateriforme*	1
		Talaromyces amestolkiae	1
		Talaromyces pinophilus	1
	Trachyspermi	*Talaromyces diversus*	3
		Talaromyces minioluteus	1
Perenniporia		*Perenniporia tenuis*	1
Phanerochaete		*Phanerochaete sordida*	1
Rhizopus		*Rhizopus microsporus*	1
		Rhizopus oryzae	3
Scedosporium	Apiospermum	*Scedosporium apiospermum*	7
		Scedosporium boydii	8
	Aurantiacum	*Scedosporium aurantiacum*	4
	Prolificans	*Lomentospora prolificans*	1
Scopulariopsis		*Scopulariopsis brevicaulis*	5
Thanatephorus		*Thanatephorus cucumeris*	2
Trichoderma		*Trichoderma harzianum*	1
		Trichoderma longibrachiatum	1
Total		124 species	633

TABLE 3 Identification performances of the different MALDI-TOF MS identification databases

Organisms	Confidence level of the identification	% identification (95% CI[a]) with:			
		Bruker (2.0)	Bruker (1.7)	MSI-1 (20)	MSI-2 (20)
All isolates ($n = 633$ isolates; 124 species)	Correct at the species level	21.8 (18.64–25.22)	38.07 (34.27–41.98)	63.19 (59.3–66.96)	83.25 (80.11–86.08)
	Correct at the complex level[b]	14.22 (11.59–17.18)	25.12 (21.78–28.69)	30.17 (26.62–33.91)	12.95 (10.44–15.82)
	Correct at the genus level[c]	0.32 (0.04–1.14)	1.74 (0.87–3.09)	3.95 (2.57–5.78)	0.16 (0–0.88)
	Incorrect at the genus level[c]	0 (0–0)	0.32 (0.04–1.14)	0.16 (0–0.88)	0 (0–0)
	Under the defined identification threshold	63.67 (59.78–67.42)	34.76 (31.05–38.61)	2.53 (1.45–4.07)	3.63 (2.32–5.4)
Aspergillus spp. ($n = 273$ isolates; 39 species in 10 complexes)	Correct at the species level	27.11 (21.92–32.79)	46.89 (40.85–53)	69.23 (63.39–74.65)	89.74 (85.52–93.08)
	Correct at the complex level[b]	18.32 (13.91–23.42)	24.54 (19.56–30.09)	30.04 (24.66–35.85)	8.42 (5.42–12.37)
	Correct at the genus level[c]	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)
	Incorrect at the genus level[d]	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)
	Under the defined identification threshold	54.58 (48.47–60.59)	28.57 (23.29–34.33)	0.73 (0.09–2.62)	1.83 (0.6–4.22)
Fusarium spp. ($n = 223$ isolates; 38 species in 8 complexes)	Correct at the species level	11.21 (7.39–16.1)	21.97 (16.72–27.99)	42.6 (36.02–49.38)	77.58 (71.53–82.88)
	Correct at the complex level[b]	16.59 (11.96–22.14)	37.67 (31.29–44.38)	46.19 (39.51–52.97)	21.52 (16.32–27.51)
	Correct at the genus level[b]	0 (0–0)	2.69 (0.99–5.76)	9.87 (6.29–14.56)	0.45 (0.01–2.47)
	Incorrect at the genus level[d]	0 (0–0)	0.9 (0.11–3.2)	0 (0–0)	0 (0–0)
	Under the defined identification threshold	72.2 (65.82–77.97)	36.77 (30.43–43.47)	1.35 (0.28–3.88)	0.45 (0.01–2.47)
Species represented in all databases ($n = 365$ isolates; 51 species)	Correct at the species level	37.81 (32.81–43)	66.03 (60.92–70.88)	83.56 (79.35–87.21)	91.51 (88.16–94.16)
	Correct at the complex level[b]	2.74 (1.32–4.98)	5.48 (3.38–8.34)	14.25 (10.83–18.26)	4.93 (2.95–7.68)
	Correct at the genus level[c]	0.55 (0.07–1.96)	1.92 (0.77–3.91)	0.55 (0.07–1.96)	0 (0–0)
	Incorrect at the genus level[d]	0 (0–0)	0 (0–0)	0.27 (0.01–1.52)	0 (0–0)
	Under the defined identification threshold	58.9 (53.66–64)	26.58 (22.11–31.42)	1.37 (0.45–3.17)	3.56 (1.91–6.01)

