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# Discrimination of *Aspergillus* isolates at the species and strain level by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry fingerprinting

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ABSTRACT

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI–TOF MS) was used to generate highly reproducible mass spectral fingerprints for 12 species of fungi of the genus *Aspergillus* and 5 different strains of *Aspergillus flavus*. Prior to MALDI–TOF MS analysis, the fungi were subjected to three 1-min bead beating cycles in an acetonitrile/trifluoroacetic acid solvent. The mass spectra contain abundant peaks in the range of 5 to 20 kDa and may be used to discriminate between species unambiguously. A discriminant analysis using all peaks from the MALDI–TOF MS data yielded error rates for classification of 0 and 18.75% for resubstitution and cross-validation methods, respectively. If a subset of 28 significant peaks is chosen, resubstitution and cross-validation error rates are 0%. Discriminant analysis of the MALDI–TOF MS data for 5 strains of *A. flavus* using all peaks yielded error rates for classification of 0 and 5% for resubstitution and cross-validation methods, respectively. These data indicate that MALDI–TOF MS data may be used for unambiguous identification of members of the genus *Aspergillus* at both the species and strain levels.

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There are approximately 180 recognized species of the genus *Aspergillus,* making it one of the most common and widely distributed genera of fungi in the world. More than 40 species of *Aspergillus* have been isolated from humans [1], and these fungi are important in industry, agriculture, and public health. Some species of *Aspergillus* (notably *A. oryzae* and *A. niger*) are used in brewing, food production, and the production of important industrial enzymes [1,2]. Others (including *A. flavus*, *A. parasiticus*) are responsible for aflatoxin production in crops (e.g., corn, peanuts, cotton) and feed that can cause injury to livestock, pets, and humans [1,3,4]. As many as 40 species of *Aspergillus* have been identified as causative agents of opportunistic infections in humans. Of these, *A. fumigatus*, *A. flavus*, and *A. niger* are the most common [1,3,5,6] and can cause allergy and/or infection in humans. Invasive aspergillosis may occur in immunocompromised hosts, and the incidence of these infections is on the rise [6].

Fungal identification is still based largely on micro- and macroscopic examination of morphological and culture characteristics [1] that require subjective evaluation and are potentially biased. Because of the large number of species in the genus *Aspergillus,* the subjective nature of current identification protocols, and the significant health and economic impact of *Aspergillus,* new molecular technologies for the identification of fungi are of significant interest. New applications, such as DNA barcoding [7] and mass spectrometry fingerprinting, may prove to be of great utility for fungal identification.

Mass spectrometry fingerprinting using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI–TOF MS)[1] [8–10] has proven to be a powerful tool for the analysis of microbiological samples such as bacteria [11–16]. Previous studies in our laboratory have demonstrated the ability to discriminate between medically relevant mycobacteria such as *Mycobacterium tuberculosis* at both the species and strain levels on the basis of MALDI–TOF MS analysis [17,18].

Although mass spectrometry-based identification has been applied extensively to bacteria, comparatively little has been published on mass spectrometry-based strategies for fungal identification [19–23]. Li and coworkers have presented the MALDI–TOF mass spectra of 4 species of *Aspergillus* (*A. flavus*, *A. oryzae*, *A. parasiticus*, and *A. sojae*) [23] and demonstrated that different

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[1] *Abbreviations used:* MALDI–TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; CHCA, α-cyano-4-hydroxycinnamic acid; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; NIOSH, National Institute for Occupational Safety and Health; ATCC, American Type Culture Collection.

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mass spectra were obtained from aflatoxigenic and nonaflatoxigenic strains and that closely related species produced similar mass spectra. In a previous article, we described a novel MALDI–TOF MS-based strategy for the identification of fungi of the genus *Penicillium* (J. M. Hettick et al., manuscript in preparation). A bead disruption step prior to MALDI matrix/sample deposition resulted in a significant improvement in number and relative abundance of observed mass-to-charge ($m/z$) ratios. Here we apply a similar methodology to an extensive analysis of 16 fungal isolates of the genus *Aspergillus,* including 12 species and 5 different isolates of *A. flavus.*

## Materials and methods

### *Reagents*

Angiotensin II (human), insulin oxidized B chain (bovine), cytochrome *c* (equine), albumin (bovine serum), α-cyano-4-hydroxycinnamic acid (CHCA), and trifluoroacetic acid (TFA) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Acetonitrile (high-performance liquid chromatography [HPLC] grade) was purchased from Fisher Scientific (Fairlawn, NJ, USA). Malt extract agar was purchased from Difco (Sparks, MD, USA). Distilled deionized water was produced by a Millipore Synthesis A-10 (Billerica, MA, USA).

