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Direct Analysis and Identification of Pathogenic *Lichtheimia* Species by Matrix-Assisted Laser Desorption Ionization–Time of Flight Analyzer-Mediated Mass Spectrometry

Wieland Schrödl,[a] Tilo Heydel,[a] Volker U. Schwartze,[b,c] Kerstin Hoffmann,[b] Anke Große-Herrenthey,[a] Grit Walther,[b,d] Ana Alastruey-Izquierdo,[e] Juan Luis Rodriguez-Tudela,[e] Philipp Olias,[f] Ilse D. Jacobsen,[c] G. Sybren de Hoog,[d] and Kerstin Voigt[b,c]

University of Leipzig, Faculty of Veterinary Medicine, Institute of Bacteriology and Mycology, Leipzig, Germany[a]; University of Jena, School of Biology and Pharmacy, Institute of Microbiology, Department of Microbiology and Molecular Biology, Jena, Germany[b]; Leibniz Institute for Natural Product Research and Infection Biology—Hans-Knöll-Institute, Jena, Germany[c]; CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands[d]; Instituto de Salud Carlos III National Centre of Microbiology, Majadahonda (Madrid), Spain[e]; and Freie Universität Berlin, Department of Veterinary Pathology, Berlin, Germany[f]

Zygomycetes of the order Mucorales can cause life-threatening infections in humans. These mucormycoses are emerging and associated with a rapid tissue destruction and high mortality. The resistance of Mucorales to antimycotic substances varies between and within clinically important genera such as *Mucor*, *Rhizopus*, and *Lichtheimia*. Thus, an accurate diagnosis before onset of antimycotic therapy is recommended. Matrix-assisted laser desorption ionization (MALDI)–time of flight (TOF) mass spectrometry (MS) is a potentially powerful tool to rapidly identify infectious agents on the species level. We investigated the potential of MALDI-TOF MS to differentiate *Lichtheimia* species, one of the most important agents of mucormycoses. Using the Bruker Daltonics FlexAnalysis (version 3.0) software package, a spectral database library with *m/z* ratios of 2,000 to 20,000 Da was created for 19 type and reference strains of clinically relevant Zygomycetes of the order Mucorales (12 species in 7 genera). The database was tested for accuracy by use of 34 clinical and environmental isolates of *Lichtheimia* comprising a total of five species. Our data demonstrate that MALDI-TOF MS can be used to clearly discriminate *Lichtheimia* species from other pathogenic species of the Mucorales. Furthermore, the method is suitable to discriminate species within the genus. The reliability and robustness of the MALDI-TOF-based identification are evidenced by high score values (above 2.3) for the designation to a certain species and by moderate score values (below 2.0) for the discrimination between clinically relevant (*Lichtheimia corymbifera*, *L. ramosa*, and *L. ornata*) and irrelevant (*L. hyalospora* and *L. sphaerocystis*) species. In total, all 34 strains were unequivocally identified by MALDI-TOF MS with score values of >1.8 down to the generic level, 32 out of 34 of the *Lichtheimia* isolates (except CNM-CM 5399 and FSU 10566) were identified accurately with score values of >2 (probable species identification), and 25 of 34 isolates were identified to the species level with score values of >2.3 (highly probable species identification). The MALDI-TOF MS-based method reported here was found to be reproducible and accurate, with low consumable costs and minimal preparation time.

Among the basal lineages of terrestrial fungi (formerly Zygomycetes), the Entomophthorales and Mucorales are known to cause infections in humans which are named entomophthoromycoses and mucormycoses, respectively. Whereas entomophthoromycoses are characterized by local infections of the skin and the gastrointestinal tract, mucormycoses comprise deep and systemic infections of the rhinocerebral and bronchorespiratory tract (20). Although both types of mycoses (formerly summarized as zygomycoses) are regarded to be comparatively uncommon, the number of patients with mucormycoses has increased during the last decades (21). These infections are associated with rapid infarct of the blood vessels and high mortality (13). Mucormycosis-inducing pathogens belong to the Mucorales, e.g., *Rhizopus*, *Apophysomyces*, *Mucor*, *Cunninghamella*, and *Lichtheimia* (formerly, *Mycocladus*, *Absidia*) (13, 20, 26).

Susceptibility of different zygomycetes to antifungal drugs varies considerably (2, 3, 4, 8). Therefore, fast and accurate identification of the pathogen is crucial for estimation of the incidence for mucormycoses caused by *Lichtheimia* species in monitoring surveys (e.g., the survey of Skiada et al. [23]) and to enrich repositories (e.g., Fungiscope) with clinical specimens. The conventional diagnosis includes the identification based on the morphology of the cultivated strains or on histopathology (7, 16). Both methods require considerable experience for correct identification of genera and species. Alternatively, molecular identification based on PCR using universal or taxon-specific primers can be used (7, 26). However, the purification of DNA followed by PCR-mediated detection is labor- and time-consuming, is highly dependent on the specificity of the oligonucleotide primers, and often requires subsequent sequencing of the PCR amplicons. Thus, identification by PCR is not easily adaptable to routine analysis in diagnostic laboratories. The requirements for a fast and accurate identification increasingly necessitate the development of more rapid, broad-spectrum identification strategies for clinical use. Diagnostic methodologies based on matrix-assisted laser desorption ionization (MALDI)–time of flight (TOF) mass spectrometry (MS) have successfully been used in recent years to discriminate clinically

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Address correspondence to Kerstin Voigt, kerstin.voigt@uni-jena.de.
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relevant Ascomycetes, e.g., *Candida*, *Aspergillus*, and *Penicillium* (1, 6, 15, 17, 24). Moreover, MALDI-TOF MS was shown to be suitable for routine identification of bacteria with an accuracy of >95% (9, 22). Importantly, it can also be used for cultivation-independent identification of bacteria in blood samples (19). However, the suitability of MALDI-TOF MS for the differentiation of zygomycetes has not been described yet.

Here we present the identification of mucoralean fungi with MALDI-TOF MS using the genus *Lichtheimia* as an example. The genus comprises three clinically relevant species (*Lichtheimia corymbifera*, *L. ramosa*, and *L. ornata*) and two species (*L. hyalospora* and *L. sphaerocystis*) which have not been associated with infections (5). Although *Lichtheimia* spp. are believed to be rare causative agents of infections, their abundance in clinical environments may be underestimated due to a lack of recognition (14). Recently, *Lichtheimia* was reported to be the second (with *Rhizopus* being the first) most common causative agent of zygomycosis in Europe (23). Furthermore, *Lichtheimia* spp. were commonly isolated in Germany from the lungs of white stork chicks with fungal pneumonia (18), suggesting frequent occurrence in the environment. Its complexity and the well-defined species designation render the *Lichtheimia* genus to be an ideal candidate for testing the high resolution and the diagnostic power of MALDI-TOF MS. Our results show that MALDI-TOF MS is a reliable, reproducible, fast, accurate, and cost-effective method for the identification of clinically important zygomycetes.

