JCM Journals.ASM.org

Use of Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry for Identification of Molds of the *Fusarium* Genus

David Triest,[a] Dirk Stubbe,[a] Koen De Cremer,[b] Denis Piérard,[c] Anne-Cécile Normand,[d,e] Renaud Piarroux,[d,e] Monique Detandt,[a] Marijke Hendrickx[a]

Service of Mycology and Aerobiology, BCCM/IHEM Fungal Collection, Scientific Institute of Public Health, Brussels, Belgium[a]; Service of Biomonitoring, Scientific Institute of Public Health, Brussels, Belgium[b]; Department of Microbiology and Infection Control, Universitair Ziekenhuis Brussel, Vrije Universiteit Brussel, Brussels, Belgium[c]; Laboratoire de Parasitologie-Mycologie, APHM, CHU Timone-Adultes, Marseille, France[d]; Aix-Marseille Université, UMR MD3 IP-TPT, Marseille, France[e]

The rates of infection with *Fusarium* molds are increasing, and a diverse number of *Fusarium* spp. belonging to different species complexes can cause infection. Conventional species identification in the clinical laboratory is time-consuming and prone to errors. We therefore evaluated whether matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) is a useful alternative. The 289 *Fusarium* strains from the Belgian Coordinated Collections of Microorganisms (BCCM)/Institute of Hygiene and Epidemiology Mycology (IHEM) culture collection with validated sequence-based identities and comprising 40 species were used in this study. An identification strategy was developed, applying a standardized MALDI-TOF MS assay and an in-house reference spectrum database. *In vitro* antifungal testing was performed to assess important differences in susceptibility between clinically relevant species/species complexes. We observed that no incorrect species complex identifications were made by MALDI-TOF MS, and 82.8% of the identifications were correct to the species level. This success rate was increased to 91% by lowering the cutoff for identification. Although the identification of the correct species complex member was not always guaranteed, antifungal susceptibility testing showed that discriminating between *Fusarium* species complexes can be important for treatment but is not necessarily required between members of a species complex. With this perspective, some *Fusarium* species complexes with closely related members can be considered as a whole, increasing the success rate of correct identifications to 97%. The application of our user-friendly MALDI-TOF MS identification approach resulted in a dramatic improvement in both time and accuracy compared to identification with the conventional method. A proof of principle of our MALDI-TOF MS approach in the clinical setting using recently isolated *Fusarium* strains demonstrated its validity.

Fusarium is a widely distributed fungal genus of soil inhabitants and plant pathogens that are important in agriculture (1). At least 70 *Fusarium* species have also been reported as opportunistic human pathogens, and infection rates have increased over the past years (2). *Fusarium* spp. can cause superficial infections, such as keratitis and onychomycosis, as well as locally invasive and disseminated infections (1, 2). These disseminated infections particularly affect immunosuppressed patients and are associated with a high mortality rate (2).

Fusarium species are grouped into several species complexes (3). Molecular evolutionary analysis has been performed for most species complexes and revealed many additional phylogenetically distinct species (4–12). These closely related species are often morphologically indistinguishable, and multilocus gene sequencing is needed to attempt species delimitation (13, 14). The most important clinically relevant *Fusarium* species complexes include the *F. solani* species complex (FSSC), *F. oxysporum* species complex (FOSC), *F. fujikuroi* species complex (FFSC), *F. dimerum* species complex (FDSC), *F. incarnatum-F. equiseti* species complex (FIESC), and *F. chlamydosporum* species complex (FCSC). Members of the FSSC are estimated to cause the majority of fusarioses (50%), followed by members of the FOSC and FFSC (both 20%) (1, 12, 15).

Compared to other molds, *Fusarium* spp., especially isolates of the FSSC, are considered relatively resistant to most antifungals. However, according to *in vitro* testing, antifungal susceptibility profiles have been shown to vary between *Fusarium* species (16–26). Correct identification to the species or species complex level may thus be crucial for patient treatment, although no *in vitro-in vivo* correlations have been demonstrated, and treatment is dependent on the type of infection (27). Identification to the species level is also essential for epidemiological purposes.

A differential diagnosis of fusariosis from other mycoses is not that straightforward, and identification of the infecting *Fusarium* sp. is even more difficult. Classically, in the clinical laboratory, the identification of a mold infection relies on a morphological examination of an isolate by expert mycologists. This approach is challenging, especially with *Fusarium*, which contains many cryptic species, and for some isolates, specific morphological traits are lacking and appear atypical in culture. Inaccurate species identifications or identifications to only the genus level are thus not uncommon. DNA sequencing, the gold standard, can complement the morphological identification but is costly, and different DNA

Received 1 August 2014 Returned for modification 3 October 2014
Accepted 14 November 2014

Accepted manuscript posted online 19 November 2014

Citation Triest D, Stubbe D, De Cremer K, Piérard D, Normand A-C, Piarroux R, Detandt M, Hendrickx M. 2015. Use of matrix-assisted laser desorption ionization–time of flight mass spectrometry for identification of molds of the *Fusarium* genus. J Clin Microbiol 53:465–476. doi:10.1128/JCM.02213-14.

Editor: D. W. Warnock

Address correspondence to David Triest, david.triest@wiv-isp.be.

Supplemental material for this article may be found at http://dx.doi.org/10.1128/JCM.02213-14.

Copyright © 2015, American Society for Microbiology. All Rights Reserved.

doi:10.1128/JCM.02213-14

markers may need to be evaluated. Moreover, routine conventional identification is a time-consuming process. It is widely accepted that the early onset of appropriate treatment for infected patients decreases the mortality rates of invasive infections and increases the chance of success. With this perspective, matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) has received major interest.

MALDI-TOF MS has improved the identification of bacteria and yeasts in the clinical setting, increasingly replacing the conventional methods (28–32). It has emerged as a rapid, simple, cost-effective, reliable, and reproducible identification tool in which a spectrum is generated based on the proteomic content of a microorganism and compared against a reference spectrum database for identification. Recently, the MALDI-TOF MS approach was also applied to mold identification and was shown to be able to accurately identify a wide array of species from various genera or groups of clinical interest, including *Aspergillus* spp. and dermatophytes (33–44). Species identification by MALDI-TOF MS within the *Fusarium* genus has not yet been thoroughly investigated. Studies have either focused on the comparison of different sample preparation procedures (45, 46) or analyzed only a limited number of *Fusarium* spp. and strains (47, 48). Moreover, it has been shown that for some mold species, the data obtained by MALDI-TOF MS are correlated with their phylogenetic data (49).

The main objective of the present study was to investigate whether MALDI-TOF MS is a useful alternative for the species identification of *Fusarium* isolates compared to identification with conventional methods. Therefore, we aimed to take into account a substantial part of the species diversity in the *Fusarium* genus. We focused on the performance of MALDI-TOF MS to accurately discriminate between species complexes and especially the members showing important differences in their antifungal susceptibility profiles.

MATERIALS AND METHODS

Taxon sampling. A total of 323 *Fusarium* strains preserved and referenced in the Belgian Coordinated Collections of Microorganisms (BCCM)/Institute of Hygiene and Epidemiology Mycology (IHEM) culture collection were used in this study (see Table S1 in the supplemental material). The strains were gathered over the previous 30 years from different origins, mostly clinical. Since many taxonomical changes have occurred during this time period and the strains were identified only by morphological examination or assigned a *Fusarium* sp. upon the time of their deposit, we reidentified all strains.

Multilocus sequencing. Our reidentification relied on BLAST analysis in GenBank and FUSARIUM-ID (50), using sequences of the internal transcribed spacer (ITS) region and part of the ribosomal large subunit (LSU), as well as sequences of a partial fragment of the beta-tubulin (BT) and translation elongation factor 1-alpha (TEF1α) gene. The choice of DNA markers was based on previous studies (11, 51). DNA extraction, PCR amplification, and sequencing were performed according to the protocol applied by Beguin et al. (52). The following primers were described previously: those for ITS by White et al. (53), for LSU by Hopple and Vilgalys (54), for BT by Glass and Donaldson (55), and for TEF1α by Carbone and Kohn (56).

Morphological characterization. The strains were also reanalyzed morphologically. This was done after cultivation on potato dextrose agar (PDA) and nutrient-poor agar at 25°C in an incubator for ≥7 days, according to The *Fusarium* laboratory manual of Leslie, Summerell, and Bullock (57).

Phylogenetic analysis. In order to determine the evolutionary species relationships in our *Fusarium* data set, phylogenetic analysis was performed. The sequences were first aligned using the Clustal W algorithm in MEGA4 (58) and edited manually. Subsequently, Bayesian inference (BI) analysis was executed on the combined sequence data set with MrBayes3.2 (59), using the Monte Carlo Markov chain method with runs of 1 million generations and sampling a tree every 500 generations. The first 25% of the sampled trees were discarded (i.e., burn-in). A consensus tree with posterior probabilities was assessed from the remaining trees. Our combined sequence data set was partitioned according to the different gene regions, and the GTR+I+Γ model of evolution was applied with parameters estimated separately for each partition. Tracer version 1.5 (60) was used to check the convergence of the likelihood scores, showing acceptable mixing of the runs and sufficient sampling. An *Acremonium* sp. (strain IHEM 7465) was selected as the outgroup.

***In vitro* antifungal susceptibility testing.** The *Fusarium* strains in the collection, which were isolated from patients, were subjected to *in vitro* antifungal susceptibility testing, according to the EUCAST E.DEF 9.1 broth microdilution method, as described previously (61). Inoculum suspensions were prepared from cultures grown on PDA for 2 days at 35°C and subsequently for 5 days at 25°C in a humidified incubator. A panel of eight commonly used antifungal agents was evaluated. Two-fold serial drug dilutions were prepared in 96-well plates, with concentrations ranging from 64 to 0.032 μg/ml for fluconazole and 5-fluorocytosine and from 16 to 0.008 μg/ml for amphotericin B, voriconazole, itraconazole, ketoconazole, posaconazole, and terbinafine. The minimum 100% inhibitory concentrations (MIC_{100}) were assessed for each strain, and internal control strains with known MIC_{100}s were included. An antifungal susceptibility profile was constructed for each species tested in the data set.

