Aspergillus ibericus: A New Species of Section *Nigri* Characterised by MALDI-TOF MS

W. Kallow[1], I. Santos[2], M. Erhard[1], R. Serra[2], A. Venâncio[2] and N. Lima[2]

[1]AnagnosTec GmbH, Im Biotechnologiepark, TGZII, 14943 Luckenwalde, Germany
[2]Centro de Engenharia Biológica, Micoteca da Universidade do Minho (MUM), Campus de Gualtar, 4710-057 Braga, Portugal

Summary

Strains from the new described species *Aspergillus ibericus* were characterised using MALDI-TOF MS and the results were compared with other related species of section *Nigri*.

Introduction

Erhard *et al.* (1997) employed for the first time Matrix Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) Mass Spectrometry fingerprinting in the characterization of toxic cyanobacteria. Subsequently, new developments and enhancements of this technology were done in order to characterise a wide spectrum of microbial cells and MALDI-TOF Mass Spectrometry has been shown high potentialities to discriminate taxa very close related. MALDI-TOF Mass Spectrometry simplified the mass spectral analysis reducing the number of signals due to gentle ionization. Thus, biomolecules to be analysed give either single and/or double charged ions. Therefore very complex samples like whole cells can be investigated. Employing unfractionated cell materials, organism-specific signal patterns ("fingerprints") in the mass range of 2000 - 20000 Da can be obtained. In filamentous fungi most signals correspond to membrane surface proteins so, their highly characteristic masses can be used for the identification and classification. Fungi have been studied using this approach (Kallow *et al.*, 2006).

Species of the *Aspergillus* section *Nigri* have been extensively used for

various biotechnological purposes and are among the fungi best studied causing biodeterioration of commodities and food spoilage. Recently, *Aspergillus ibericus* was described as a new species in the section (Serra *et al.*, 2006). This new species was not only separated from their relatives in the section by morphological distinction but also from molecular point of view: briefly, *A. ibericus* among other morphological differences has 5-7 μm conidia size which allows separate this taxon from *A. carbonarius* (7-9 μm) and *A. niger* and its aggregate species (3-5 μm); the analysis of the ITS-5.8S rDNA, calmodulin gene sequences and AFLP patterns allowed consistently discriminate this taxon. Taking into account the potentialities of MALDI-TOF Mass Spectrometry as a novel identification method based on a phenotype characterisation, *A. ibericus* was studied and compared with other black aspergilli.

Materials and Methods

The fungal strains used in this study are listed in Table 1. Fungi were grown 3 days in liquid medium and after the mycelia were washed with distillated water and lyophilised. The dried mycelia were transferred as a thin film to the MALDI stainless steel template and mixed with 1 ml MALDI matrix solution (10 mg/ml 2,5-dihydroxybenzoic acid in water/acetonitrile [1:1] with 0.03% trifluoroacetic acid). The sample mixtures were air dried at room temperature. The analysis were performed using a MALDI-TOF mass spectrometer with a 337 nm nitrogen laser (Voyager DE-PRO, Applied

Table 1. List of strains used for MALDI-TOF Mass Spectrometry analysis.

Species	Isolate number	Geographical origin	Source
A. ibericus	MUM 03.49 (=IMI 391429, ITEM 4776) (**T**)	Portugal	Wine grapes
	MUM 03.50 (=IMI 391430, ITEM 6601)	Portugal	Wine grapes
	MUM 03.51 (=IMI 39143,1 ITEM 6602)	Portugal	Wine grapes
A. carbonarius	MUM 03.06 (=IMI 016136, NRRL 369) (**T**)	Unknown	Paper
	MUM 05.18 (=IMI 387223)	Portugal	Wine grapes
	MUM 03.59 (=IMI 387242)	Portugal	Wine must
A. niger	MUM 03.01 (=IMI 050566, NRRL 326) (**T**)	USA	Tannin-gallic acid fermentation
	MUM 03.57 (molecular pattern N)	Portugal	Wine grapes
	MUM 05.13 (molecular pattern T)	Portugal	Wine grapes
A. sclerotioniger	CBS 115572 (=MUM 06.151) (**T**)	India	Arabic coffee, green
A. lacticoffeatus	CBS 101883 (=MUM 06.150) (**T**)	Indonesia	Coffee robusta, surface sterlized beans
A. tubingensis	CBS 134.48 (=MUM 06.152) (**T**)	Unknown	Unknown
A. vadensis	CBS 113365 (=MUM 06.153) (**T**)	Unknown	Dead plant tissue
A. ellipticus	MUM 03.12 (=IMI 172283, NRRL 5120) (**T**)	Costa Rica	Soil
A. japonicus	MUM 03.02 (=ATCC 1042) (**T**)	Puorto Rico	Soil
A. aculeatus	MUM 03.11 (=IMI 211388) (**T**)	Unknown	Tropical soil
A. phoenicis	MUM 03.05 (=NRRL 365)	Unknown	Unknown
A. flavus (outgroup)	MUM 00.06	Portugal	Cheese repining chamber

(**T**) Type strain.