MATERIALS AND METHODS

Strains and cultivation. A total of 53 fungal strains were used in this study (Table 1) and deposited in the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands (CBS); the Mold Collection of the Spanish National Center for Microbiology, Instituto de Salud Carlos III, Spain (CNM-CM); the strain collection of the Institute for Bacteriology and Mycology, Faculty of Veterinary Medicine at the University of Leipzig (IBML), and the Jena Microbial Resource Collection (FSU). Forty-six strains of different *Lichtheimia* species were used, including 34 clinical isolates, with 21 and 13 isolates being from human and animal hosts, respectively. *Rhizopus oryzae* and *Mucor circinelloides* f. *circinelloides* were used as negative controls/outgroups in Fig. 1, Fig. 2, and Table 2. The species designation was determined or confirmed via molecular identification on the basis of the internal transcribed spacer 1 (ITS1)-5.8S-ITS2 ribosomal DNA (rDNA) sequence. The fungal strains were cultivated in liquid Sabouraud glucose (2%) medium (SIFIN, Berlin, Germany) supplemented with penicillin (100 units/ml) and streptomycin (100 μg/ml) for 24 h at 22°C on a shaker (200 rpm).

Sample preparation for MALDI-TOF MS analysis. One milliliter of each culture was centrifuged for 10 min at 6,500 × *g*. The pellet was resuspended in 1 ml of water (high-pressure liquid chromatography [HPLC] quality; Merck, Germany), mixed thoroughly, centrifuged for 5 min at 6,500 × *g*, resuspended in a mixture of 200 μl bidistilled water and 600 μl absolute ethanol, and stored in tubes (1.5 ml; Eppendorf, Germany) at −75°C overnight. After thawing and collection via centrifugation, the fungal cells were dried at 37°C for 1 h and mixed thoroughly with 50 μl of 70% formic acid (Merck, Germany) and 50 μl acetonitrile (Merck, Germany), followed by a centrifugation step for 2 min at 6,500 × *g*. A volume of 1 μl clear supernatant was placed onto a ground steel MALDI target plate (Bruker Daltonik GmbH, Germany) and allowed to dry at room temperature. Five MALDI target positions per strain were prepared in parallel. Subsequently, each sample was overlaid with 1 μl of matrix, a saturated solution of α-cyano-hydroxy-cinnamic acid in 2.5% trifluoroacetic acid and 50% acetonitrile in water (CHCA; Bruker Daltonik GmbH, Germany), and air dried at room temperature. All chemicals used (Merck, Germany) were designated to be especially suitable for HPLC- or MALDI-based techniques according to the recommendations of the manufacturer. This procedure, including fungal cultivation, was repeated to ensure reproducibility of results.

MALDI-TOF measurement and database creation. Nineteen strains comprising 12 well-characterized strains of *Lichtheimia* species, including type strains of all five species of *Lichtheimia* and 7 additional strains of *Apophysomyces elegans*, *Cokeromyces recurvatus*, *Mucor circinelloides* f. *circinelloides*, *Syncephalastrum racemosum*, *Rhizomucor miehei*, *Rhizopus microsporus* var. *microsporus*, and *R. oryzae*, were used to create the database. The MALDI-TOF MS measurement was performed using a Microflex LT mass spectrometer (Bruker Daltonik GmbH, Germany). The method is proteomics based and displays the overall protein spectra mainly represented by ribosomal proteins. Spectra were recorded in linear positive mode within a mass range from 2,000 to 20,000 Da. Spectrum acquisition was performed automatically using the software FlexControl (Bruker Daltonik GmbH, Germany) in autoexecute mode by rastering the target spot with the default pattern, including spectrum collection 40 times with a laser frequency of 60 Hz. All collected spectra were subjected to analysis with the software package FlexAnalysis (version 3.0; Bruker Daltonik GmbH, Germany). The software performs steps of smoothing, normalization, baseline correction, identification of the most significant peaks, and comparison of all collected spectra to each other (*m/z* values). Five reference spectra were generated per sample, and finally, a master spectrum was created and transferred into our database.

Testing of the database using *Lichtheimia* strains. To test the created database, spectra of 34 additional isolates of *Lichtheimia* species were generated as described above and identified using the database as a library for identification purposes. Results of the pattern-matching process were expressed as proposed by the manufacturer with scores ranging from 0 to 3.0. Interpretation of the log (score [*S*]) values was performed as described by Marklein et al. (17) and van Veen et al. (25). Log (score) values of >1.7 generally indicated relationships on the genus level, and values of >2.0 indicated relationships on the species level. In particular, the meaning of the score values is as follows: 2.300 to 3.000, highly probable species identification; 2.000 to 2.299, secure genus identification, probable species identification; 1.700 to 1.999, probable genus identification; and 0.000 to 1.699, identification not reliable. The dendrograms shown in Fig. 2 and 3 were generated by using the software MALDI Biotyper (version 2.0; Bruker Daltonik GmbH, Germany).

Phylogenetic multilocus analysis. The diagnostic resolution of the MALDI-TOF-generated dendrogram was validated with a multilocus phylogram using a representative set of 12 *Lichtheimia* strains and 1 strain of *Rhizopus oryzae* as the outgroup shown in Fig. 2. Nucleotide sequences for the nuclear large subunit (LSU) ribosomal DNA, the ITS region spanning ITS1-5.8S-ITS2, and the gene encoding actin were generated in one of our previous studies on *Lichtheimia* (5). The server version of MAFFT (www.ebi.ac.uk/Tools/mafft) was used for nucleotide sequence alignment. The combined alignment consists of nucleotide sequences of LSU, ITS, and actin with 648, 889, and 730 characters, respectively. Phylogenetic reconstruction was done using the online version of RaxML HPC-2 on Abe on the CIPRES portal (http://www.phylo.org). The GTRGAMMA model was used for the bootstrapping phase and the final tree. Bootstrap iterations were set to 1,000 replicates.