MALDI-TOF MS assay. Our MALDI-TOF MS assay was based on the optimized procedure developed by Cassagne et al. (33). This assay has already been applied in several studies for MALDI-TOF MS identification of molds from different genera (40, 42–44). Some adaptations were made to the protocol. The strains were cultivated on Sabouraud-chloramphenicol agar plates at 25°C in a humidified incubator for 3 days. The incubation times of the sample in formic acid and acetonitrile for protein extraction were both augmented to 15 min, and the volume administered was increased to 50 μl. The acquisition of mass spectra was performed on a microflex LT mass spectrometer (Bruker Daltonics, Bremen, Germany), using the default settings of the manufacturer. Instrument calibration was achieved with a Bacterial Test Standard (Bruker Daltonics). The time of flight measurements were converted to *m/z* values, and all raw spectra were automatically processed by the Flexcontrol version 2.4 AutoXecute software (Bruker Daltonics). The resulting peak lists were exported to the MALDI Biotyper 3.0 software (Bruker Daltonics).

Construction of reference spectrum database. A reference spectrum was constructed for each *Fusarium* strain, as described by Cassagne et al. (33), taking into account 4 culture replicates per strain for separate protein extraction and spotting each extract 10 times onto a MALDI 96 polished steel target plate (Bruker Daltonics). The resulting *Fusarium* reference spectra were implemented in an in-house database, which already contained the reference spectra of 1,021 other validated strains comprising 152 different fungal genera from the BCCM/IHEM collection.

Identification strategy. In order to make unbiased MALDI-TOF MS identifications for our *Fusarium* strains, we applied the following strategy. For each strain, 4 additional raw spectra (i.e., the identification spectra) were collected from a separate culture. These identification spectra were subsequently challenged against the reference spectrum database using the MALDI Biotyper 3.0 software and excluding the reference spectrum of the analyzed strain to determine its identity. The second step was done to ensure that the strain would not be recognized by its own reference spectrum. The results of the matching process were expressed as logarithmic scores from 0 (no spectrum match) to 3 (a perfect match). For identification in the present study we took into account the reference strain associated with the highest logarithmic score (i.e., the top score). Analogous to the methodology of Cassagne et al. (33), the identification of a strain by MALDI-TOF MS was considered interpretable when two criteria were

fulfilled: (i) at least three out of four identification spectra were identified by the reference spectrum of the same species, and (ii) at least one of their top scores had a value above the standard manufacturer-defined 2.0 cutoff for reliable species identification. The MALDI-TOF MS identification of a strain was found to be correct when the obtained identity matched that determined from the gold standard multilocus sequence analysis. Correct species complex identifications were also taken into account. When the criteria for identification were not fulfilled, it was evaluated whether lowering the cutoff value for reliable identification would allow the strain to be identified; otherwise, the strain remained unidentified by MALDI-TOF MS. Per *Fusarium* species in the data set for which more than one strain could be analyzed, we calculated the percentages of correct species identifications, correct species complex identifications, erroneous identifications, and identifications in which the criteria were not fulfilled (for both the standard 2.0 and a lowered cutoff value). Species represented by only one strain in the data set were not included in the calculations, since, in our setup, they were always misidentified (seeing as their own reference spectrum is not taken into account). For these one-strain species, it was checked whether they were identified by MALDI-TOF MS as a phylogenetically closely related species (i.e., within the correct species complex).

Comparison. In order to visualize the species relationships in the *Fusarium* reference spectrum data set, a dendrogram was constructed. Therefore, the distance matrix of the MALDI Biotyper 3.0 software was used, in which distance values are relative and normalized to a maximum value of 1,000. The topology of the dendrogram was compared with that of the BI consensus tree. In this way, we evaluated if there was a correlation between the MALDI-TOF MS data and the phylogenetic data.

Proof of principle. The validity of our MALDI-TOF MS identification approach was assessed in a clinical routine by screening 20 recent clinical isolates with a presumable *Fusarium* identity. The isolates were recovered from patient samples between 2012 and 2013 in the medical mycology laboratories of the Universitair Ziekenhuis Brussel (Belgium) and the Universitair Medisch Centrum Sint-Pieter (Belgium). We subjected them to MALDI-TOF MS identification, according to the criteria described above, and in parallel to a DNA sequence-based identification using ITS or, when ITS was not found to be sufficiently discriminatory, TEF1α.

Nucleotide sequence accession numbers. All sequences were deposited in GenBank with the accession numbers given in Table 1; see also the accession numbers in Table S1 in the supplemental material.

RESULTS

Taxon sampling, multilocus sequencing, and morphological characterization. While performing multilocus sequencing, we encountered difficulties or inconsistencies with 61 of the 323 *Fusarium* strains (see Table S1 in the supplemental material). For 4 strains, no viable or pure culture was obtained. Sequencing failed for one of the DNA markers in 4 strains, and 5 strains were reclassified in a *Fusarium*-like genus that previously belonged to *Fusarium sensu stricto*. Eight other strains were identified as a species not belonging to *Fusarium* or a *Fusarium*-like genus (mostly *Acremonium*). The remaining 40 strains showed a different *Fusarium* identity than the one under which it was preserved in the collection. Twenty-seven of these 40 strains were retained in the collection and this study, with a corrected identity, since the species concerned had not yet been defined upon the time of deposit of the strain or a misidentification on the basis of morphology seemed likely. For the other 13 strains, a misidentification seemed unlikely, and they were rejected from this study together with the abovementioned 21 unviable, impure, incompletely sequenced, or reclassified strains. A total of 289 strains were thus available for further analysis. These 289 validated strains contained 40 species of *Fusarium sensu stricto* divided across 9 species complexes: the FSSC, FOSC, FFSC, FDSC, FIESC, FCSC, *F. sambucinum* species complex (FSAMSC), *F. tricinctum* species complex (FTSC), and the *F. lateritium* species complex (FLSC). There were 21 one-strain species, of which five remained as *Fusarium* due to phylogenetic and morphological considerations (i.e., insufficient sequence similarity and morphological similarity with known formally described *Fusarium* species). Nineteen species were represented by more than one strain (i.e., a total of 268 strains). In summary, 8.2% of these strains were initially misidentified by classical morphological examination and 35.1% were classified as *Fusarium* sp. only. The other 56.7% of the strains had been correctly identified to the species level.

Phylogenetic analysis. A representation of the obtained BI consensus tree, showing the evolutionary relationships between the species clades in our *Fusarium* data set, is given in Fig. 1a. The effective sample sizes were >100 for most parameters of the BI analysis, and the consensus tree was inferred from the 3,002 remaining trees after burn-in.

***In vitro* antifungal susceptibility testing.** Of the 289 validated *Fusarium* strains, 180 had been isolated from patients (blood samples or biopsy specimens from infected sites). This included 15 species and 4 species complexes. An antifungal susceptibility profile was constructed for each species represented (Table 2). We found that for the azoles, except for voriconazole and ketoconazole, as well as for 5-fluorocytosine, all strains had MIC_{100}s higher than the maximum concentration tested (i.e., 16 μg/ml or 64 μg/ml). For ketoconazole, most strains also displayed a high MIC_{100}. In the FSSC and FOSC as well as for *Fusarium verticillioides* and *F. dimerum*, voriconazole had no or only a limited effect compared to the effects of other antifungals. Amphotericin B was the only drug active against all *Fusarium* spp., except for some strains, especially *F. verticillioides*, which showed a high MIC_{100}. For these *F. verticillioides* strains, terbinafine generally displayed the lowest MIC_{100}. This was also the case in the FDSC and with *Fusarium sacchari* and the 3 species for which only one strain was analyzed. In the FSSC and FOSC, terbinafine had little or no activity. For *Fusarium proliferatum* and *Fusarium musae*, similar MIC_{100}s were observed with terbinafine, amphotericin B, and voriconazole.

MALDI-TOF MS assay, construction of reference spectrum database, and identification strategy. MALDI-TOF MS reference spectra were created for the 289 validated *Fusarium* strains and implemented in our in-house database. Each strain was subsequently challenged against this database according to well-defined criteria, and the individual identification results are shown in Table S1 in the supplemental material. It should be noted that the identification spectra of each strain matched their own reference spectrum with the highest logarithmic scores observed, always well above the standard 2.0 cutoff value. For the 19 species represented by more than one strain, the percentages of strains with a correct species identification, correct species complex identification, and incorrect species complex identification, as well as the percentage of unidentified strains were calculated (Fig. 2). Using 2.0 as a cutoff, we found that 82.8% of the MALDI-TOF MS-based identifications were correct to the species level, whereas 3% of the strains were incorrectly identified, although always within the correct species complex. The incorrectly identified strains include 8 strains (see Table S2 in the supplemental material), with seven of them belonging to the FSSC and the other to the FFSC. For 14.2% of the strains (i.e., 38 strains), the criteria for identification were not fulfilled. Lowering the cutoff value to 1.4 would allow 22 of

TABLE 1 Assessment of the validity of our MALDI-TOF MS approach by screening recent clinical isolates, with a presumable *Fusarium* identity, against our in-house reference spectrum database, using 4 identification spectra, and comparison of the MALDI-TOF MS identification with the one obtained by DNA sequence analysis, using ITS or, when ITS was not sufficiently discriminatory, TEF1α