### *Fungal culture*

A total of 12 species of *Aspergillus* (Table 1) and 5 isolates of *A. flavus* (Table 2) were subcultured from National Institute for Occupational Safety and Health (NIOSH) or American Type Culture Collection (ATCC) stock sources and grown for 14 days on malt extract agar. To ensure reproducibility, 8 independent cultures were performed for each isolate for a total of 128 individual fungal cultures. Conidia and hyphae from one culture plate ($\sim 10^8$ cells) were transferred to 100 μl of 0.1-mm zirconium beads (Biospec, Bartlesville, OK, USA) and 1 ml of acetonitrile/4% TFA (50:50). The samples were subjected to three 1-min bead beating cycles. The resulting solution was centrifuged at 14,500 rpm for 10 min, and the supernatant was taken for MALDI–TOF MS analysis.

### *Mass spectrometry*

MALDI–TOF MS samples were prepared by mixing supernatant 1:1 with 10 mg/ml CHCA. Then 1 μl of the resulting solution was deposited on a gold sample stage (Bio-Rad, Hercules, CA, USA) and allowed to air dry. Each sample was analyzed in duplicate for a total of 16 composite MALDI–TOF mass spectra per isolate. MALDI–TOF mass spectra were acquired using a Ciphergen PBS-IIc linear TOF mass spectrometer (Bio-Rad) with a flight path of 0.8 m, capable of mass resolution ($m/\Delta m$) of 500 to 1000 and mass accuracy of ±1000 ppm. Spectra were acquired over the $m/z$ range of 0 to 100 kDa, with the delayed extraction parameters set to optimally focus the 10- to 20-kDa range. Composite mass spectra are the average of 100 laser shots taken from 20 distinct positions across the sample deposit. These positions were held constant for all samples used in this study, and 337-nm $N_2$ laser intensity was maintained just above the threshold for ion production. Mass spectra were externally calibrated using a set of peptide and protein calibrants that covered the range of 1 to 66 kDa.

**Table 1**
Species of the genus *Aspergillus* used in this study

| Species | NIOSH or ATCC catalog number | Label |
|---|---|---|
| *A. candidus* | 172824 | A |
| *A. chevalieri* | 198601 | B |
| *A. flavus* | (16883) | C |
| *A. fumigatus* | 173037 | D |
| *A. nidulans* | 152208 | E |
| *A. niger* | 172804 | F |
| *A. parasiticus* | (26691) | G |
| *A. repens* | 198603 | H |
| *A. sydowii* | 324605 | I |
| *A. terreus* | 173031 | J |
| *A. ustus* | 198608 | K |
| *A. versicolor* | 324606 | L |

*Note.* ATCC catalog numbers are in parentheses.

**Table 2**
Strains of the species *A. flavus* used in this study

| Species | NIOSH or ATCC catalog number | Label |
|---|---|---|
| *A. flavus* | 15224 | a |
| *A. flavus* | 15417 | b |
| *A. flavus* | (16883) | c |
| *A. flavus* | 34689 | d |
| *A. flavus* | PRC86N | e |

*Note.* ATCC catalog number is in parentheses.

### *Data analysis*

Initial data analysis was performed using the Biomarker Wizard (Bio-Rad) software suite. Spectra were baseline corrected and normalized to total ion current. "Clusters" of peaks common to a given isolate were generated by selecting all peaks with signal-to-noise (S/N) ratios greater than 5 that occurred in each spectrum from that isolate. The mass tolerance for each cluster was set to 0.3% of the $m/z$. Linear discriminant functions were analyzed using SAS/STAT software (version 9.1 of the SAS system for Windows, SAS Institute, Cary, NC, USA). The intensity values were first tested for their distribution and found to be log normal, so a natural log transformation was used on the intensity values. A stepwise variable selection method using the "PROC STEPDISC" procedure, which selects a subset of the variables of interest using a stepwise discriminant analysis (keeping the most significant variables from iterative *F* tests), was then performed to select a subset of variables that could serve as predictor variables for class membership. Using this new subset of significant peaks, "PROC DISCRIM" was used to determine the classification error rate between species and strains of *Aspergillus.* This is done by calculating a discriminant function that allows each data point to be compared with all other data points for cross-validation classification. This process is done iteratively for each data point individually. This procedure was followed by the "PROC CANDISC" procedure, which reduces the number of dimensions to find linear combinations of the variable set that also summarizes between-class variation, to perform a canonical discriminant analysis on that subset of variables. This creates new variables by taking linear combinations of the original variables and aids in determining the true underlying dimension of the data space. The canonical functions generated allow the calculation of canonical scores, which can be used to discriminate between the various strains. Using the same subsets of variables, cluster analysis was then performed using the "PROC CLUSTER" procedure, which uses distances between data points to form hierarchical clusters, and a dendrogram was generated using the "PROC TREE" procedure, which uses the data set from "PROC CLUSTER" to produce a dendrogram.