[a]CI, confidence interval.
[b]MS identification was different from DNA-based identification at the species level but belonged to the same complex of species as the sequencing gold standard.
[c]Both species and complexes of species were incorrect, but identification belonged to the same genus as the sequencing gold standard.
[d]Genus difference.

carry out DNA sequence analysis only for isolates belonging to unusual species or to formally confirm identifications proposed in scientific studies. Therefore, this panel consisted only of isolates obtained from cultures of human samples and was not representative of routine activity. The panel underrepresents the most common molds (*Aspergillus flavus* and *A. fumigatus*, for example) and overrepresents those molds that are rarer and more complicated to identify. With this panel of difficult isolates containing only molds, we challenged the most recent Bruker filamentous database, the now-unavailable MSI-1 application, and the newly developed MSI-2 application.

The results show that the new MSI-2 application allows the identification of both the filamentous fungi usually identified in the daily activity of a clinical mycology laboratory and rarer cryptic mold species with high success rates at the species level, exhibiting better performances than either the previous MSI-1 application or the different Bruker databases.

The greatest improvements in identification were observed within the *Aspergillus* and *Fusarium* genera, which have been a particular focus for the implementation of the MSI database between the two versions. Indeed, in a previous study, the difficulty of identifying cryptic species of *Aspergillus* at the species level was stated (19). In the current study, we used a panel of mold species that was rigorously selected based on the availability and correctness of their DNA sequences (only isolates with sequences of genes that allowed reliable identification at the species level).

Obtaining an accurate and reliable identification, in addition to its obvious necessity for the development of knowledge and for epidemiology, may be of clinical interest for the management of patients suffering from fungal infections. Indeed, there might be a difference in susceptibility to a particular antifungal drug among species complexes, such as those reported by Imbert et al. for *Aspergillus* species in the section Fumigati (20) or by Carrara et al. for species in the section Nigri (21). In these sections, species identification relates to the intrinsic antifungal susceptibility profile, suggesting the usefulness of accurate identification for disease management.

In the present study, we focused on molds because the main manufacturers' databases are exhaustive enough for yeast identification. In contrast, improvements are still necessary for dermatophyte identification, and we plan to set up a new database for these specific fungi. This database was not presented here, as the work is still in progress.

The best performances of the official Bruker RUO and filamentous-fungus databases reached 66% correct identification at the species level when considering only species that are represented in the database (i.e., without most of the cryptic *Aspergillus* and *Fusarium* species). The percentages of identification at the species level within the genus *Fusarium* with the Bruker system remained low regardless of the threshold utilized.

Even when we focused on the 51 species (365 isolates) of our panel that are represented in the three databases, i.e., isolates that are likely to be identified regardless of the database tested, the performances of the three applications were significantly different, indicating that mold identification is affected not only by the database content (i.e., the number of references per species) but also by the preparation method of the protein extract, the type of culture (the mold references by Bruker were acquired from liquid cultures), the spectrum acquisition conditions, and the identification algorithms, which play important roles in the performances. We previously observed this while locally implementing the Bruker database and comparing the identification results with those produced with the same references but implemented on the MSI-1 application (7). In this panel of 365 isolates, only three isolates were correctly identified by the Bruker application while being identified only at the species complex level by MSI-2. Those three isolates were two *Alternaria alternata* isolates that were identified as *Alternaria arborescens* by MSI-2 and one *Geosmithia argillacea* isolate that was misidentified as *Geosmithia piperina* by MSI-2. Regarding the *Alternaria* misidentifications, the two species are closely related, and the introduction of *A. arborescens* for phytopathology purposes into the database might be a source of confusion. The reference that misidentified the *G. argillacea* isolate as *G. piperina* (IHEM16128) was recently renamed by the Belgian collection and was labeled *G. argillacea* until recently.