						Identification spectrum 1		
Reference no.	Source of isolation of the strain (substrate), patient pathology, hospital	Identification by ITS	ITS GenBank accession no.	Identification by TEF1α	TEF1α GenBank accession no.	Top score	Associated IHEM strain in database	Identified as:
12/0930	Human nail, onychomycosis, Universitair Ziekenhuis Brussel	*F. petroliphilum*	KJ173886			1.288	IHEM 2813	*F. petroliphilum*
12/1188	Human nail, onychomycosis, Universitair Ziekenhuis Brussel	*F. oxysporum*	KJ173887			1.768	IHEM 15652	*F. oxysporum*
12/1342	Human nail, contaminant ?, Universitair Ziekenhuis Brussel	*F. equiseti*	KJ173888	*F. equiseti*	KJ173883	1.330	IHEM 3571	*F. equiseti*
12/1573	Human nail, onychomycosis, Universitair Ziekenhuis Brussel	*F. oxysporum*	KJ173889			1.929	IHEM 22005	*F. oxysporum*
13/0077	Human nail, onychomycosis, Universitair Ziekenhuis Brussel	*F. oxysporum*	KJ173890			1.682	IHEM 17811	*F. oxysporum*
13/0080	Human nail, onychomycosis, Universitair Ziekenhuis Brussel	*F. oxysporum*	KJ173891			1.891	IHEM 21643	*F. oxysporum*
13/0091	Human nail, onychomycosis, Universitair Ziekenhuis Brussel	*F. oxysporum*	KJ173892			1.949	IHEM 18448	*F. oxysporum*
13/0093	Human nail, onychomycosis, Universitair Ziekenhuis Brussel	*F. oxysporum*	KJ173893			1.872	IHEM 25352	*F. oxysporum*
13/0158	Human nail, onychomycosis, Universitair Ziekenhuis Brussel	*F. petroliphilum*	KJ173894			2.104	IHEM 22467	*F. petroliphilum*
13/0585	Human tracheal secretions, cardiac arrest and prolonged resuscitation at intensive care unit, Universitair Ziekenhuis Brussel	*F. oxysporum*	KJ173895			2.181	IHEM 15652	*F. oxysporum*
13/0638	Human tracheal secretions, cardiac arrest and prolonged resuscitation at intensive care unit, Universitair Ziekenhuis Brussel	*F. oxysporum*	KJ173896			2.099	IHEM 18037	*F. oxysporum*
13/0882	Human nail, onychomycosis, Universitair Ziekenhuis Brussel	*F. oxysporum*	KJ173897			2.001	IHEM 18037	*F. oxysporum*
13/0889	Human tracheal secretions, cardiac arrest, Universitair Ziekenhuis Brussel	*F. oxysporum*	KJ173898			2.042	IHEM 18037	*F. oxysporum*
13/1134	Human nail, onychomycosis, Universitair Ziekenhuis Brussel	*F. oxysporum*	KJ173899			2.026	IHEM 15652	*F. oxysporum*
13/1149	Human nail, onychomycosis, Universitair Ziekenhuis Brussel	*F. oxysporum*	KJ173900			2.450	IHEM 23153	*F. oxysporum*
13/1159	Human nail, onychomycosis, Universitair Ziekenhuis Brussel	*F. proliferatum*	KJ173901	*F. proliferatum*	KJ173884	1.688	IHEM 25354	*F. proliferatum*
13/1161	Human nail, onychomycosis, Universitair Ziekenhuis Brussel	*F. petroliphilum*	KJ173902			2.353	IHEM 18411	*F. petroliphilum*
12/0547	Human toe, lymphoblastic lymphoma, Universitair Medisch Centrum Sint-Pieter	*F. solani*	KJ173903			2.387	IHEM 19488	*F. solani*
13/0176	Human brain biopsy specimen, transplant patient, Universitair Medisch Centrum Sint-Pieter	*F. oxysporum*	KJ173904			1.885	IHEM 17811	*F. oxysporum*
13/0678	Human blood, acute leukemia and chemotherapy, Universitair Medisch Centrum Sint-Pieter	*F. verticillioides*	KJ173905	*F. musae*	KJ173885	2.080	IHEM 18495	*F. musae*

[a] MALDI-TOF MS identification of a strain was considered interpretable when two criteria were fulfilled: (i) at least three out of four identification spectra are identified by the reference spectrum of the same species, and (ii) at least one of their top scores meets the cutoff listed here (i.e., >2.0 or >1.8).

these strains to be identified correctly to the species level and 7 to the species complex level (see Table S2), resulting in an overall success rate of 91% correct species identifications and 5.6% correct species complex identifications. When we also consider the FSSC and the FIESC as a whole, an additional 5 strains would have been identified correctly, leading to an overall success rate of 97% correct identifications. The inability of MALDI-TOF MS to achieve identification would then concern 4 strains: one *F. incarnatum* strain, one *F. equiseti* strain, one *Fusarium sporotrichioides* strain, and one *F. sacchari* strain. No incorrect species complex or non-*Fusarium* spp. identifications occurred by MALDI-TOF MS. Thirteen of the 21 species represented by only one strain in the data set were identified as a phylogenetically closely related species with our MALDI-TOF MS approach when using 1.4 as the cutoff (see Table S2). The other one-strain species remained unidentified or were misidentified.

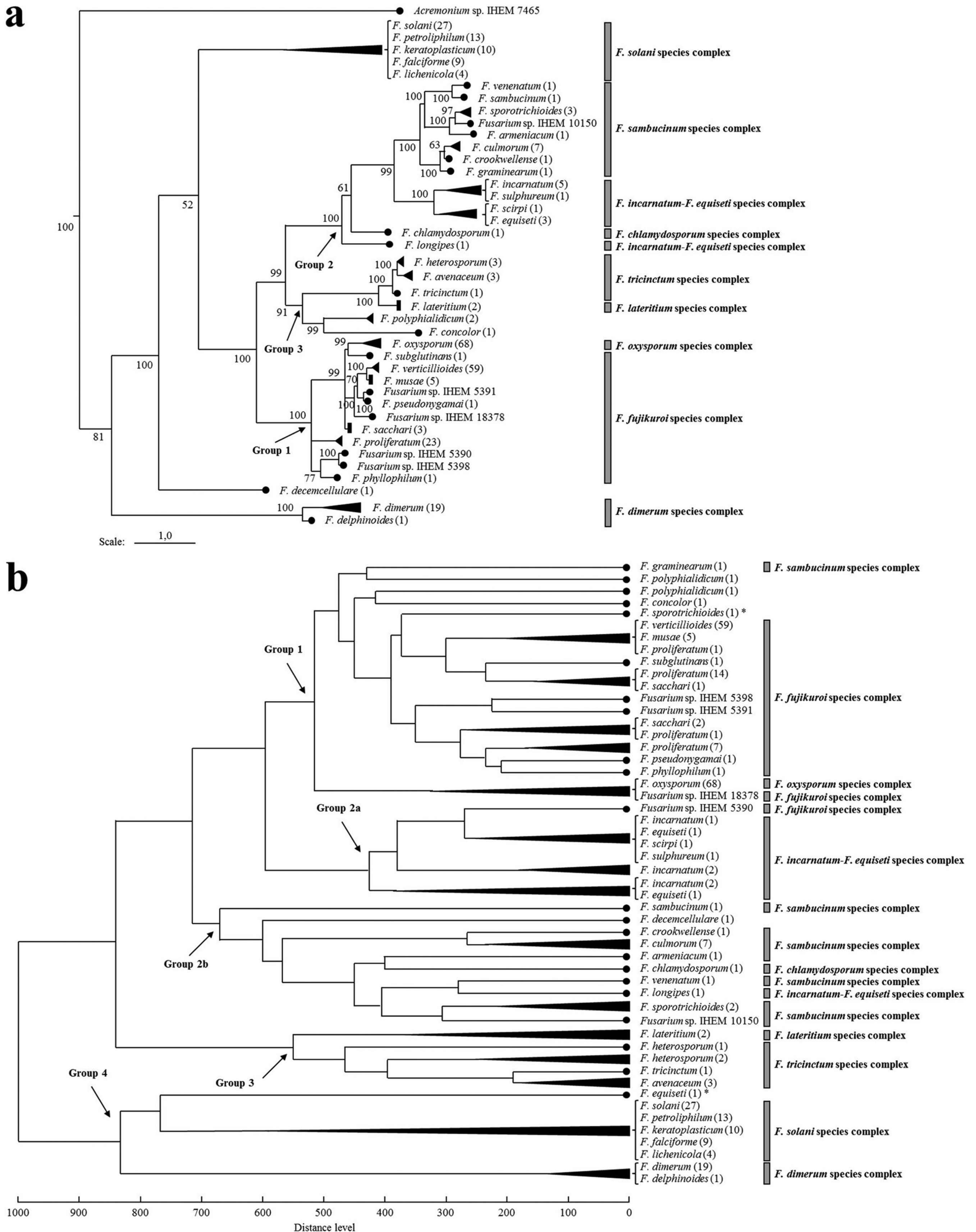
Comparison. A distance matrix dendrogram of the 289 *Fusarium* reference spectra was constructed, showing the relationships between the different strains and species (Fig. 1b). We observed that with exception of a *F. equiseti* (IHEM 3571) and a *F. sporotrichioides* (IHEM 3235) strain, all strains of a same species clustered together into a single clade or closely related clades. The two aberrant strains are also the ones that were not identified by MALDI-TOF MS. The topology of the dendrogram appeared to be similar

TABLE 1 (Continued)