## Results and discussion

The MALDI–TOF fingerprint mass spectra for 12 species of *Aspergillus* are presented in Figs. 1–3. In general, the fingerprint mass spectra contain abundant peaks covering the $m/z$ range of

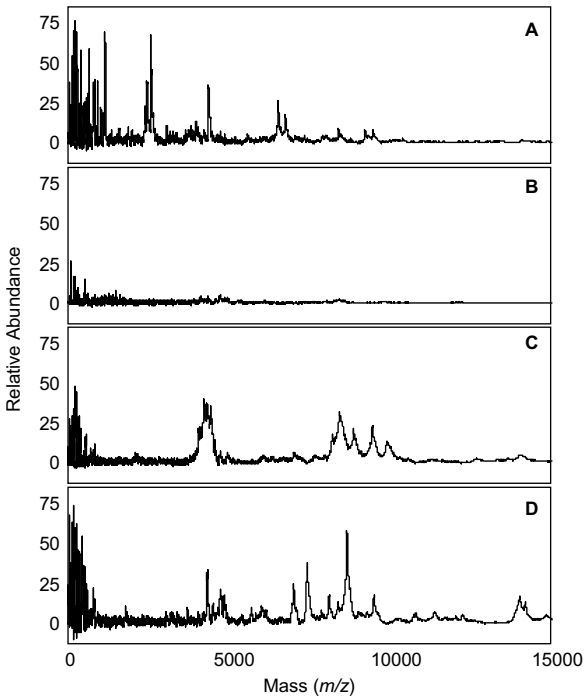


**Fig. 1.** MALDI–TOF MS fingerprint spectra of *A. candidus* (A), *A. chevalieri* (B), *A. flavus* (C), and *A. fumigatus* (D).

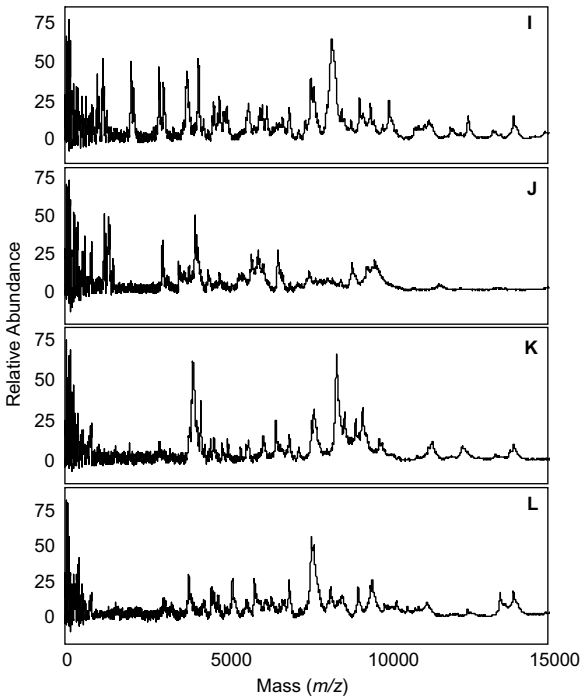


**Fig. 3.** MALDI–TOF MS fingerprint spectra of *A. sydowii* (I), *A. terreus* (J), *A. ustus* (K), and *A. versicolor* (L).