The references that were proposed in the MSI-1 application were thoroughly cleaned up for MSI-2. In some cases, this might lead to a decrease in performance with the new application (Table S2), as MSI-1 contained references that have been deleted in MSI-2, even if some of them contributed to the performance of MSI-1. These references corresponded to spectra obtained in various collaborating centers and were obtained from isolates morphologically identified. However, those isolates were never stored in a collection nor sequenced. Hence, their identification could not be verified with a DNA-based method, and we decided to remove these uncertain references from the MSI-2 application. To further improve the MSI-2 application and to fill the gaps

resulting from the removal of these references, as shown for nine species in Table S2 (*Aspergillus flavus*, *Aspergillus parasiticus*, *Cladosporium cladosporioides*, *Fusarium incarnatum*, *Fusarium oxysporum*, *Geosmithia argillacea*, *Geotrichum capitatum*, *Penicillium glabrum*, and *Lomentospora prolificans*), we encourage MSI-2 users to send us any isolates that could not be identified, when possible, so that we can perform in-depth identifications of the isolates prior to including them in the new reference database. In addition to these changes, some strains have been renamed due to changes in the fungal taxonomy, as it is the case for the *Geosmithia piperina* IHEM16128 reference, and others were deleted from the database, as they were excluded from the BCCM/IHEM collection. All those modifications globally improve the quality of the identifications obtained with MSI-2, even though a few isolates of our panel (16/365) were better identified with the former MSI-1 application.

Using the new application, we achieved approximately 78% correct identifications at the species level for the *Fusarium* isolates. This result is still unsatisfactory even if it is 35% better than the first MSI application and 65% better than the Bruker database with a threshold of 2.0. The new application comprises many references of new species to take into account new developments of the *Fusarium* taxonomy, especially regarding the *Fusarium solani* species complex (FSSC) and the *Fusarium oxysporum* species complex (FOSC) (11, 22). This increase in the number of species made it more difficult to obtain accurate identification results. Moreover, as these new species are almost impossible to distinguish from each other and are rarely encountered in clinical practice, they have not been considered in clinical studies in terms of specific pathogenicity or antifungal susceptibility profiles, which raises the question of the significance of these new species in the context of routine practice.

In conclusion, the MSI-2 application constitutes a reliable identification tool for most filamentous species identified on a daily basis in a clinical mycology laboratory. The improvements of the reference database regarding the *Aspergillus* and *Fusarium* genera now allow us to identify clinical specimens from these genera at the species level. These improvements may have an impact on the treatment given to the patient, as the most effective treatment may vary from one cryptic species to another; moreover, the improvements may decrease the delay before the prescription of adequate antifungal treatment.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

SUPPLEMENTAL FILE 2, PDF file, 0.1 MB.

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REFERENCES

1. Wilkendorf LS, Bowles E, Buil JB, van der Lee HAL, Posteraro B, Sanguinetti M, Verweij PE. 2020. Update on matrix-assisted laser desorption ionization–time of flight mass spectrometry identification of filamentous fungi. J Clin Microbiol 58:e01263-20. https://doi.org/10.1128/JCM.01263-20.
2. Jing R, Yang WH, Xiao M, Li Y, Zou GL, Wang CY, Li XW, Xu YC, Hsueh PR. 29 March 2021. Species identification and antifungal susceptibility testing of Aspergillus strains isolated from patients with otomycosis in northern China. J Microbiol Immunol Infect https://doi.org/10.1016/j.jmii.2021.03.011.
3. Normand AC, Imbert S, Brun S, Al-Hatmi AMS, Chryssanthou E, Cassaing S, Schuttler C, Hasseine L, Mahinc C, Costa D, Bonnal C, Ranque S, Sautour M, Rubio E, Delhaes L, Riat A, Sendid B, Kristensen L, Brandenberger M, Guitard J, Packeu A, Piarroux R, Fekkar A. 2021. Clinical origin and species distribution of Fusarium spp. isolates identified by molecular sequencing