Identification spectrum 2			Identification spectrum 3			Identification spectrum 4			MALDI-TOF MS identification with cutoff of[a]:	
Top score	Associated IHEM strain in database	Identified as:	Top score	Associated IHEM strain in database	Identified as:	Top score	Associated IHEM strain in database	Identified as:	>2.0	>1.8
1.649	IHEM 18411	*F. petroliphilum*	1.704	IHEM 18411	*F. petroliphilum*	1.844	IHEM 18411	*F. petroliphilum*	Criteria not fulfilled	*F. petroliphilum*
1.940	IHEM 15652	*F. oxysporum*	1.817	IHEM 19994	*F. oxysporum*	1.839	IHEM 23185	*F. oxysporum*	Criteria not fulfilled	*F. oxysporum*
1.384	IHEM 20883	*F. incarnatum*	1.344	IHEM 15929	*F. sporotrichioides*	1.332	IHEM 20883	*F. incarnatum*	Criteria not fulfilled	Criteria not fulfilled
1.643	IHEM 15895	*F. oxysporum*	1.658	IHEM 22005	*F. oxysporum*	1.709	IHEM 22005	*F. oxysporum*	Criteria not fulfilled	*F. oxysporum*
1.701	IHEM 18037	*F. oxysporum*	1.803	IHEM 19992	*F. oxysporum*	1.747	IHEM 19992	*F. oxysporum*	Criteria not fulfilled	*F. oxysporum*
1.971	IHEM 21643	*F. oxysporum*	1.948	IHEM 21643	*F. oxysporum*	1.844	IHEM 21643	*F. oxysporum*	Criteria not fulfilled	*F. oxysporum*
1.930	IHEM 25352	*F. oxysporum*	2.080	IHEM 18448	*F. oxysporum*	2.047	IHEM 18448	*F. oxysporum*	*F. oxysporum*	*F. oxysporum*
1.984	IHEM 25352	*F. oxysporum*	2.081	IHEM 19994	*F. oxysporum*	2.079	IHEM 19994	*F. oxysporum*	*F. oxysporum*	*F. oxysporum*
1.586	IHEM 18410	*F. petroliphilum*	1.959	IHEM 18410	*F. petroliphilum*	1.594	IHEM 18528	*F. solani*	*F. petroliphilum*	*F. petroliphilum*
2.102	IHEM 19994	*F. oxysporum*	1.936	IHEM 25352	*F. oxysporum*	2.246	IHEM 15652	*F. oxysporum*	*F. oxysporum*	*F. oxysporum*
2.021	IHEM 18037	*F. oxysporum*	2.091	IHEM 18037	*F. oxysporum*	2.042	IHEM 18037	*F. oxysporum*	*F. oxysporum*	*F. oxysporum*
2.093	IHEM 18037	*F. oxysporum*	2.041	IHEM 18037	*F. oxysporum*	2.084	IHEM 18037	*F. oxysporum*	*F. oxysporum*	*F. oxysporum*
2.077	IHEM 18037	*F. oxysporum*	2.022	IHEM 18037	*F. oxysporum*	2.091	IHEM 18037	*F. oxysporum*	*F. oxysporum*	*F. oxysporum*
1.793	IHEM 15652	*F. oxysporum*	1.918	IHEM 15652	*F. oxysporum*	2.070	IHEM 15652	*F. oxysporum*	*F. oxysporum*	*F. oxysporum*
2.381	IHEM 13316	*F. oxysporum*	2.396	IHEM 13316	*F. oxysporum*	2.378	IHEM 13316	*F. oxysporum*	*F. oxysporum*	*F. oxysporum*
1.905	IHEM 25671	*F. proliferatum*	1.708	IHEM 9534	*F. verticillioides*	1.790	IHEM 25671	*F. proliferatum*	Criteria not fulfilled	*F. proliferatum*
2.312	IHEM 18411	*F. petroliphilum*	2.123	IHEM 18411	*F. petroliphilum*	2.311	IHEM 18411	*F. petroliphilum*	*F. petroliphilum*	*F. petroliphilum*
2.270	IHEM 19488	*F. solani*	2.316	IHEM 19488	*F. solani*	2.263	IHEM 19488	*F. solani*	*F. solani*	*F. solani*
1.861	IHEM 17890	*F. oxysporum*	1.547	IHEM 23189	*F. oxysporum*	1.955	IHEM 9896	*F. oxysporum*	Criteria not fulfilled	*F. oxysporum*
2.137	IHEM 19881	*F. musae*	2.170	IHEM 18495	*F. musae*	2.104	IHEM 19667	*F. musae*	*F. musae*	*F. musae*

to that of the BI consensus tree (Fig. 1a). To emphasize this, we defined 4 groups of species forming a well-supported lineage in the phylogeny (group 4, comprising the species at the basis of the tree). These groups of species were also distinguished, with few inconsistencies, in the dendrogram. Differences with the BI consensus tree were noticeable for the species *Fusarium graminearum* (in group 1 instead of group 2), *Fusarium polyphialidicum* (in group 1 instead of group 3), *Fusarium concolor* (in group 1 instead of group 3), *Fusarium* sp. strain IHEM 5390 (in group 2a instead of group 1), *Fusarium decemcellulare* (in group 2b instead of group 4), and the aberrant strains IHEM 3235 and IHEM 3571 (in groups 1 and 4, respectively, instead of group 2). In addition, we found that most members of the same species complex also clustered together in the dendrogram, with the exception of *Fusarium longipes*, *F. graminearum*, *Fusarium* sp. strain IHEM 5390, *Fusarium* sp. strain IHEM 18378, and the two aberrant strains.

Proof of principle. A screening of the 20 recent clinical isolates against our in-house database revealed a *Fusarium* identity for each of them (Table 1). Nineteen isolates received a species identity by MALDI-TOF MS, though only when a cutoff of 1.8 instead of the standard 2.0 was used for accepting identification; otherwise, eight isolates could not be identified. For isolate 12/1342, 3 of the 4 identification spectra corresponded to a member of the FIESC (i.e., *F. incarnatum* or *F. equiseti*) but with top scores of <1.4 (Table 1). The MALDI-TOF MS identifications were all confirmed by the identifications made with DNA sequencing. In three



a
Acremonium sp. IHEM 7465
F. solani (27)
F. petroliphilum (13)
F. keratoplasticum (10)
F. falciforme (9)
F. lichenicola (4)
F. solani species complex
F. venenatum (1)
F. sambucinum (1)
F. sporotrichioides (3)
Fusarium sp. IHEM 10150
F. armeniacum (1)
F. culmorum (7)
F. crookwellense (1)
F. graminearum (1)
F. sambucinum species complex
F. incarnatum (5)
F. sulphureum (1)
F. scirpi (1)
F. equiseti (3)
F. incarnatum-F. equiseti species complex
F. chlamydosporum (1)
F. chlamydosporum species complex
F. longipes (1)
F. incarnatum-F. equiseti species complex
Group 2
F. heterosporum (3)
F. avenaceum (3)
F. tricinctum (1)
F. tricinctum species complex
F. lateritium (2)
F. lateritium species complex
F. polyphialidicum (2)
F. concolor (1)
Group 3
F. oxysporum (68)
F. oxysporum species complex
F. subglutinans (1)
F. verticillioides (59)
F. musae (5)
Fusarium sp. IHEM 5391
F. pseudonygamai (1)
Fusarium sp. IHEM 18378
F. sacchari (3)
F. proliferatum (23)
Group 1
Fusarium sp. IHEM 5390
Fusarium sp. IHEM 5398
F. phyllophilum (1)
F. fujikuroi species complex
F. decemcellulare (1)
F. dimerum (19)
F. delphinoides (1)
F. dimerum species complex
100 52 100 100 81 99 91 99 100 61 100 99 100 100 97 100 63 100 100 100 100 100 99 99 100 70 100 100 100 100 77
Scale: 1,0
b
F. graminearum (1)
F. sambucinum species complex
F. polyphialidicum (1)
F. polyphialidicum (1)
F. concolor (1)
F. sporotrichioides (1) *
F. verticillioides (59)
F. musae (5)
F. proliferatum (1)
F. subglutinans (1)
F. proliferatum (14)
F. sacchari (1)
Fusarium sp. IHEM 5398
Fusarium sp. IHEM 5391
F. sacchari (2)
F. proliferatum (1)
F. proliferatum (7)
F. pseudonygamai (1)
F. phyllophilum (1)
F. fujikuroi species complex
Group 1
F. oxysporum (68)
F. oxysporum species complex
Fusarium sp. IHEM 18378
F. fujikuroi species complex
Fusarium sp. IHEM 5390
F. fujikuroi species complex
Group 2a
F. incarnatum (1)
F. equiseti (1)
F. scirpi (1)
F. sulphureum (1)
F. incarnatum (2)
F. incarnatum (2)
F. equiseti (1)
F. incarnatum-F. equiseti species complex
F. sambucinum (1)
F. sambucinum species complex
F. decemcellulare (1)
F. crookwellense (1)
F. culmorum (7)
F. armeniacum (1)
F. sambucinum species complex
Group 2b
F. chlamydosporum (1)
F. chlamydosporum species complex
F. venenatum (1)
F. sambucinum species complex
F. longipes (1)
F. incarnatum-F. equiseti species complex
F. sporotrichioides (2)
Fusarium sp. IHEM 10150
F. sambucinum species complex
F. lateritium (2)
F. lateritium species complex
F. heterosporum (1)
F. heterosporum (2)
F. tricinctum (1)
F. avenaceum (3)
F. tricinctum species complex
F. equiseti (1) *
Group 3
Group 4
F. solani (27)
F. petroliphilum (13)
F. keratoplasticum (10)
F. falciforme (9)
F. lichenicola (4)
F. solani species complex
F. dimerum (19)
F. delphinoides (1)
F. dimerum species complex
1000 900 800 700 600 500 400 300 200 100 0
Distance level

cases, TEF1α sequencing was deemed necessary for reliable DNA sequence-based identification. For isolate 13/0678, this resulted in an identification of *F. musae* instead of *F. verticillioides* using ITS only, which was concordant with the obtained MALDI-TOF MS identity. A total of 6 distinct *Fusarium* spp. were identified: *F. petroliphilum* (3 strains), *F. oxysporum* (13 strains), *F. proliferatum* (1 strain), *F. solani* (1 strain), *F. musae* (1 strain), and a member of the FIESC (i.e., *F. equiseti*).