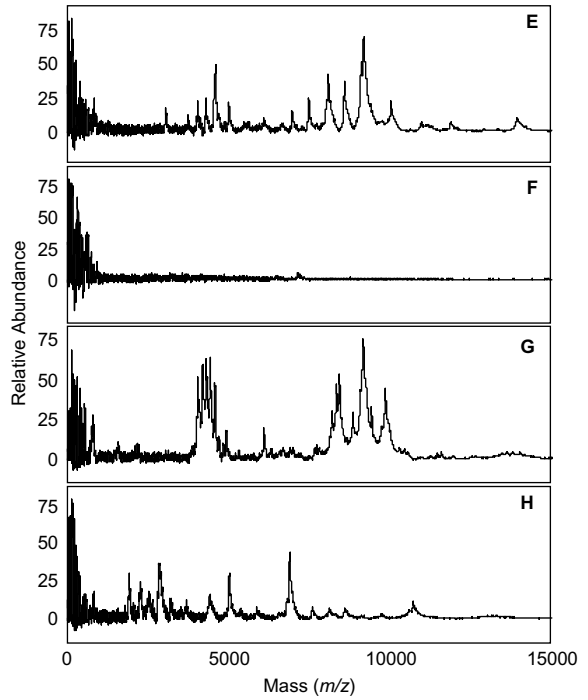


**Fig. 2.** MALDI–TOF MS fingerprint spectra of *A. nidulans* (E), *A. niger* (F), *A. parasiticus* (G), and *A. repens* (H).

1 to 20 kDa with the exception of *A. chevalieri* and *A. niger* (Figs. 1B and 2F, respectively). The fingerprint mass spectrum of *A. chevalieri* has only a few low-abundance peaks in the *m*/*z* range of 5 to 15 kDa. The fingerprint mass spectrum of *A. niger* has only two low-abundance peaks at approximately *m*/*z* 7 kDa. These observations are consistent with previous studies in our laboratory and others demonstrating that melanized (dark-spored) fungi, such as *A. niger* and many species of *Stachybotrys* and *Epicoccum,* produce poor fingerprint mass spectra. In 2002, Valentine and coworkers [21] reported difficulty in observing biomarkers in the MALDI–TOF mass spectra of *A. niger* even with sample pretreatment. These researchers did not report a mass spectrum for *A. niger,* making it difficult to compare with the current results. It is important to note that in spite of the poor relative abundance and paucity of peaks observed in the fingerprint mass spectra of *A. chevalieri* and *A. niger,* the spectra are reproducible and can be used to discriminate the 2 species from each of the others in this study.

Li and coworkers presented the MALDI–TOF MS fingerprint mass spectra of 4 species of *Aspergillus* (*A. flavus*, *A. parasiticus*, *A. oryzae*, and *A. sojae*) [23]. Of these species, we examined *A. flavus* (Fig. 1C) and *A. parasiticus* (Fig. 2G). Although the fingerprint mass spectra from the two reports are similar, there are some significant differences. The spectra reported by Li and coworkers contain fewer *m*/*z* peaks than do those presented here. This is most likely due to the bead disruption sample pretreatment step employed in our laboratory. Early experiments in our laboratory yielded much less complex fingerprint mass spectra for fungi than for bacteria. Thus, we have incorporated bead disruption in an effort to increase the information content of our MALDI–TOF MS data (J. M. Hettick et al., manuscript in preparation). Other factors that may influence the appearance of MALDI–TOF fingerprint data include culture conditions, MALDI sample preparation, and mass spectrometry instrumentation. The MALDI fingerprint mass spectra of microorganisms have been demonstrated to vary with the choice of growth media [24,25]. For this reason, it is important to keep culture conditions constant when developing a database of fingerprint mass spectra.

The error rates for species prediction based on the MALDI–TOF MS dataset were calculated using both resubstitution and cross-validation methods. In resubstitution, the discriminant function is fitted to the dataset and then applied to each observation. In contrast, cross-validation deletes the observation, fits the discriminant function to the remaining dataset, and then applies the function to the deleted observation. Resubstitution tends to underestimate classification error, whereas cross-validation is unbiased and preferred

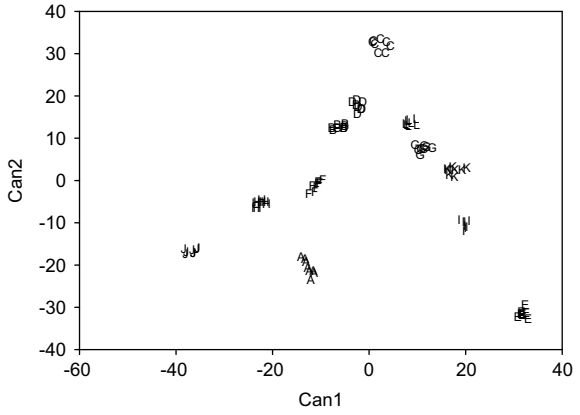


**Fig. 4.** Two-dimensional canonical discriminant plot for MALDI–TOF MS data from 12 species of *Aspergillus*: (A) *A. candidus*; (B) *A. chevalieri*; (C) *A. flavus*; (D) *A. fumigatus*; (E) *A. nidulans*; (F) *A. niger*; (G) *A. parasiticus*; (H) *A. repens*; (I) *A. sydowii*; (J) *A. terreus*; (K) *A. ustus*; (L) *A. versicolor*.