RESULTS

Comparison of phylogenies based on DNA sequence and mass spectrometric data. Mass spectra were obtained from a total of 53 strains comprising 12 mucoralean species (Table 1). Seven species (each species represented by one strain) known to be clinically relevant (26) were used as outgroup taxa. These are *Apophysomyces elegans*, *Cokeromyces recurvatus*, *Mucor circinelloides* f. *circinelloides*, *Syncephalastrum racemosum*, *Rhizomucor miehei*, *Rhizopus microsporus* var. *microsporus*, and *R. oryzae*. The remaining 46 strains investigated in this study encompass the 5 species of the genus *Lichtheimia*, namely, *L. corymbifera*, *L. ramosa*, *L. ornata*, *L. hyalospora*, and *L. sphaerocystis*, comprising 19, 17, 3, 4, and 3

TABLE 1 Fifty-three strains investigated with present molecular identification based on MALDI-TOF analyses[a]

Species	Strain	Type designation	Equivalent strain designation	Substrate	Country
Lichtheimia corymbifera	**CBS 429.75***	NT	FSU 9682	Soil	Afghanistan
L. corymbifera	**CBS 519.71***	T of *Absidia griseola*	FSU 10164	Kurone developed during the manufacture of soy sauce (koji)	Japan
L. corymbifera	CNM-CM 3346		FSU 10239	Human, sputum	Spain
L. corymbifera	CNM-CM 3415		FSU 10240	Human, ear swab	Spain
L. corymbifera	CNM-CM 4671		FSU 10243	Human, sputum	Spain
L. corymbifera	CNM-CM 4738		FSU 10244	Human, bronchoalveolar lavage fluid	Spain
L. corymbifera	CNM-CM 5039		FSU 10247	Human, peritoneal drainage	Spain
L. corymbifera	CNM-CM 5538		FSU 10252	Human, sputum	Spain
L. corymbifera	CNM-CM 5637		FSU 10253	Human, skin	Spain
L. corymbifera	CNM-CM 5738		FSU 10255	Human, abscess	Spain
L. corymbifera	CNM-CM 5861		FSU 10257	Human, cutaneous wound	Spain
L. corymbifera	FSU 6250			Human, scale	Germany
L. corymbifera	FSU 10563		623	Stork, chick	Germany
L. corymbifera	FSU 10564		829	Stork, chick	Germany
L. corymbifera	FSU 10565		612B	Stork, chick	Germany
L. corymbifera	FSU 10567		909B	Stork, chick	Germany
L. corymbifera	IBML 004-M10012		FSU 10178	Cattle, gut	Germany
L. corymbifera	IBML 006-M10005		FSU 10179	Horse, gut	Germany
L. corymbifera	IBML 007-D10005		FSU 10180	Horse, gut	Germany
L. ramosa	**CBS 103.35***	T of *Absidia gracilis*	FSU 9927	Fruit, *Musa sapientum*	NA
L. ramosa	**CBS 582.65***	NT	FSU 10166	Seed, *Theobroma cacao*	Ghana
L. ramosa	CNM-CM 3013		FSU 10238	Human, wound	Spain
L. ramosa	CNM-CM 4228		FSU 10241	Human, face skin	Spain
L. ramosa	CNM-CM 4849		FSU 10245	Human, skin biopsy	Spain
L. ramosa	CNM-CM 5111		FSU 10248	Human, sputum	Spain
L. ramosa	CNM-CM 5396		FSU 10258	Human, bronchoalveolar lavage fluid	Spain
L. ramosa	CNM-CM 5398		FSU 10250	Human, bronchioaspirate	Spain
L. ramosa	CNM-CM 5399		FSU 10251	Human, bronchioaspirate	Spain
L. ramosa	CNM-CM 5400		FSU 10242	Human, bronchioaspirate	Spain
L. ramosa	CNM-CM 5677		FSU 10254	Human, tracheal aspirate	Spain
L. ramosa	**FSU 6197***	T of *Absidia idahoensis* var. *thermophila*	As 3.4808	Soil	China
L. ramosa	FSU 10156			Human, stool	Germany
L. ramosa	FSU 10566		905A	Stork, chick	Germany
L. ramosa	FSU 10568		909A	Stork, chick	Germany
L. ramosa	IBML 003-D10007		FSU 10175	Cattle, gut	Germany
L. ramosa	IBML 014-M10036			Horse, fur and skin	Germany
L. ornata	**CBS 291.66***	T of *Absidia ornata*	FSU 10165	Bird, dung	India
L. ornata	CBS 958.68*		FSU 10167	NA	NA
L. ornata	CNM-CM 4978		FSU 10246	Human, wound	Spain
L. hyalospora	**CBS 100.28***	T of *Absidia blakesleeana*	FSU 10160	Nut, *Bertholletia excelsa*	USA
L. hyalospora	**CBS 102.36***	T of *Absidia cristata*	FSU 10161	Stem, *Manihot esculenta*	Ghana
L. hyalospora	**CBS 173.67***	NT of *Tieghemella hyalospora*	FSU 10163	Fermented food, taosi	Philippines
L. hyalospora	**CBS 518.71***	T of *Absidia blakesleeana* var. *atrospora*	FSU 10162	Kurone developed during the manufacture of soy sauce (koji)	Japan
L. sphaerocystis	**CBS 420.70***	T	FSU 10079	NA	India
L. sphaerocystis	CBS 647.78		FSU 10638	Dung of mouse	India
L. sphaerocystis	CBS 648.78		FSU 10640	Soil	India
Apophysomyces elegans	**CBS 476.78**	T	FSU 789	Soil, mango orchard	India
Cokeromyces recurvatus	**CBS 158.50**	T	FSU 793	Rabbit, dung	USA
Mucor circinelloides f. circinelloides	**CBS 195.68**	NT of *M. circinelloides*	FSU 10232	Air	The Netherlands

(Continued on following page)

TABLE 1 (Continued)

Species	Strain	Type designation	Equivalent strain designation	Substrate	Country
Rhizomucor miehei	**CBS 182.67**	T of *Mucor miehei*	FSU 9680	Rotting plant, *Parthenium argentatum*	USA
Rhizopus oryzae	**CBS 112.07***	T of *R. oryzae*	FSU 10159	Human, lung	The Netherlands
R. microsporus var. *microsporus*	CBS 308.87		FSU 5255	Human, necrotic skin (hand after a spider bite)	Australia
Syncephalastrum racemosum	DSM 859		FSU 762	NA	NA