DISCUSSION

The present study demonstrates the validity of the standardized MALDI-TOF MS assay for the identification of molds developed by Cassagne et al. (33), to identify *Fusarium* isolates in the clinical setting. No incorrect species complex or non-*Fusarium* identifications were made by MALDI-TOF MS and our in-house reference spectrum database. However, identification of the correct species complex member was not always guaranteed, and some strains remained unidentified. The problem of unidentified strains can be resolved without increasing the number of misidentifications by lowering the cutoff value for accepting identification, the feasibility of which has been demonstrated in previous studies (42). Moreover, taking into account the FSSC and the FIESC as a whole and not discriminating between its members would increase the percentage of correct identifications even more. This leaves only 4 unidentified strains of species rarely or not yet encountered as human pathogen (1).

In vitro antifungal susceptibility testing showed that discriminating between *Fusarium* species complexes can indeed be important for appropriate patient treatment, as previously indicated (27). Although amphotericin B was active against all strains analyzed and was the drug of choice for all members of the FSSC and FOSC (i.e., the other antifungals tested had no or only a limited effect), our results suggest alternatives for members of the FFSC and FDSC. For *F. verticillioides*, *F. sacchari*, *F. dimerum*, and *Fusarium delphinoides*, terbinafine generally showed the lowest MIC_{100}, and for *F. sacchari* and *F. delphinoides*, voriconazole might also be a good alternative. Similar MIC_{100}s for amphotericin B, terbinafine, and voriconazole were observed with *F. proliferatum* and *F. musae* strains. Discriminating between members of the same species complex thus appeared not to be that clinically relevant, with the exception of the FFSC.

Since our MALDI-TOF MS approach did not make any incorrect species complex identifications, and most incorrect species identifications occurred within the FSSC, the most resistant species complex for which only amphotericin B seems effective, these misidentifications are likely to have no clinical implications (i.e., no repercussion on the treatment of the patient). Moreover, in the FSSC, many new species formerly identified as *F. solani sensu stricto* are defined or may be defined in the near future based upon the phylogenetic species concept (13). The species *Fusarium keratoplasticum* and *F. petroliphilum* are examples of this. They were described by Short et al. (13) as phylogenetically distinct species, despite the fact that they cannot be discriminated from each other or from other FSSC members on the basis of morphology, niche, or clinical appearance. It can be questioned whether identification to the species level is clinically relevant in this species complex, though from an epidemiological point of view, it is always recommended. When we consider the FSSC as a whole and no distinction is made between its members, a success ratio of 100% correct identifications would be obtained with MALDI-TOF MS for this species complex, comparable to that of *F. oxysporum*.

In the FFSC, for which correct species identification seems important due to differences in antifungal susceptibility profiles, the applied MALDI-TOF MS assay works well. Only for the *F. sacchari* strains and one *F. musae* strain we were unable to obtain a correct species identification by MALDI-TOF MS. Yet, the clinical significance of these species is rather limited, and they are recognized as a phylogenetically closely related species (i.e., *F. proliferatum* and *F. verticillioides*, respectively) (1, 62). Other incorrect species identifications occurred with members of species complexes that are of less clinical importance (i.e., the FIESC) or that have not been found to be associated with human pathogenesis (i.e., the FSAMSC).

With respect to the complexity of the *Fusarium* genus and its often closely related species, our identification results are comparable to those obtained in other studies using the same standardized MALDI-TOF MS procedure. Cassagne et al. (33), L'Ollivier et al. (40), Gautier et al. (43), and Ranque et al. (44) correctly identified 87%, 97.8%, 98.8%, and 89% of their clinical mold isolates, respectively. Although the assay was optimized on a large panel of clinically relevant molds and several genera have been analyzed, its feasibility for identification within the *Fusarium* genus was not yet established in detail. To our knowledge, Marinach-Patrice et al. (48) was the only study to focus on the capacity of MALDI-TOF MS to identify *Fusarium* isolates. However, the reference spectrum database used in this study contained only 5 *Fusarium* spp., represented by a few strains, and species other than *Fusarium* were not included. In contrast, our applied in-house database contained 40 different *Fusarium* spp., of which 19 had more than one strain, as well as various other species from 152 different fungal genera. Moreover, it is well known that the currently available Bruker database contains only a limited number of mold reference spectra, insufficiently capturing the diversity in clinically relevant species. Using the Bruker database, identification within *Fusarium* seems extremely problematic, since only one species (i.e., *F. proliferatum*) is represented.

The robustness of our reference spectrum database and principle of our MALDI-TOF MS approach were proven by successfully screening recent clinical *Fusarium* isolates. All isolates except one were correctly identified to the species level by MALDI-TOF MS. For one isolate, MALDI-TOF MS even outperformed ITS sequencing. Another isolate appeared to be *F. equiseti*, but

FIG 1 (a) Representation of our BI consensus tree of combined sequences (ITS, BT, TEF1α, and LSU). The different species clades are shown. Our *Fusarium* data set consisted of 289 validated strains, containing 40 species (the number of strains per species is indicated in parentheses) divided across 9 species complexes. Species with an unresolved phylogenetic relationship are combined with a bracket. Posterior probabilities (in percentages) are indicated at the nodes of the tree. An *Acremonium* sp. was applied to root the tree. (b) Representation of the distance matrix dendrogram of our *Fusarium* reference spectra. The different species clades are shown. Most strains of a same species fall into a single clade or closely related clades. The topology of this dendrogram looks similar to that of the BI consensus tree, as the major species groups in phylogeny (indicated by an arrow and with group 4 comprising the species at the basis of the tree) were also found to be distinguishable and with few inconsistencies in the dendrogram. Most members of a same species complex also clustered together in the dendrogram. *, misidentified strain.

TABLE 2 *In vitro* antifungal susceptibility profiles (distribution of the MIC_{100} per antifungal tested) of the *Fusarium* spp. represented in the 180 patient-isolated strains

Antifungal agent[a]	*Fusarium* species (no. of isolates)	Species complex	No. of strains with MIC_{100} (μg/ml) of[b]:							
			0.25	0.5	1	2	4	8	16	>16
Amphotericin B	*F. solani* (21)	*F. solani*			3	3	10	4	1	
	F. falciforme (6)	*F. solani*			2	3	1			
	F. petroliphilum (10)	*F. solani*				5	5			
	F. keratoplasticum (10)	*F. solani*				1	5	3	1	
	F. lichenicola (3)	*F. solani*				1	2			
	F. oxysporum (47)	*F. oxysporum*				1	25	19	1	1
	F. verticillioides (39)	*F. fujikuroi*				1	3	20	14	1
	F. proliferatum (16)	*F. fujikuroi*					8	7	1	
	F. musae (4)	*F. fujikuroi*					1	3		
	F. sacchari (3)	*F. fujikuroi*				2		1		
	F. pseudonygamai (1)	*F. fujikuroi*						1		
	F. dimerum (17)	*F. dimerum*			3	7	7			
	F. delphinoides (1)	*F. dimerum*				1				
	F. concolor (1)						1			
	F. decemcellulare (1)						1			
Voriconazole	*F. solani* (21)	*F. solani*				1	1		5	14
	F. falciforme (6)	*F. solani*						2	2	2
	F. petroliphilum (10)	*F. solani*							3	7
	F. keratoplasticum (10)	*F. solani*						1	4	5
	F. lichenicola (3)	*F. solani*					1	1	1	
	F. oxysporum (47)	*F. oxysporum*					8	20	10	9
	F. verticillioides (39)	*F. fujikuroi*				6	3	2	1	27
	F. proliferatum (16)	*F. fujikuroi*				1	4	6	4	1
	F. musae (4)	*F. fujikuroi*					2	2		
	F. sacchari (3)	*F. fujikuroi*					2	1		
	F. pseudonygamai (1)	*F. fujikuroi*					1			
	F. dimerum (17)	*F. dimerum*					2	10	5	
	F. delphinoides (1)	*F. dimerum*				1				
	F. concolor (1)							1		
	F. decemcellulare (1)					1				
Terbinafine	*F. solani* (21)	*F. solani*								21
	F. falciforme (6)	*F. solani*								6
	F. petroliphilum (10)	*F. solani*								10
	F. keratoplasticum (10)	*F. solani*								10
	F. lichenicola (3)	*F. solani*								3
	F. oxysporum (47)	*F. oxysporum*					6	4	4	33
	F. verticillioides (39)	*F. fujikuroi*		2	3	13	12	2	3	4
	F. proliferatum (16)	*F. fujikuroi*				5	5	5		1
	F. musae (4)	*F. fujikuroi*				1	2		1	
	F. sacchari (3)	*F. fujikuroi*			2	1				
	F. pseudonygamai (1)	*F. fujikuroi*			1					
	F. dimerum (17)	*F. dimerum*	3	12	2					
	F. delphinoides (1)	*F. dimerum*			1					
	F. concolor (1)					1				
	F. decemcellulare (1)			1						
Ketoconazole	*F. solani* (21)	*F. solani*							1	20
	F. falciforme (6)	*F. solani*								6
	F. petroliphilum (10)	*F. solani*								10
	F. keratoplasticum (10)	*F. solani*								10
	F. lichenicola (3)	*F. solani*								3
	F. oxysporum (47)	*F. oxysporum*								47
	F. verticillioides (39)	*F. fujikuroi*					3	3	4	29
	F. proliferatum (16)	*F. fujikuroi*					2	1		13
	F. musae (4)	*F. fujikuroi*							3	1
	F. sacchari (3)	*F. fujikuroi*								3
	F. pseudonygamai (1)	*F. fujikuroi*								1
	F. dimerum (17)	*F. dimerum*								17
	F. delphinoides (1)	*F. dimerum*								1
	F. concolor (1)									1
	F. decemcellulare (1)									1

[a] For itraconazole, posaconazole, fluconazole, and 5-fluorocytosine, all strains had a MIC_{100} higher than the maximum concentration tested.
[b] MIC_{100}s of $<$0.25 μg/ml were not observed.