for large datasets [26]. A discriminant analysis using all peaks from the MALDI–TOF MS data yielded error rates for classification of 0 and 18.75% for resubstitution and cross-validation methods, respectively. If a subset of 28 significant peaks is chosen, resubstitution and cross-validation error rates are 0%. The *m*/*z* values of these peaks (in order of decreasing significance) are 9192.55, 8648.68, 8410.89, 7703.10, 6881.57, 10029.54, 2563.83, 4036.72, 8328.31, 4298.99, 9878.48, 46559.12, 9593.48, 13475.39, 12262.94, 4349.47, 8862.55, 6585.83, 3040.53, 7412.16, 11870.63, 6517.06, 21821.91, 3557.38, 2126.38, 5859.69, 4131.42, and 8575.93.

The results of a canonical discriminant analysis of the MALDI–TOF MS fingerprint data are presented in Fig. 4. In this figure, the results of the canonical discriminant analysis are plotted for each of 8 replicate cultures of each species. Each culture is represented by a capital letter corresponding to that species. All 8 spectra from each species cluster together and are spatially resolved from those of other species. This is a useful tool for reducing the complex set of *m*/*z* and relative abundance values into an easily understandable two-dimensional scatterplot highlighting the differences between species.

In a similar fashion, Fig. 5 presents the cluster analysis dendrogram derived from this same dataset. Here each spectrum for the 8 replicate cultures is represented by a point on the *x* axis and is grouped by lateral brackets with the next most similar MALDI–TOF mass spectrum or group of spectra. In most cases, each MALDI–TOF mass spectrum for a species clusters with the other 7 spectra from that species. For example, the mass spectrum for each individual culture of *A. repens* is clustered most closely with spectra from other *A. repens* cultures (Fig. 5, H1–H8); similarly, each culture of *A. versicolor* clusters with other cultures of *A. versicolor* (Fig. 5, L1–L8). Fig. 5 may be examined in a hierarchical manner. For example, the MALDI–TOF mass spectra of *A. repens* and *A. versicolor* (Figs. 5H and 5L, respectively) are more similar to one another than to other species. Continuing "up" the cluster chart, the mass spectra of *A. repens* and *A. versicolor* are more similar to *A. candidus* and so on. Examination of the MALDI–TOF fingerprint mass spectra of these species in Figs. 1–3 suggests that these species share many *m*/*z* in common and with similar relative abundances. As the MALDI–TOF fingerprint mass spectra become more dissimilar, the species move apart from one another along the *x* axis of Fig. 5. It should be noted that 2 species, *A. chevalieri* and *A. niger*, are not completely resolved by the cluster analysis. These are the 2 melanized species with relatively poor MALDI–TOF fingerprint mass spectra. Because these 2 species have comparatively little data on which to base the clustering, it is perhaps not surprising that the algorithm does not resolve them.

Previous studies in our laboratory with mycobacteria have demonstrated the ability to discriminate between bacteria at the strain level [18]. Fig. 6 presents the MALDI–TOF fingerprint mass spectra of 5 strains of the invasive pathogenic and aflatoxin-producing fungus *A. flavus* (4 from geographically distinct