and mass spectrometry: a European multicenter hospital prospective study. J Fungi 7:246. https://doi.org/10.3390/jof7040246.
4. Gnat S, Łagowski D, Nowakiewicz A, Dyląg M, Osińska M, Sawicki M. 2021. Detection and identification of dermatophytes based on currently available methods—a comparative study. J Appl Microbiol 130:278–291. https://doi.org/10.1111/jam.14778.
5. Becker P, Normand AC, Vanantwerpen G, Vanrobaeys M, Haesendonck R, Vercammen F, Stubbe D, Piarroux R, Hendrickx M. 2019. Identification of fungal isolates by MALDI-TOF mass spectrometry in veterinary practice: validation of a web application. J Vet Diagn Invest 31:471–474. https://doi.org/10.1177/1040638719835577.
6. Bartosch T, Heydel T, Uhrlaß S, Nenoff P, Müller H, Baums CG, Schrödl W. 2018. MALDI-TOF MS analysis of bovine and zoonotic Trichophyton verrucosum isolates reveals a distinct peak and cluster formation of a subgroup with Trichophyton benhamiae. Med Mycol 56:602–609. https://doi.org/10.1093/mmy/myx084.
7. Normand AC, Becker P, Gabriel F, Cassagne C, Accoceberry I, Gari-Toussaint M, Hasseine L, De Geyter D, Pierard D, Surmont I, Djenad F, Donnadieu JL, Piarroux M, Ranque S, Hendrickx M, Piarroux R. 2017. Validation of a new web application for identification of fungi by use of matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol 55:2661–2670. https://doi.org/10.1128/JCM.00263-17.
8. Irinyi L, Serena C, Garcia-Hermoso D, Arabatzis M, Desnos-Ollivier M, Vu D, Cardinali G, Arthur I, Normand AC, Giraldo A, da Cunha KC, Sandoval-Denis M, Hendrickx M, Nishikaku AS, de Azevedo Melo AS, Merseguel KB, Khan A, Parente Rocha JA, Sampaio P, da Silva Briones MR, e Ferreira RC, de Medeiros Muniz M, Castañón-Olivares LR, Estrada-Barcenas D, Cassagne C, Mary C, Duan SY, Kong F, Sun AY, Zeng X, Zhao Z, Gantois N, Botterel F, Robbertse B, Schoch C, Gams W, Ellis D, Halliday C, Chen S, Sorrell TC, Piarroux R, Colombo AL, Pais C, de Hoog S, Zancopé-Oliveira RM, Taylor ML, Toriello C, de Almeida Soares CM, Delhaes L, Stubbe D, et al. 2015. International Society of Human and Animal Mycology (ISHAM)-ITS reference DNA barcoding database—the quality controlled standard tool for routine identification of human and animal pathogenic fungi. Med Mycol 53:313–337. https://doi.org/10.1093/mmy/myv008.
9. Hoang MTV, Irinyi L, Chen SCA, Sorrell TC, Meyer W, ISHAM Barcoding of Medical Fungi Working Group. 2019. Dual DNA barcoding for the molecular identification of the agents of invasive fungal infections. Front Microbiol 10:1647. https://doi.org/10.3389/fmicb.2019.01647.
10. Houbraken J, Kocsubé S, Visagie CM, Yilmaz N, Wang XC, Meijer M, Kraak B, Hubka V, Bensch K, Samson RA, Frisvad JC. 2020. Classification of Aspergillus, Penicillium, Talaromyces and related genera (Eurotiales): an overview of families, genera, subgenera, sections, series and species. Stud Mycol 95:5–169. https://doi.org/10.1016/j.simyco.2020.05.002.
11. Lombard L, Sandoval-Denis M, Lamprecht SC, Crous PW. 2019. Epitypification of Fusarium oxysporum—clearing the taxonomic chaos. Persoonia 43:1–47. https://doi.org/10.3767/persoonia.2019.43.01.
12. O'Donnell K, Sutton DA, Fothergill A, McCarthy D, Rinaldi MG, Brandt ME, Zhang N, Geiser DM. 2008. Molecular phylogenetic diversity, multilocus haplotype nomenclature, and in vitro antifungal resistance within the Fusarium solani species complex. J Clin Microbiol 46:2477–2490. https://doi.org/10.1128/JCM.02371-07.