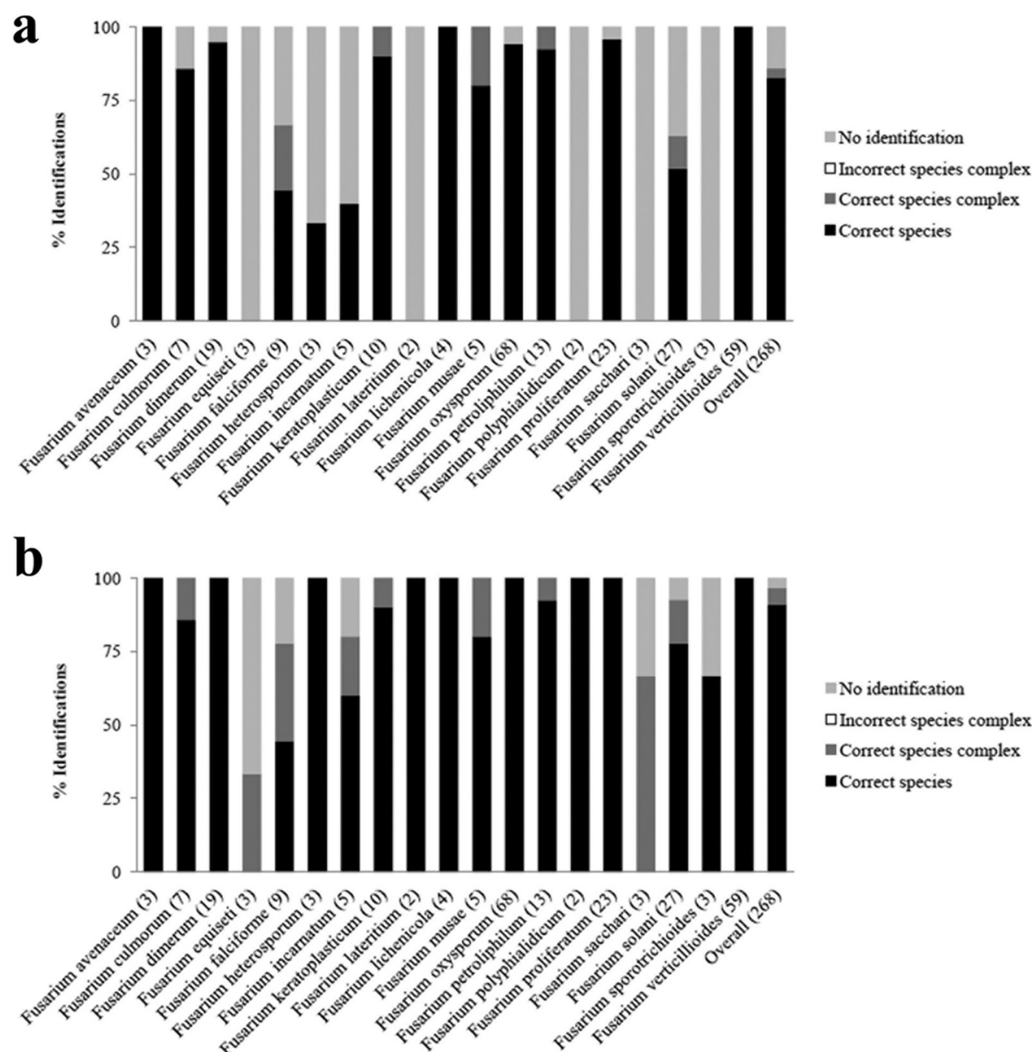


FIG 2 Summary of the identification results with the MALDI-TOF MS assay. The validated *Fusarium* strains were screened against our in-house reference spectrum database, according to well-defined identification criteria using a cutoff of either 2.0 (a) or 1.4 (b). The MALDI-TOF MS identifications were compared with those obtained by the multilocus sequence analysis. For the 19 species represented by more than one strain in the data set, the percentages of strains with a correct species identification, correct species complex identification, and incorrect species complex identification, as well as the percentage of unidentified strains were assessed. The number of strains per species is indicated in parentheses.

MALDI-TOF MS identified it only as a member of the FIESC, with low identification scores. The identification of FIESC members seems difficult with the applied MALDI-TOF MS approach. Also, when screening our collection material, only 37.5% of the FIESC strains were identified correctly to the species level, even when the cutoff value for accepting identification was lowered. When the FIESC was taken as a whole and no distinction was made between its members, the success rate was increased to 75%. Nevertheless, the identification scores were often low, especially with *F. equiseti* strains, indicating that the extraction procedure is not that stable for members of this species complex.

Accurate identification by MALDI-TOF MS is largely limited by the species diversity in the reference spectrum database. This is emphasized by the one-strain species in our data set, which could not be correctly identified according to the identification strategy. These species were generally identified as phylogenetically closely related species. This observation triggered a comparison of the available phylogenetic and MALDI-TOF MS data. We saw an interesting similarity in the topology of a BI phylogenetic tree and that of a dendrogram outlining the relationships between the *Fusarium* reference spectra. Such a correlation was also demonstrated in the study of Packeu et al. (49) with molds of the *Trichophyton mentagrophytes* species complex and indicates that phylogenetically closely related species also have similar protein spectra.

In the routine clinical laboratory, the advantages of MALDI-TOF MS for the identification of *Fusarium* isolates and molds in general are that it is easy to perform, requires no expert mycologists, and is faster than the conventional identification methods. Furthermore, it allows an objective identification not prone to interpretation by the examiner. With the MALDI-TOF MS assay of Cassagne et al. (33), an identification was generated within 1 h after the 3 days of culture, whereas with DNA sequence analysis, ≥2 additional days are needed after culturing. Moreover, after 3 days of culture, certain characteristics needed for morphological

identification may not yet be developed. Reducing the time frame for mold identification is of major clinical interest, especially with invasive infections for which patient prognosis depends on a timely onset of appropriate treatment. Even more important is that MALDI-TOF MS outperforms the conventional identification of molds. Ranque et al. (44) found a 31% to 61% increase in correct identifications with MALDI-TOF MS compared to the conventional method for their non-*Aspergillus* isolates. After the implementation of MALDI-TOF MS in a clinical routine, Gautier et al. (43) observed a dramatic increase in mold identifications at the species level and a decrease in the rate of misidentifications. In our study, the morphological examination of the *Fusarium* strains upon the time of deposit in the collection showed a correct identification in only 56.7% of the cases to the species level. This percentage was dramatically increased by using MALDI-TOF MS technology, rendering a success rate of 82.8% and even 97% when the cutoff value for accepting identification was lowered and some species complexes were taken as a whole.

In conclusion, our study highlights once again the usefulness of MALDI-TOF MS and in particular the user-friendly standardized procedure of Cassagne et al. (33) for mold identification in the clinical setting. In combination with the constructed in-house reference spectrum database, identification within the *Fusarium* genus was found to be highly accurate, taking into account the complexity of the genus. Indeed, the availability of an extended database of reference spectra is indispensable for routine use, and the database currently provided by the manufacturer might be too limited for mold identification. Therefore, we are currently working on creating an online portal that will allow MALDI-TOF MS identifications to be performed by querying our reference spectrum database.

ACKNOWLEDGMENTS

We thank the Belgian Science Policy (Belspo) for funding, Annelies De Bel for sending us clinical isolates from the Universitair Ziekenhuis Brussel, and Delphine Martiny for sending us clinical isolates from the Universitair Medisch Centrum Sint-Pieter.