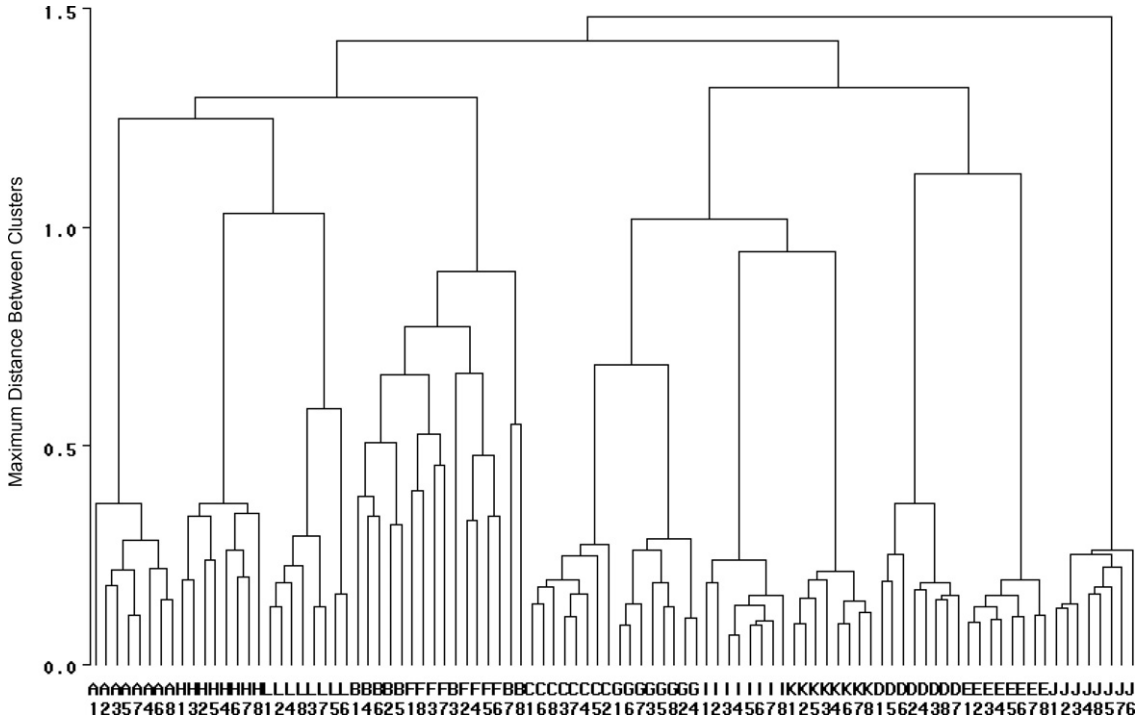


**Fig. 5.** Cluster analysis dendrogram for MALDI–TOF MS data from 12 species of *Aspergillus:* (A) *A. candidus*; (B) *A. chevalieri*; (C) *A. flavus*; (D) *A. fumigatus*; (E) *A. nidulans*; (F) *A. niger*; (G) *A. parasiticus*; (H) *A. repens*; (I) *A. sydowii*; (J) *A. terreus*; (K) *A. ustus*; (L) *A. versicolor*.

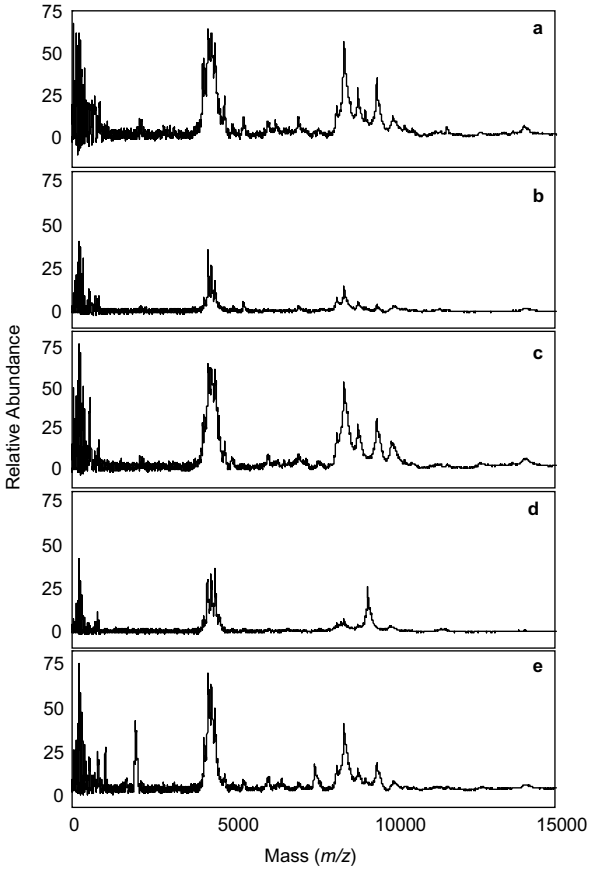


**Fig. 6.** MALDI–TOF MS fingerprint spectra of 5 strains of *A. flavus*: (a) NIOSH no. 15224; (b) NIOSH no. 15417; (c) ATCC no. 16883; (d) NIOSH no. 34689; (e) NIOSH no. PRC86N.