[a] Ex-type strains are printed in bold and indicated by T (type strain) or NT (neotype strain). Thirteen strains (marked with asterisks) were used as representative type/reference material and included in the comparative phylogenetic study (as shown in Fig. 2). CBS, Centraalbureau voor Schimmelcultures Utrecht, The Netherlands; CNM-CM, Mold Collection of the Spanish National Center for Microbiology, Instituto de Salud Carlos III, Spain; IBML, Institute for Bacteriology and Mycology, Faculty of Veterinary Medicine at the University of Leipzig, Leipzig, Germany; FSU, Jena Microbial Resource Collection (formerly Fungal Reference Centre of the Friedrich Schiller University Jena, Germany); NA, not available.

strains, respectively. The mass spectra showed clear differences (Fig. 1). The type strains of all five species of *Lichtheimia* (Fig. 1A to E) could be easily distinguished from all other clinically important mucoralean genera, like, e.g., *Rhizopus oryzae* (Fig. 1F). To test the topology-compliance of the cladograms derived from mass spectra, 12 well-characterized strains of *Lichtheimia*, including the type material from all 5 species (analyzed in Fig. 1), were subjected to comparative analyses using 28S (LSU) rDNA, ITS1-5.8S-ITS2, and the actin gene and *Rhizopus oryzae* as the outgroup (Fig. 2). The clear separation of all five *Lichtheimia* species, the split of *L. ramosa* into two subgroups, and the close genetic relatedness of the three clinical species as shown by Alastruey-

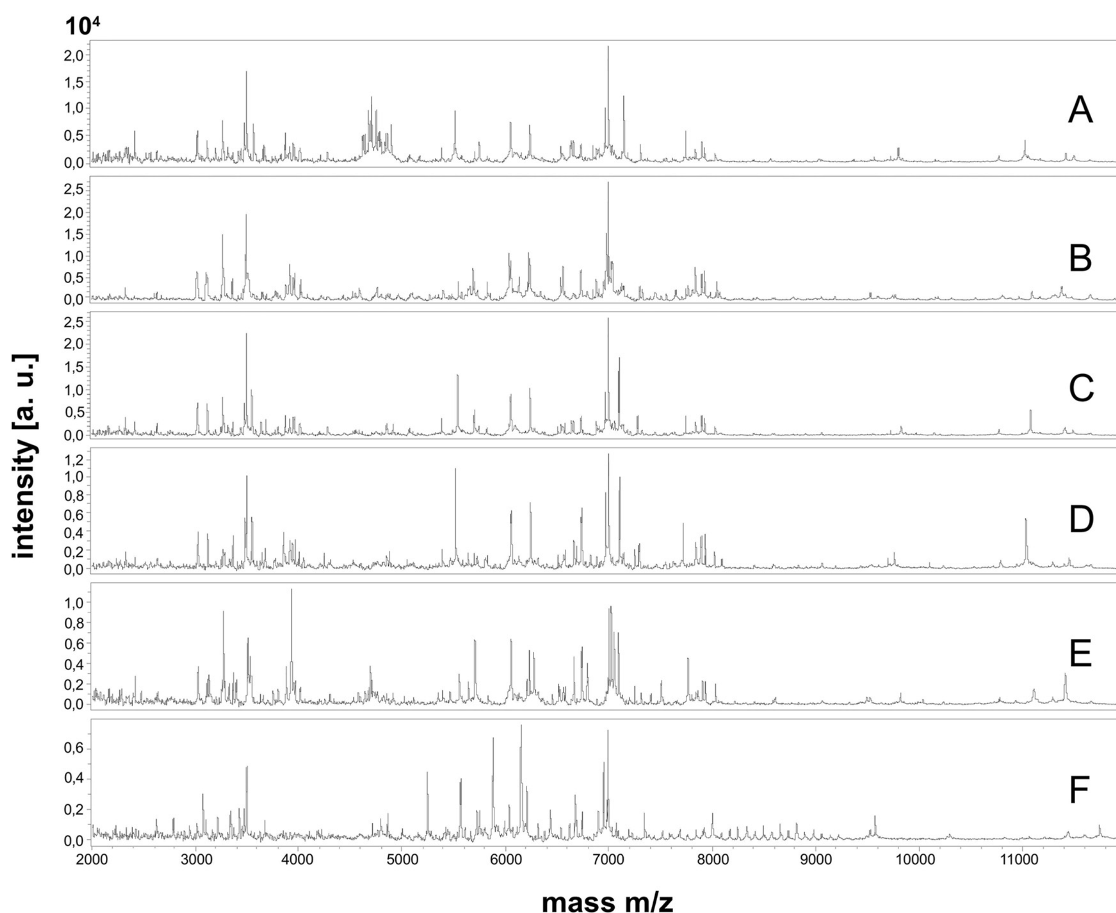

FIG 1 MALDI-TOF spectra (*m/z* 2,000 to 12,000) of *Lichtheimia corymbifera* CBS 429.75^{NT} (A), *L. ramosa* CBS 582.65^{NT} (B), *L. ornata* CBS 291.66^{T} (C), *L. hyalospora* CBS 173.67^{NT} (D), *L. sphaerocystis* CBS 420.70^{T} (E), and *Rhizopus oryzae* CBS 112.07 ^{T} (F). a.u., arbitrary units.