REFERENCES

1. **Nucci M, Anaissie E.** 2007. *Fusarium* infections in immunocompromised patients. Clin Microbiol Rev **20:**695–704. http://dx.doi.org/10.1128/CMR.00014-07.
2. **Miceli MH, Lee SA.** 2011. Emerging moulds: epidemiological trends and antifungal resistance. Mycoses **54:**666–678. http://dx.doi.org/10.1111/j.1439-0507.2011.02032.x.
3. **Geiser DM, Aoki T, Bacon CW, Baker SE, Bhattacharyya MK, Brandt ME, Brown DW, Burgess LW, Chulze S, Coleman JJ, Correll JC, Covert SF, Crous PW, Cuomo CA, De Hoog GS, Di Pietro A, Elmer WH, Epstein L, Frandsen RJ, Freeman S, Gagkaeva T, Glenn AE, Gordon TR, Gregory NF, Hammond-Kosack KE, Hanson LE, Jímenez-Gasco Mdel M, Kang S, Kistler HC, Kuldau GA, Leslie JF, Logrieco A, Lu GZ, Lysøe E, Ma LJ, McCormick SP, Migheli Q, Moretti A, Munaut F, O'Donnell K, Pfenning L, Ploetz RC, Proctor RH, Rehner SA, Robert VA, Rooney AP, Bin Salleh B, Scandiani MM, Scauflaire J, Short DP, et al.** 2013. One fungus, one name: defining the genus *Fusarium* in a scientifically robust way that preserves the longstanding use. Phytopathology **103:**400–408. http://dx.doi.org/10.1094/PHYTO-07-12-0150-LE.
4. **O'Donnell K, Cigelnik E, Nirenberg HI.** 1998. Molecular systematics and phylogeography of the *Gibberella fujikuroi* species complex. Mycologia **90:**465–493. http://dx.doi.org/10.2307/3761407.
5. **O'Donnell K.** 2000. Molecular phylogeny of the *Nectria haematococca-Fusarium solani* species complex. Mycologia **92:**919–938. http://dx.doi.org/10.2307/3761588.
6. **O'Donnell K, Nirenberg HI, Aoki T, Cigelnik E.** 2000. A multigene phylogeny of the *Gibberella fujikuroi* species complex: detection of additional phylogenetically distinct species. Mycoscience **41:**61–78. http://dx.doi.org/10.1007/BF02464387.
7. **Summerbell RC, Schroers HJ.** 2002. Analysis of phylogenetic relationship of *Cylindrocarpon lichenicola* and *Acremonium falciforme* to the *Fusarium solani* species complex and a review of similarities in the spectrum of opportunistic infections caused by these fungi. J Clin Microbiol **40:**2866–2875. http://dx.doi.org/10.1128/JCM.40.8.2866-2875.2002.
8. **O'Donnell K, Sutton DA, Rinaldi MG, Magnon KC, Cox PA, Revankar SG, Sanche S, Geiser DM, Juba JH, van Burik JA, Padhye A, Anaissie E, Francesconi A, Walsh TJ, Robinson JS.** 2004. Genetic diversity of human pathogenic members of the *Fusarium oxysporum* complex inferred multilocus DNA sequence data and amplified fragment length polymorphism analyses: evidence for the recent dispersion of a geographically widespread clonal lineage and nosocomial origin. J Clin Microbiol **42:**5109–5120. http://dx.doi.org/10.1128/JCM.42.11.5109-5120.2004.
9. **Zhang N, O'Donnell K, Sutton DA, Nalim FA, Summerbell RC, Padhye A, Geiser DM.** 2006. Members of the *Fusarium solani* species complex that cause infections in both humans and plants are common in the environment. J Clin Microbiol **44:**2186–2190. http://dx.doi.org/10.1128/JCM.00120-06.
10. **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. http://dx.doi.org/10.1128/JCM.02371-07.
11. **Schroers HJ, O'Donnell K, Lamprecht SC, Kammeyer PL, Johnson S, Sutton DA, Rinaldi MG, Geiser DM, Summerbell RC.** 2009. Taxonomy and phylogeny of the *Fusarium dimerum* species group. Mycologia **101:**44–70. http://dx.doi.org/10.3852/08-002.
12. **O'Donnell K, Sutton DA, Rinaldi MG, Gueidan C, Crous PW, Geiser DM.** 2009. Novel multilocus sequence typing scheme reveals high genetic diversity of human pathogenic members of the *Fusarium incarnatum-F. equiseti* and *F. chlamydosporum* species complexes within the United States. J Clin Microbiol **47:**3851–3861. http://dx.doi.org/10.1128/JCM.01616-09.
13. **Short DP, O'Donnell K, Thrane U, Nielsen KF, Zhang N, Juba JH, Geiser DM.** 2013. Phylogenetic relationships among members of the *Fusarium solani* species complex in human infections and the descriptions of *F. keratoplasticum* sp. nov. and *F. petroliphilum* stat. nov. Fungal Genet Biol **53:**59–70. http://dx.doi.org/10.1016/j.fgb.2013.01.004.
14. **Laurence MH, Summerell BA, Burgess LW, Liew EC.** 2014. Genealogical concordance phylogenetic species recognition in the *Fusarium oxysporum* species complex. Fungal Biol **118:**374–384. http://dx.doi.org/10.1016/j.funbio.2014.02.002.
15. **O'Donnell K, Sarver BA, Brandt M, Chang DC, Noble-Wang J, Park BJ, Sutton DA, Benjamin L, Lindsley M, Padhye A, Geiser DM, Ward TJ.** 2007. Phylogenetic diversity and microsphere array-based genotyping of human pathogenic fusaria, including isolates from the multistate contact lens-associated U.S. keratitis outbreaks of 2005 and 2006. J Clin Microbiol **45:**2235–2248. http://dx.doi.org/10.1128/JCM.00533-07.
16. **Azor M, Gené J, Cano J, Guarro J.** 2007. Universal *in vitro* antifungal resistance of genetic clades of the *Fusarium solani* species complex. Antimicrob Agents Chemother **51:**1500–1503. http://dx.doi.org/10.1128/AAC.01618-06.
17. **Azor M, Gené J, Cano J, Sutton DA, Fothergill AW, Rinaldi MG, Guarro J.** 2008. *In vitro* antifungal susceptibility and molecular characterization of clinical isolates of *Fusarium verticillioides* (*F. moniliforme*) and *Fusarium thapsinum*. Antimicrob Agents Chemother **52:**2228–2231. http://dx.doi.org/10.1128/AAC.00176-08.
18. **Alastruey-Izquierdo A, Cuenca-Estrella M, Monzón A, Mellado E, Rodríguez-Tudela JL.** 2008. Antifungal susceptibility profile of clinical *Fusarium* spp. isolates identified by molecular methods. J Antimicrob Chemother **61:**805–809. http://dx.doi.org/10.1093/jac/dkn022.
19. **Iqbal NJ, Boey A, Park BJ, Brandt ME.** 2008. Determination of *in vitro* susceptibility of ocular *Fusarium* spp. isolates from keratitis cases and comparison of Clinical and Laboratory Standards Institute M38-A2 and E test methods. Diagn Microbiol Infect Dis **62:**348–350. http://dx.doi.org/10.1016/j.diagmicrobio.2008.07.003.
20. **Tortorano AM, Prigitano A, Dho G, Esposto MC, Gianni C, Grancini A, Ossi C, Viviani MA.** 2008. Species distribution and *in vitro* antifungal susceptibility patterns of 75 clinical isolates of *Fusarium* spp. from northern Italy. Antimicrob Agents Chemother **52:**2683–2685. http://dx.doi.org/10.1128/AAC.00272-08.