13. Najafzadeh MJ, Dolatabadi S, de Hoog S, Esfahani MK, Haghani I, Aghili SR, Ghazvini RD, Rezaei-Matehkolaei A, Abastabar M, Al-Hatmi AMS. 2020. Phylogenetic analysis of clinically relevant Fusarium species in Iran. Mycopathologia 185:515–525. https://doi.org/10.1007/s11046-020-00460-x.
14. Al-Hatmi AMS, Hagen F, Menken SB, Meis JF, de Hoog GS. 2016. Global molecular epidemiology and genetic diversity of Fusarium, a significant emerging group of human opportunists from 1958 to 2015. Emerg Microbes Infect 5:e124. https://doi.org/10.1038/emi.2016.126.
15. Dupont D, Normand AC, Persat F, Hendrickx M, Piarroux R, Wallon M. 2019. Comparison of matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) systems for the identification of moulds in the routine microbiology laboratory. Clin Microbiol Infect 25:892–897. https://doi.org/10.1016/j.cmi.2018.10.013.
16. Normand AC, Cassagne C, Gautier M, Becker P, Ranque S, Hendrickx M, Piarroux R. 2017. Decision criteria for MALDI-TOF MS-based identification of filamentous fungi using commercial and in-house reference databases. BMC Microbiol 17:25. https://doi.org/10.1186/s12866-017-0937-2.
17. Schulthess B, Ledermann R, Mouttet F, Zbinden A, Bloemberg GV, Böttger EC, Hombach M. 2014. Use of the Bruker MALDI Biotyper for identification of molds in the clinical mycology laboratory. J Clin Microbiol 52:2797–2803. https://doi.org/10.1128/JCM.00049-14.
18. Sun Y, Guo J, Chen R, Hu L, Xia Q, Wu W, Wang J, Hu F. 2021. Multicenter evaluation of three different MALDI-TOF MS systems for identification of clinically relevant filamentous fungi. Med Mycol 59:81–86. https://doi.org/10.1093/mmy/myaa037.
19. Imbert S, Normand AC, Gabriel F, Cassaing S, Bonnal C, Costa D, Lachaud L, Hasseine L, Kristensen L, Schuttler C, Raberin H, Brun S, Hendrickx M, Stubbe D, Piarroux R, Fekkar A. 2019. Multi-centric evaluation of the online MSI platform for the identification of cryptic and rare species of Aspergillus by MALDI-TOF. Med Mycol 57:962–968. https://doi.org/10.1093/mmy/myz004.
20. Imbert S, Normand AC, Cassaing S, Gabriel F, Kristensen L, Bonnal C, Lachaud L, Costa D, Guitard J, Hasseine L, Palous M, Piarroux M, Hendrickx M, Piarroux R, Fekkar A. 2020. Multicentric analysis of the species distribution and antifungal susceptibility of cryptic isolates from Aspergillus section Fumigati. Antimicrob Agents Chemother 64:e01374-20. https://doi.org/10.1128/AAC.01374-20.
21. Carrara B, Richards R, Imbert S, Morio F, Sasso M, Zahr N, Normand AC, Pape PL, Lachaud L, Ranque S, Maubon D, Piarroux R, Fekkar A. 2020. Species distribution and comparison between EUCAST and gradient concentration strips methods for antifungal susceptibility testing of 112 Aspergillus section Nigri isolates. Antimicrob Agents Chemother 64:e02510-19. https://doi.org/10.1128/AAC.02510-19.
22. O'Donnell K, Al-Hatmi AMS, Aoki T, Brankovics B, Cano-Lira JF, Coleman JJ, de Hoog GS, Di Pietro A, Frandsen RJN, Geiser DM, Gibas CFC, Guarro J, Kim HS, Kistler HC, Laraba I, Leslie JF, López-Berges MS, Lysøe E, Meis JF, Monod M, Proctor RH, Rep M, Ruiz-Roldán C, Šišić A, Stajich JE, Steenkamp ET, Summerell BA, van der Lee TAJ, van Diepeningen AD, Verweij PE, Waalwijk C, Ward TJ, Wickes BL, Wiederhold NP, Wingfield MJ, Zhang N, Zhang SX. 2020. No to Neocosmospora: phylogenomic and practical reasons for continued inclusion of the Fusarium solani species complex in the genus Fusarium. mSphere 5:e00810-20. https://doi.org/10.1128/mSphere.00810-20.