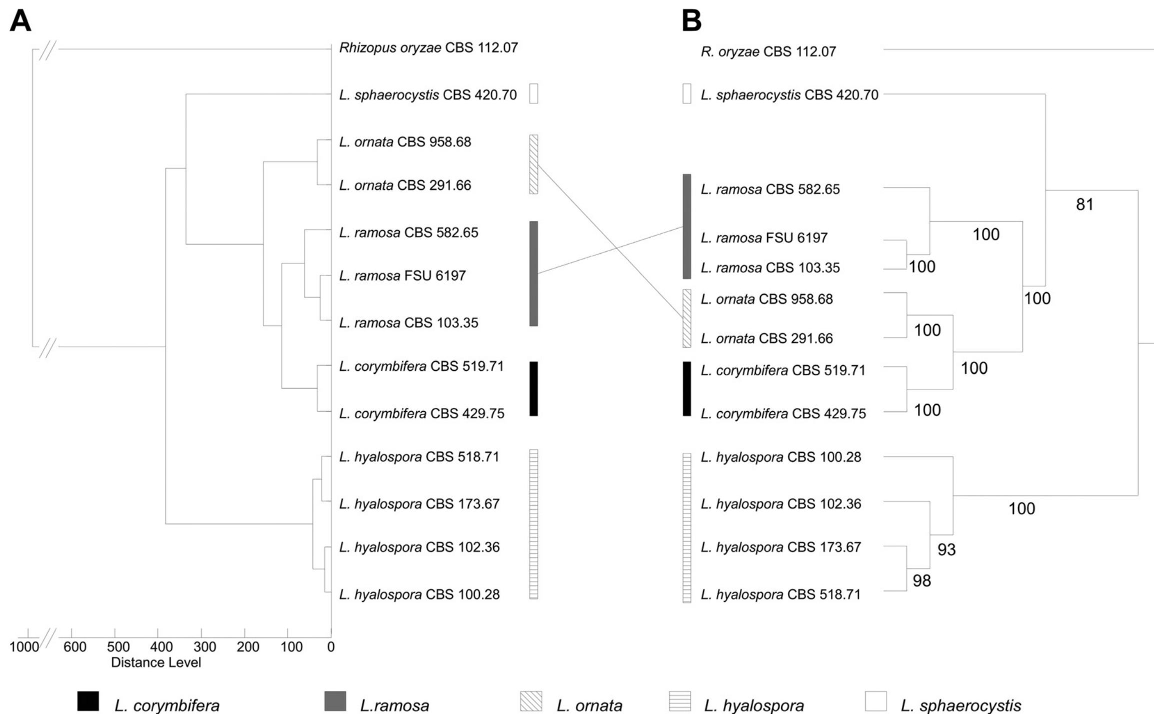

FIG 2 Comparative distance analyses based on mass spectra (A) and combined nucleotide sequences of LSU, ITS, and the actin gene (B) using identical sets of strains. The phylogenetic relationships among the clinically relevant species are supported by bootstrap values of 100%; bootstrap values of >80% support phylogenetic relationships at deeper branches.

Izquierdo et al. (5) could be confirmed in both analyses (Fig. 2A versus B). The phylogenetic tree based on a combined data set of ITS, LSU, and actin sequences revealed a robust branching pattern with bootstrap (BS) values of >80%. The clinically relevant species *L. corymbifera*, *L. ramosa*, and *L. ornata* form a monophyletic group supported by the maximum BS of 100% in the phylogenetic tree (Fig. 2B). Topologies of mass spectrometry-based cladograms and multigene genealogy were almost identical; only the positions of *L. ramosa* and *L. ornata* changed. In the MALDI-TOF MS analysis (Fig. 2A), *L. ornata* is basal to the clade consisting of *L. ramosa* and *L. corymbifera*, while *L. ramosa* appears to be paraphyletic to the monophyletic clade consisting of *L. ornata* and *L. corymbifera* in the phylogenetic analysis (Fig. 2B). This sister group relationship of *L. ornata* and *L. corymbifera* has previously been described on the basis of single-locus analyses for ITS and actin (5).

Identification of clinical isolates of *Lichtheimia* spp. based on comparison of MALDI-TOF mass spectra of the strains with type strain spectra. The reliability of the log (score) values obtained by the MALDI-TOF analyses on *Lichtheimia* was validated in intergeneric comparisons using the mucoralean species *Apophysomyces elegans*, *Mucor circinelloides* f. *circinelloides*, *Rhizopus microsporus* var. *microsporus*, *Rhizopus oryzae*, *Rhizomucor miehei*, and *Syncephalastrum racemosum*, which were reported to be human pathogenic (13, 16, 21, 26). *Rhizopus oryzae* and *Mucor circinelloides* were used as representative outgroup taxa in Fig. 2 and 3. On average, score values were <0.5 when *Lichtheimia* spp. were compared with *Apophysomyces elegans*, *Rhizopus microsporus* var. *microsporus*, *Rhizomucor miehei*, and *Syncephalastrum racemosum* (data not shown). The highest intergeneric score value was 0.902 when *Apophysomyces* was compared with *Syncephalastrum*. Forty-six isolates of *Lichtheimia* (including 5 type strains) were subjected to iterative MALDI-TOF analysis at the intrageneric level (Table 2). Score values were <0.8 when the *Lichtheimia* isolates were compared to *Rhizopus oryzae* and *Mucor circinelloides*. Out of 41 *Lichtheimia* isolates subjected to MALDI-TOF-based species delimitation analyses, 18, 16, 2, 3, and 2 isolates were unambiguously designated to *L. corymbifera*, *L. ramosa*, *L. ornata*, *L. hyalospora*, and *L. sphaerocystis* with score values of >2.5, >1.8, >2.7, >2.5, and >2.0, respectively, compared to the type strains of *L. corymbifera* CBS 429.75, *L. ramosa* CBS 582.65, *L. ornata* CBS 291.66, *L. hyalospora* CBS 173.67, and *L. sphaerocystis* CBS 420.70, respectively. In contrast, interspecies variation did not exceed 2.5 and ranged from 1.1 to 2.4, 1.0 to 2.0, 1.5 to 2.3, 1.2 to 2.0, and 0.4 to 1.1 for *L. corymbifera*, *L. ramosa*, *L. ornata*, *L. hyalospora*, and *L. sphaerocystis*, respectively, providing moderate to low support for designation to species other than the original one. All strains ($n = 19$) of *L. corymbifera* were homogeneously identified with S values of >2.5 compared with the neotype (NT) strain CBS 429.75. It is proposed that only one top match be used for species identification, even if other matches fall into the highly probable species range. However, intraspecific variation appears to be higher in *L. ramosa* than in *L. corymbifera*. Four (all from clinical samples) of the 17 tested strains were identified with S values of >2.5, 11 strains gained score values of $2.0 > S < 2.5$, and strains FSU 10566 and CNM-CM 5399 (recovered from stork chick and human bronchioaspirate, respectively) had score values of $1.8 > S < 2.0$ compared to the type strain of *L. ramosa* CBS 582.65. On the other hand, all *L. ornata* ($n = 3$) isolates were identified cor-

TABLE 2 Matrix of score values calculated for 46 strains of *Lichtheimia* against the type and reference strains of *Lichtheimia corymbifera*, *L. ramosa*, *L. ornata*, *L. hyalospora*, *L. sphaerocystis*, *Rhizopus oryzae*, and *Mucor circinelloides* used as comparative measures