areas and 1 ATCC-type strain). Although the MALDI–TOF fingerprint mass spectra are very similar, some differences are observed, most notably in the region of 7 to 10 kDa. In spite of the high degree of similarity between strains, a canonical discriminant analysis was able to resolve the 8 spectra from each strain (Fig. 7). The spectra for *A. flavus* strain D (NIOSH isolate no. 34689) are particularly well resolved due to the contribution of the strong peak at *m*/*z* 9158. Discriminant analysis of the MALDI–TOF MS data for 5 strains of *A. flavus* using all peaks yielded error rates for classification of 0 and 5% for resubstitution and cross-validation methods, respectively. Although using a subset of significant peaks for identification was preferred for the larger dataset of 12 species, in this smaller dataset that method gives 100% error for strain identification. This is because the subset of 7 peaks selected is shared in common between all strains of *A. flavus*. These common *m*/*z* values are 9158.80, 5303.71, 4041.14, 4084.56, 4200.13, 8191.02, and 4421.21. In this case, all peaks in the fingerprint spectra must be considered to correctly identify the individual *A. flavus* strains.

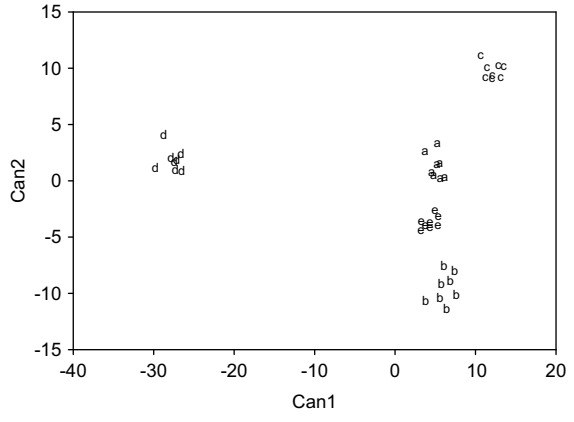


**Fig. 7.** Two-dimensional canonical discriminant plot for MALDI–TOF MS data from 5 strains of *A. flavus*: (a) NIOSH no. 15224; (b) NIOSH no. 15417; (c) ATCC no. 16883; (d) NIOSH no. 34689; (e) NIOSH no. PRC86N.

## Conclusions

MALDI–TOF MS was used to generate highly reproducible mass spectral fingerprints for 12 species of fungi of the genus *Aspergillus* and 5 different strains of *A. flavus*. Discriminant analysis of the MALDI–TOF MS data was able to correctly classify each *Aspergillus* species with 100% accuracy and was able to correctly classify strains of *A. flavus* with 95 to 100% accuracy. These data indicate that MALDI–TOF MS data may be used for unambiguous objective identification of members of the genus *Aspergillus* at both the species and strain levels. For large datasets with significant differences in MALDI–TOF mass spectra between species, a subset of "most significant" peaks allows 100% identification accuracy; however, all of the peaks in the dataset must be included to accurately identify highly similar fingerprint mass spectra such as those from different strains of the same species. To correctly identify unknown species and strains, a comprehensive database of fungal fingerprint mass spectra of the approximately 180 species of *Aspergillus* will need to be established. However, the current study clearly demonstrates the utility of the methodology for discrimination between species and strains of *Aspergillus*. MALDI–TOF MS-based fingerprinting is an objective analytical methodology that will complement current subjective identification techniques, which are based on observation of colony morphology.

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## References

[1] H.C. Gugnani, Ecology and taxonomy of pathogenic aspergilli, Front. Biosci. 8 (2003) S346–S357.
[2] W.M. Fogarty, Enzymes of the genus Aspergillus, in: J.E. Smith (Ed.), *Aspergillus*, Plenum, New York, 1994, pp. 177–218.
[3] J.I. Pitt, Toxigenic fungi: Which are important?, Med Mycol. 38 (Suppl. 1) (2000) 17–22.
[4] J.L. Richard, R.J. Cole, Mycotoxins: Economic and Health Risks, Council for Agricultural Science and Technology, Ames, IA, 1989.
[5] M.T. Hedayati, A.C. Pasqualotto, P.A. Warn, P. Bowyer, D.W. Denning, *Aspergillus flavus:* Human pathogen, allergen, and mycotoxin producer, Microbiology 153 (2007) 1677–1692.
[6] M.D. Richardson, *Aspergillus* and *Penicillium*, in: L. Ajello, R.J. Hay (Eds.), Topley and Wilson's Microbiology and Microbial Infections, Oxford University Press, London, 1998, pp. 282–312.
[7] K.A. Seifert, R.A. Samson, J.R. Dewaard, J. Houbraken, C.A. Levesque, J.M. Moncalvo, G. Louis-Seize, P.D. Hebert, Prospects for fungus identification using CO1 DNA barcodes, with *Penicillium* as a test case, Proc. Natl. Acad. Sci. USA 104 (2007) 3901–3906.
[8] M. Karas, D. Bachmann, U. Bahr, F. Hillenkamp, Matrix-assisted ultraviolet–laser desorption of nonvolatile compounds, Intl. J. Mass Spectrom. 78 (1987) 53–68.
[9] M. Karas, F. Hillenkamp, Laser desorption/ionization of proteins with molecular masses exceeding 10,000 Daltons, Anal. Chem. 60 (1988) 2299–2301.
[10] R.J. Cotter, The new time-of-flight mass spectrometry, Anal. Chem. 71 (1999) 445A–451A.