21. **Xie L, Zhai H, Zhao J, Sun S, Shi W, Dong X.** 2008. Antifungal susceptibility for common pathogens of fungal keratitis in Shandong Province, China. Am J Ophthalmol **146:**260–265. http://dx.doi.org/10.1016/j.ajo.2008.04.019.
22. **Azor M, Gené J, Cano J, Manikandan P, Venkatapathy N, Guarro J.** 2009. Less-frequent *Fusarium* species of clinical interest: correlation between morphological and molecular identification and antifungal susceptibility. J Clin Microbiol **47:**1463–1468. http://dx.doi.org/10.1128/JCM.02467-08.
23. **Azor M, Cano J, Gené J, Guarro J.** 2009. High genetic diversity and poor *in vitro* response to antifungals of clinical strains of *Fusarium oxysporum*. J Antimicrob Chemother **63:**1152–1155. http://dx.doi.org/10.1093/jac/dkp095.
24. **Wang H, Xiao M, Kong F, Chen S, Dou HT, Sorrell T, Li RY, Xu YC.** 2011. Accurate and practical identification of 20 *Fusarium* species by seven-locus sequence analysis and reverse line blot hybridization, and an *in vitro* antifungal susceptibility study. J Clin Microbiol **49:**1890–1898. http://dx.doi.org/10.1128/JCM.02415-10.
25. **Homa M, Shobana CS, Singh YR, Manikandan P, Selvam KP, Kredics L, Narendran V, Vágvölgyi C, Galgóczy L.** 2013. *Fusarium* keratitis in South India: causative agents, their antifungal susceptibilities and a rapid identification method for the *Fusarium solani* species complex. Mycoses **56:**501–511. http://dx.doi.org/10.1111/myc.12062.
26. **Oechsler RA, Feilmeier MR, Miller D, Shi W, Hofling-Lima AL, Alfonso EC.** 2013. *Fusarium keratitis: genotyping*, *in vitro* susceptibility and clinical outcomes. Cornea **32:**667–673. http://dx.doi.org/10.1097/ICO.0b013e318277ac74.
27. **Tortorano AM, Prigitano A, Esposto MC, Arsic Arsenijevic V, Kolarovic J, Ivanovic D, Paripovic L, Klingspor L, Nordøy I, Hamal P, Arikan Akdagli S, Ossi C, Grancini A, Cavanna C, Lo Cascio G, Scarparo C, Candoni A, Caira M, Drogari Apiranthitou M, ECMM Working Group.** 2014. European Confederation of Medical Mycology (ECMM) epidemiological survey on invasive infections due to *Fusarium* species in Europe. Eur J Clin Microbiol Infect Dis **33:**1623–1630. http://dx.doi.org/10.1007/s10096-014-2111-1.
28. **Marklein G, Josten M, Klanke U, Müller E, Horré R, Maier T, Wenzel T, Kostrzewa M, Bierbaum G, Hoerauf A, Sahl HG.** 2009. Matrix-assisted laser desorption ionization-time of flight mass spectrometry for fast and reliable identification of clinical yeast isolates. J Clin Microbiol **47:**2912–2917. http://dx.doi.org/10.1128/JCM.00389-09.
29. **Seng P, Drancourt M, Gouriet F, La Scola B, Fournier PE, Rolain JM, Raoult D.** 2009. Ongoing revolution in bacteriology: routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Clin Infect Dis **49:**543–551. http://dx.doi.org/10.1086/600885.
30. **Dhiman N, Hall L, Wohlfiel SL, Buckwalter SP, Wengenack NL.** 2011. Performance and cost analysis of matrix-assisted laser desorption ionization-time of flight mass spectrometry for routine identification of yeast. J Clin Microbiol **49:**1614–1616. http://dx.doi.org/10.1128/JCM.02381-10.
31. **Martiny D, Busson L, Wybo I, El Haj RA, Dediste A, Vandenberg O.** 2012. Comparison of the Microflex LT and Vitek MS systems for routine identification of bacteria by matrix-assisted laser desorption ionization–time of flight mass spectrometry. J Clin Microbiol **50:**1313–1325. http://dx.doi.org/10.1128/JCM.05971-11.
32. **Schmidt V, Jarosch A, März P, Sander C, Vacata V, Kalka-Moll W.** 2012. Rapid identification of bacteria in positive blood culture by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Eur J Clin Microbiol Infect Dis **31:**311–317. http://dx.doi.org/10.1007/s10096-011-1312-0.
33. **Cassagne C, Ranque S, Normand AC, Fourquet P, Thiebault S, Planard C, Hendrickx M, Piarroux R.** 2011. Mould routine identification in the clinical laboratory by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. PLoS One **6:**e28425. http://dx.doi.org/10.1371/journal.pone.0028425.
34. **Santos C, Paterson RR, Venâncio A, Lima N.** 2010. Filamentous fungal characterizations by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. J Appl Microbiol **108:**375–385. http://dx.doi.org/10.1111/j.1365-2672.2009.04448.x.
35. **Alanio A, Beretti JL, Dauphin B, Mellado E, Quesne G, Lacroix C, Amara A, Berche P, Nassif X, Bougnoux ME.** 2011. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry for fast and accurate identification of clinically relevant *Aspergillus* species. Clin Microbiol Infect **17:**750–755. http://dx.doi.org/10.1111/j.1469-0691.2010.03323.x.
36. **Theel ES, Hall L, Mandrekar J, Wengenack NL.** 2011. Dermatophyte identification using matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol **49:**4067–4071. http://dx.doi.org/10.1128/JCM.01280-11.
37. **Alshawa K, Beretti JL, Lacroix C, Feuilhade M, Dauphin B, Quesne G, Hassouni N, Nassif X, Bougnoux ME.** 2012. Successful identification of clinical dermatophyte and *Neoscytalidium* species by matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol **50:**2277–2281. http://dx.doi.org/10.1128/JCM.06634-11.
38. **De Carolis E, Posteraro B, Lass-Flörl C, Vella A, Florio AR, Torelli R, Girmenia C, Colozza C, Tortorano AM, Sanguinetti M, Fadda G.** 2012. Species identification of *Aspergillus*, *Fusarium* and *Mucorales* with direct surface analysis by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Clin Microbiol Infect **18:**475–484. http://dx.doi.org/10.1111/j.1469-0691.2011.03599.x.
39. **Chalupová J, Raus M, Sedlářová M, Sebela M.** 2014. Identification of fungal microorganisms by MALDI-TOF mass spectrometry. Biotechnol Adv **32:**230–241. http://dx.doi.org/10.1016/j.biotechadv.2013.11.002.
40. **L'Ollivier C, Cassagne C, Normand AC, Bouchara JP, Contet-Audonneau N, Hendrickx M, Fourquet P, Coulibaly O, Piarroux R, Ranque S.** 2013. A MALDI-TOF MS procedure for clinical dermatophyte species identification in the routine laboratory. Med Mycol **51:**713–720. http://dx.doi.org/10.3109/13693786.2013.781691.
41. **Nenoff P, Erhard M, Simon JC, Muylowa GK, Herrmann J, Rataj W, Gräser Y.** 2013. MALDI-TOF mass spectrometry—a rapid method for the identification of dermatophyte species. Med Mycol **51:**17–24. http://dx.doi.org/10.3109/13693786.2012.685186.
42. **Normand AC, Cassagne C, Ranque S, L'Ollivier C, Fourquet P, Roesems S, Hendrickx M, Piarroux R.** 2013. Assessment of various parameters to improve MALDI-TOF MS reference spectra libraries constructed for the routine identification of filamentous fungi. BMC Microbiol **13:**76. http://dx.doi.org/10.1186/1471-2180-13-76.
43. **Gautier M, Ranque S, Normand AC, Becker P, Packeu A, Cassagne C, L'Ollivier C, Hendrickx M, Piarroux R.** 2014. MALDI-TOF mass spectrometry: revolutionising clinical laboratory diagnosis of mould infections. Clin Microbiol Infect http://dx.doi.org/10.1111/1469-0691.12750.
44. **Ranque S, Normand AC, Cassagne C, Murat JB, Bourgeois N, Dalle F, Gari-Toussaint M, Fourquet P, Hendrickx M, Piarroux R.** 2014. MALDI-TOF mass spectrometry identification of filamentous fungi in the clinical laboratory. Mycoses **57:**135–140. http://dx.doi.org/10.1111/myc.12115.
45. **Kemptner J, Marchetti-Deschmann M, Mach R, Druzhinina IS, Kubicek CP, Allmaier G.** 2009. Evaluation of matrix-assisted laser desorption/ionization (MALDI) preparation techniques for surface characterization of intact *Fusarium* spores by MALDI linear time-of flight mass spectrometry. Rapid Commun Mass Spectrom **23:**877–884. http://dx.doi.org/10.1002/rcm.3949.
46. **Dong HJ, Kemptner J, Marchetti-Deschmann M, Kubicek CP, Allmaier G.** 2009. Development of a MALDI two-layer volume sample preparation technique for analysis of colored conidia spores of *Fusarium* by MALDI linear TOF mass spectrometry. Anal Bioanal Chem **395:**1373–1383. http://dx.doi.org/10.1007/s00216-009-3067-3.
47. **Seyfarth F, Ziemer M, Sayer HG, Burmester A, Erhard M, Welker M, Schliemann S, Straube E, Hipler UC.** 2008. The use of ITS DNA sequence analysis and MALDI-TOF mass spectrometry in diagnosing an infection with *Fusarium proliferatum*. Exp Dermatol **17:**965–971. http://dx.doi.org/10.1111/j.1600-0625.2008.00726.x.
48. **Marinach-Patrice C, Lethuillier A, Marly A, Brossas JY, Gené J, Symoens F, Datry A, Guarro J, Mazier D, Hennequin C.** 2009. Use of mass spectrometry to identify clinical *Fusarium* isolates. Clin Microbiol Infect **15:**634–642. http://dx.doi.org/10.1111/j.1469-0691.2009.02758.x.
49. **Packeu A, Hendrickx M, Beguin H, Martiny D, Vandenberg O, Detandt M.** 2013. Identification of the *Trichophyton mentagrophytes* complex species using MALDI-TOF mass spectrometry. Med Mycol **51:**580–585. http://dx.doi.org/10.3109/13693786.2013.770605.
50. **O'Donnell K, Humber RA, Geiser DM, Kang S, Park B, Robert VA, Crous PW, Johnston PR, Aoki T, Rooney AP, Rehner SA.** 2012. Phylogenetic diversity of insecticolous fusaria inferred from multilocus DNA sequence data and their molecular identification via FUSARIUM-ID and *Fusarium* MLST. Mycologia **104:**427–445. http://dx.doi.org/10.3852/11-179.
51. **Gräfenhan T, Schroers HJ, Nirenberg HI, Seifert KA.** 2011. An overview of the taxonomy, phylogeny, and typification of nectriaceous fungi in *Cosmospora*, *Acremonium*, *Fusarium*, *Stilbella*, and *Volutella*. Stud Mycol **68:**79–113. http://dx.doi.org/10.3114/sim.2011.68.04.
52. **Beguin H, Pyck N, Hendrickx M, Planard C, Stubbe D, Detandt M.**

2012. The taxonomic status of *Trichophyton quinckeanum* and *T. interdigitale* revisited: a multigene phylogenetic approach. Med Mycol **50:**871–882. http://dx.doi.org/10.3109/13693786.2012.684153.

53. **White TJ, Bruns TD, Lee SB, Taylor JW.** 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, p 315–322. *In* Innis MA, Gelfand DH, Sninsky JJ, White TJ (ed), PCR protocols: a guide to methods and applications. Academic Press, New York, NY.
54. **Hopple JS, Jr, Vilgalys R.** 1999. Phylogenetic relationships in the mushroom genus *Coprinus* and dark-spored allies based on sequence data from the nuclear gene coding for the large ribosomal subunit RNA: divergent domains, outgroups, and monophyly. Mol Phylogenet Evol **13:**1–19. http://dx.doi.org/10.1006/mpev.1999.0634.
55. **Glass NL, Donaldson GC.** 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Appl Environ Microbiol **61:**1323–1330.
56. **Carbone I, Kohn LM.** 1999. A method for designing primer sets for speciation studies in ascomycetes. Mycologia **91:**553–556. http://dx.doi.org/10.2307/3761358.
57. **Leslie JF, Summerell BA, Bullock S.** 2006. The *Fusarium* laboratory manual. Wiley-Blackwell, Hoboken, NJ.
58. **Tamura K, Dudley J, Nei M, Kumar S.** 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol **24:**1596–1599. http://dx.doi.org/10.1093/molbev/msm092.
59. **Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Höhna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP.** 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst Biol **61:**539–542. http://dx.doi.org/10.1093/sysbio/sys029.
60. **Rambaut A, Suchard M, Drummond AJ.** 2007. Tracer v1.5. http://tree.bio.ed.ac.uk/software/tracer/.
61. **Rodriguez-Tudela JL, Arendrup MC, Arikan S, Barchiesi F, Bille J, Chryssanthou E, Cuenca-Estrella M, Dannaoui E, Denning DW, Donnelly JP, Fegeler W, Lass-Flörl C, Moore C, Richardson M, Gaustad P, Schmalreck A, Velegraki A, Verweij P.** 2008. EUCAST DEFINITIVE DOCUMENT E.DEF 9.1: method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for conidia forming moulds. Subcommittee on Antifungal Susceptibility Testing (AFST) of the ESCMID European Committee for Antimicrobial Susceptibility Testing (EUCAST), London, United Kingdom.
62. **Van Hove F, Waalwijk C, Logrieco A, Munaut F, Moretti A.** 2011. *Gibberella musae* (*Fusarium musae*) sp. nov., a recently discovered species from banana is sister to *F. verticillioides*. Mycologia **103:**570–585. http://dx.doi.org/10.3852/10-038.