Strain	Score value						
	L. corymbifera CBS 429.75[NT]	*L. ramosa* CBS 582.65[NT]	*L. ornata* CBS 291.66[T]	*L. hyalospora* CBS 173.67[NT]	*L. sphaerocystis* CBS 420.70[T]	*Rhizopus oryzae* CBS 112.07[T]	*Mucor circinelloides* f. *circinelloides* CBS 195.68[NT]
L. corymbifera CBS 429.75	3.000	1.756	2.388	1.881	1.474	0.229	<0
L. corymbifera CBS 519.71	2.720	2.036	2.429	1.754	1.470	0.190	<0
L. corymbifera CNM-CM 3346	2.717	1.700	2.347	1.525	1.266	0.468	<0
L. corymbifera CNM-CM 3415	2.605	1.951	2.407	1.632	1.429	0.556	<0
L. corymbifera CNM-CM 4671	2.646	1.993	2.356	1.795	1.179	0.306	<0
L. corymbifera CNM-CM 4738	2.637	2.046	2.377	1.778	1.562	0.160	<0
L. corymbifera CNM-CM 5039	2.769	1.473	2.122	1.775	1.368	0.718	<0
L. corymbifera CNM-CM 5538	2.688	1.983	2.354	1.641	1.506	0.452	<0
L. corymbifera CNM-CM 5637	2.653	2.007	2.365	1.783	1.339	0.373	0.085
L. corymbifera CNM-CM 5738	2.578	2.030	2.268	1.658	1.574	0.576	0.036
L. corymbifera CNM-CM 5861	2.608	1.903	2.283	1.709	1.298	0.381	<0
L. corymbifera FSU 6250	2.627	2.107	2.441	1.720	1.346	0.001	<0
L. corymbifera FSU 10563	2.700	2.094	2.358	1.853	1.483	0.221	<0
L. corymbifera FSU 10564	2.712	1.959	2.406	1.706	1.547	0.567	<0
L. corymbifera FSU 10565	2.670	1.988	2.349	1.833	1.244	0.509	<0
L. corymbifera FSU 10567	2.749	2.027	2.397	1.846	1.494	0.430	<0
L. corymbifera IBML 004-M10012	2.616	1.959	2.381	1.774	1.446	0.358	<0
L. corymbifera IBML 006-M10005	2.595	2.170	2.375	1.830	1.522	0.410	<0
L. corymbifera IBML 007-D10005	2.729	2.056	2.424	1.883	1.475	0.649	<0
L. ramosa CBS 103.35	2.000	2.300	2.014	1.773	1.428	0.364	0.203
L. ramosa CBS 582.65	1.828	3.000	2.005	1.509	1.420	0.297	0.245
L. ramosa CNM-CM 3013	1.915	2.039	1.865	1.760	1.145	0.567	0.423
L. ramosa CNM-CM 4228	1.935	2.500	1.974	1.699	1.301	0.396	<0
L. ramosa CNM-CM 4849	1.781	2.381	1.988	1.725	1.447	<0	<0
L. ramosa CNM-CM 5111	1.710	2.575	1.968	1.620	1.360	0.311	<0
L. ramosa CNM-CM 5396	1.822	2.016	1.893	1.691	1.307	0.110	0.401
L. ramosa CNM-CM 5398	1.917	2.203	2.002	1.808	1.396	0.780	0.357
L. ramosa CNM-CM 5399	1.831	1.856	1.960	1.841	1.125	<0	0.225
L. ramosa CNM-CM 5400	1.948	2.182	2.075	1.780	1.302	0.569	0.212
L. ramosa CNM-CM 5677	1.916	2.055	2.072	1.832	1.350	0.806	0.274
L. ramosa FSU 6197	2.084	2.253	2.091	1.860	1.370	0.480	0.308
L. ramosa FSU 10156	1.914	2.025	1.967	1.540	1.271	0.442	0.171
L. ramosa FSU 10566	2.046	1.887	2.101	1.818	1.262	0.153	0.228
L. ramosa FSU 10568	2.048	2.330	2.155	1.801	1.009	0.705	0.824
L. ramosa IBML 003-D10007	1.953	2.538	2.073	1.779	1.309	0.273	<0
L. ramosa IBML 014-M10036	1.736	2.466	1.880	1.624	1.299	<0	<0
L. ornata CBS 291.66	2.387	2.059	3.000	2.051	1.734	0.469	<0
L. ornata CBS 958.68	2.268	2.102	2.829	1.997	1.734	<0	<0
L. ornata CNM-CM 4978	2.170	1.853	2.705	1.824	1.568	0.590	<0
L. hyalospora CBS 100.28	1.698	1.575	2.074	2.596	1.558	0.005	0.395
L. hyalospora CBS 102.36	1.776	1.269	2.050	2.712	1.511	<0	0.270
L. hyalospora CBS 173.67	1.804	1.670	2.049	3.000	1.477	<0	0.708
L. hyalospora CBS 518.71	1.758	1.570	1.980	2.856	1.566	<0	0.404
L. sphaerocystis CBS 420.70	1.436	1.424	1.692	1.481	3.000	0.172	0.351
L. sphaerocystis CBS 647.78	1.044	0.757	1.095	0.521	2.308	<0	<0
L. sphaerocystis CBS 648.78	0.564	0.418	0.748	0.941	2.073	0.233	<0
Rhizopus oryzae CBS 112.07	0.470	0.530	0.465	0.145	0.129	3.000	<0
Mucor circinelloides f. *circinelloides* CBS 195.68	<0	0.378	<0	0.046	<0	<0	3.000

rectly with *S* values of >2.7. In general, the clinically relevant species *L. corymbifera*, *L. ramosa*, and *L. ornata* were clearly discriminated from the nonclinically relevant type strains of *L. hyalospora* CBS 173.67 and *L. sphaerocystis* CBS 420.70, as indicated by low score values of $1.18 < S > 2.07$. Comparison with *L. sphaerocystis* CBS 420.70 revealed *S* values of <1.5 in 31 out of a total of 39 strains from the clinically important species (Table 2). Two additionally tested strains of *L. sphaerocystis* gained score values of <1.1 compared to *Lichtheimia* type strains (Table 2). Taking into consideration the fact that log (score) values of >1.7 generally indicate relationships on the genus level (17, 25), *L. sphaerocystis* is taxonomically at the edge of