[11] T.C. Cain, D.M. Lubman, W.J. Weber Jr., Differentiation of bacteria using protein profiles from matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, Rapid Commun. Mass Spectrom. 8 (1994) 1026–1030.
[12] M.A. Claydon, S.N. Davey, V. Edwards-Jones, D.B. Gordon, The rapid identification of intact microorganisms using mass spectrometry, Nat. Biotechnol. 14 (1996) 1584–1586.
[13] T. Krishnamurthy, P.L. Ross, Rapid identification of bacteria by direct matrix-assisted laser desorption/ionization mass spectrometric analysis of whole cells, Rapid Commun. Mass Spectrom. 10 (1996) 1992–1996.
[14] K.J. Welham, M.A. Domin, D.E. Scannell, E. Cohen, D.S. Ashton, The characterization of micro-organisms by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, Rapid Commun. Mass Spectrom. 12 (1998) 176–180.
[15] C. Fenselau, P.A. Demirev, Characterization of intact microorganisms by MALDI mass spectrometry, Mass Spectrom. Rev. 20 (2001) 157–171.
[16] J.O. Lay Jr., MALDI–TOF mass spectrometry of bacteria, Mass Spectrom. Rev. 20 (2001) 172–194.
[17] J.M. Hettick, M.L. Kashon, J.P. Simpson, P.D. Siegel, G.H. Mazurek, D.N. Weissman, Proteomic profiling of intact mycobacteria by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, Anal. Chem. 76 (2004) 5769–5776.
[18] J.M. Hettick, M.L. Kashon, J.E. Slaven, Y. Ma, J.P. Simpson, P.D. Siegel, G.N. Mazurek, D.N. Weissman, Discrimination of intact mycobacteria at the strain level: A combined MALDI–TOF MS and biostatistical analysis, Proteomics 6 (2006) 6416–6425.
[19] K.J. Welham, M.A. Domin, K. Johnson, L. Jones, D.S. Ashton, Characterization of fungal spores by laser desorption/ionization time-of-flight mass spectrometry, Rapid Commun. Mass Spectrom. 14 (2000) 307–310.
[20] B. Amiri-Eliasi, C. Fenselau, Characterization of protein biomarkers desorbed by MALDI from whole fungal cells, Anal. Chem. 73 (2001) 5228–5231.
[21] N.B. Valentine, J.H. Wahl, M.T. Kingsley, K.L. Wahl, Direct surface analysis of fungal species by matrix-assisted laser desorption/ionization mass spectrometry, Rapid Commun. Mass Spectrom. 16 (2002) 1352–1357.
[22] H.Y. Chen, Y.C. Chen, Characterization of intact *Penicillium* spores by matrix-assisted laser desorption/ionization mass spectrometry, Rapid Commun. Mass Spectrom. 19 (2005) 3564–3568.
[23] T. Li, B. Liu, Y. Chen, Characterization of *Aspergillus* spores by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, Rapid Commun. Mass Spectrom. 14 (2000) 2393–2400.
[24] K. Bernardo, N. Pakulat, M. Macht, O. Krut, H. Seifert, S. Fleer, F. Hünger, M. Krönke, Identification and discrimination of *Staphylococcus aureus* strains using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, Proteomics 2 (2002) 747–753.
[25] R.J. Arnold, J.A. Karty, A.D. Ellington, J.P. Reilly, Monitoring the growth of a bacteria culture by MALDI–MS of whole cells, Anal. Chem. 71 (1999) 1990–1996.
[26] U. Braga-Neto, R. Hashimoto, E.R. Dougherty, D.V. Nguyen, R.J. Carroll, Is cross-validation better than resubstitution for ranking genes?, Bioinformatics 20 (2004) 253–258