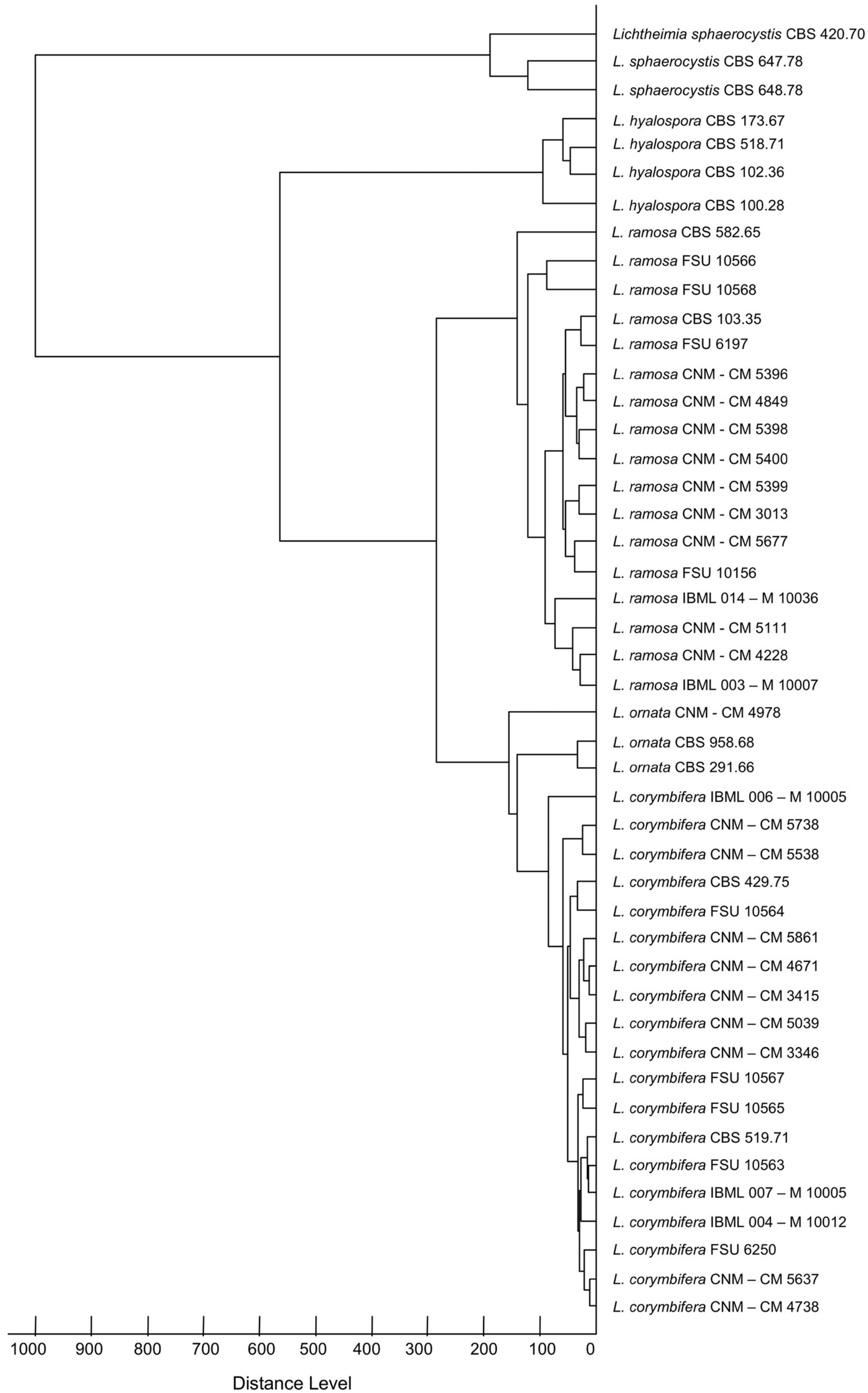

FIG 3 Cladogram of a total of 46 isolates from *Lichtheimia* species based on mass spectra.

the generic border. In total, 95% of all tested strains (39 out of 41) were identified to have score values of >2 (probable species identification) and 77% were identified to have score values of >2.3 (highly probable species identification) down to the species level. Just 2 isolates (FSU 10566 and CNM-CM 5399) showed moderate species identification, as indicated by score values of >1.8. A cladogram based on the mass spectra presents the correct placement in accordance with their species affiliation (Fig. 3).

DISCUSSION

We showed that MALDI-TOF mass spectrometry is a convenient technique and powerful tool for the identification of human-pathogenic Mucorales at both intergeneric and intrageneric levels. However, a standardized sample preparation equally applied to all strains is crucial for reproducible measurement. Multiple techniques for the cultivation and the sample preparation for mass spectrometry of filamentous fungi have been published (1, 15). However, direct comparison of the spectra obtained by different protocols is not possible (9, 25). Therefore, a standardized procedure for sample preparation which should be applied to all mucoralean fungi subjected to MALDI-TOF analyses is proposed in this study.

Mass spectrum-based distance cladograms are phylogeny compliant compared to the phylogeny published for *Lichtheimia* by Alastruey-Izquierdo et al. (5). At the intergeneric level, the three clinically most important species, *Rhizopus oryzae*, *Mucor circinelloides*, and *Lichtheimia corymbifera*, could be clearly identified. In contrast, *L. ramosa* exhibits higher intraspecific heterogeneity, which is concordant with the findings of previous analyses (5, 11, 12). The identification of the pathogen and knowledge of the susceptibility to antifungal drugs are crucial for efficient treatment and therapy. There are significant differences in susceptibility to itraconazole, posaconazole, terbinafine, and amphotericin B between *Rhizopus* species and *Lichtheimia* species (2, 3, 4, 8). While *Rhizopus* species appear to be more resistant to amphotericin B than *Mucor* species (8), *Lichtheimia* species respond well to amphotericin B (4). Among the species of *Lichtheimia*, *L. ramosa* showed slightly higher MICs than the other species for all drugs (4). Therefore, in the future, identification at the species level may be helpful for determination of the antifungal therapy based on the susceptibility profile of the individual pathogen. The high resolution and discriminative power of MALDI-TOF mass spectrometry facilitate differentiation of closely related species. Thus, it represents an important prerequisite for future refinements and monitoring of efficiency control in antifungal therapy strategies, on the one hand, and for the detection of fungi with mycotoxin-producing or allergy-causing potential, on the other hand. The current procedure requires isolation and cultivation of the fungus prior to species determination. Direct identification of *Lichtheimia* in biopsy material has not been established yet. If the necessary mass spectrometer is available, MALDI-TOF is the fastest (5.1 min of hands-on time/identification) and cheapest ($0.50/sample) strategy among all the other cultivation-dependent methods of molecular detection (e.g., PCR, restriction fragment length polymorphism analysis, DNA fingerprinting) for fungal identification (10).

In summary, MALDI-TOF MS has been implemented as a rapid, simple, cost-effective, and high-throughput proteomic technique for routine identification of *Lichtheimia* isolates in our laboratory. Although five reference spectra were generated for the creation of master spectra, two spots (equivalent to two technical replications) would be sufficient for reliable species identification in routine diagnosis, because of the high reproducibility of the spectra. MALDI-TOF mass spectrometry yielded 100% accurate identification on the genus level (spectral scores, $\geq$1.8) for 41 strains of *Lichtheimia*. The technique has a high accuracy for microbial identification in general and performs as well as or better than conventional techniques. The performance can be significantly improved when more spectra of appropriate reference strains are added to the database (25). The data set presented in this study can be used as a reference set which can be included in the database of any MALDI-TOF equipment. Furthermore, the protocol for sample preparation used in this study provides a robust approach which eliminates the strong influences of culture media and culture conditions, as previously discussed for bacteria, yeasts and filamentous fungi (6, 7, 14, 15, 17, 19, 22, 24, 25). MALDI-TOF MS revolutionizes the identification of pathogens in clinical laboratories but requires a good knowledge of taxonomy when this technique is implemented in a routine clinical biology laboratory (9).

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REFERENCES

1. **Alanio A, et al.** 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.
2. **Alastruey-Izquierdo A, et al.** 2009. Activity of posaconazole and other antifungal agents against Mucorales strains identified by sequencing of internal transcribed spacers. Antimicrob. Agents Chemother. **53**:1686–1689.
3. **Alastruey-Izquierdo A, et al.** 2009. *In vitro* activity of antifungals against Zygomycetes. Clin. Microbiol. Infect. **15**(Suppl 5):71–76.
4. **Alastruey-Izquierdo A, Cuesta I, Walther G, Cuenca-Estrella M, Rodriguez-Tudela JL.** 2010. Antifungal susceptibility profile of human-pathogenic species of *Lichtheimia*. Antimicrob. Agents Chemother. **54**: 3058–3060.
5. **Alastruey-Izquierdo A, et al.** 2010. Species recognition and clinical relevance of the zygomycetous genus *Lichtheimia* (syn. *Absidia* pro parte, *Mycocladus*). J. Clin. Microbiol. **48**:2154–2170.
6. **Bader O, et al.** 2010. Improved clinical laboratory identification of human pathogenic yeasts by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Clin. Microbiol. Infect. **17**: 1359–1365.
7. **Dannaoui E.** 2009. Molecular tools for identification of Zygomycetes and the diagnosis of zygomycosis. Clin. Microbiol. Infect. **15**(Suppl 5):66–70.
8. **Dannaoui E, et al.** 2003. *In vitro* susceptibilities of zygomycetes to conventional and new antifungals. J. Antimicrob. Chemother. **51**:45–52.
9. **De Bel A, et al.** 2010. Correct implementation of matrix-assisted laser desorption ionization–time of flight mass spectrometry in routine clinical microbiology. J. Clin. Microbiol. **48**:1991–1992.
10. **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.
11. **Ellis JJ, Hesseltine GW.** 1966. Species of *Absidia* with ovoid sporangiospores. II. Sabouraudia **5**:59–77.
12. **Garcia-Hermoso D, et al.** 2009. Molecular and phenotypic evaluation of *Lichtheimia corymbifera* (formerly *Absidia corymbifera*) complex isolates associated with human mucormycosis: rehabilitation of *L. ramosa*. J. Clin. Microbiol. **47**:3862–3870.
13. **Greenberg RN, Scott LJ, Vaughn HH, Ribes JA.** 2004. Zygomycosis (mucormycosis): emerging clinical importance and new treatments. Curr. Opin. Infect. Dis. **17**:517–525.
14. **Grosse-Herrenthey A, et al.** 2008. Challenging the problem of clostridial identification with matrix-assisted laser desorption and ionization-time-of-flight mass spectrometry (MALDI-TOF MS). Anaerobe **14**:242–249.
15. **Hettick JM, et al.** 2008. Discrimination of *Penicillium* isolates by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry fingerprinting. Rapid Commun. Mass Spectrom. **22**:2555–2560.

16. **Lass-Flörl C.** 2009. Zygomycosis: conventional laboratory diagnosis. Clin. Microbiol. Infect. **15**(Suppl 5):60–65.
17. **Marklein G, et al.** 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.
18. **Olias P, Gruber AD, Winfried B, Hafez HM, Lierz M.** 2010. Fungal pneumonia as a major cause of mortality in white stork (*Ciconia ciconia*) chicks. Avian Dis. **54**:94–98.
19. **Prod'hom G, Bizzini A, Durussel C, Bille J, Greub G.** 2010. Matrix-assisted laser desorption ionization–time of flight mass spectrometry for direct bacterial identification from positive blood culture pellets. J. Clin. Microbiol. **48**:1481–1483.
20. **Ribes JA, Vanover-Sams CL, Baker DJ.** 2000. Zygomycetes in human disease. Clin. Microbiol. Rev. **13**:236–301.
21. **Roden MM, et al.** 2005. Epidemiology and outcome of zygomycosis: a review of 929 reported cases. Clin. Infect. Dis. **41**:634–653.
22. **Seng P, et al.** 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.
23. **Skiada A, et al.** 2011. Zygomycosis in Europe: analysis of 230 cases accrued by the registry of the European Confederation of Medical Mycology (ECMM) Working Group on Zygomycosis between 2005 and 2007. Clin. Microbiol. Infect. **17**:1859–1867.
24. **Stevenson LG, Drake SK, Shea YR, Zelazny AM, Murray PR.** 2010. Evaluation of matrix-assisted laser desorption ionization–time of flight mass spectrometry for identification of clinically important yeast species. J. Clin. Microbiol. **48**:3482–3486.
25. **Van Veen SQ, Claas EC, Kuijper EJ.** 2010. High-throughput identification of bacteria and yeast by matrix-assisted laser desorption ionization–time of flight mass spectrometry in conventional medical microbiology laboratories. J. Clin. Microbiol. **48**:900–907.
26. **Voigt K, Cigelnik E, O'Donnell K.** 1999. Phylogeny and PCR identification of clinically important Zygomycetes based on nuclear ribosomal-DNA sequence data. J. Clin. Microbiol. **37**:3957–3964.