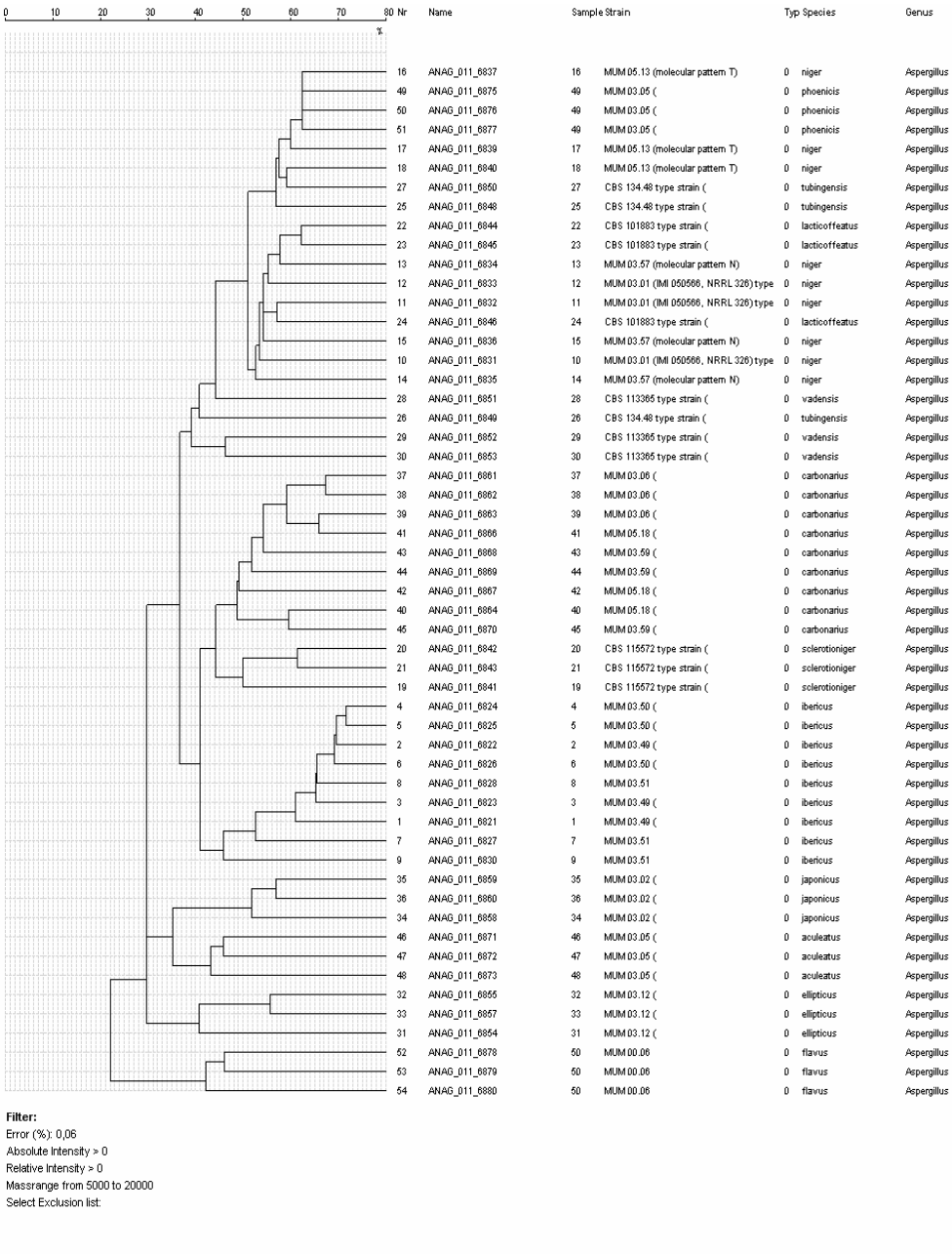

Figure 1. Dendrogram of relatedness between members of section Nigri *based on MALDI-TOF MS analysis.*

Biosystems, Foster City, CA) and a linear and delay extraction mode giving separation of proteins peaks and a mass accuracy of at least 200 ppm. Routinely the mass range from m/z = 2000 to 20000 Da was recorded.

Escherichia coli strain DH5a with known mass values of ribosomal proteins was used for external calibration. Following smoothing, baseline correction and peak detection steps, the peak lists of studied strains were directly transferred into the SARAMIS (Spectral ARchiving And Microbial Identification System) software where it is matched against the SARAMIS database for spectra comparison. This software allows the classification of microorganisms and, additionally theoretical spectra (superspectra) help to multiply the efficiency of the microbial identification and the grouping of samples.

Results

A. ibericus is closed related to *A. carbonarius* but was readily distinguished on the basis of morphological and molecular analysis. In the mass spectra dendrogram (Fig.1) shows two distinct clades for these two species. Additionally, *A. sclerotioniger* and *A. lacticoffeatus* show also relatedness with *A. carbonarius* and *A. niger*, respectively. This was obtained before when β-tubulin gene sequence was used. *A. niger* aggregate studied is composed by *A. niger*, *A. phoenicis*, and *A. tubingensis*. *A. vadensis* is now described as a new species which came from the *A. niger* aggregate but, from a chemotaxonomical point of view, it is considered the most related species with *A. tubingensis*. As it is observed in the dendrogram all these species are well related and aggregated in a clade. Furthermore, *A. japonicus* and *A. aculeatus* are the only uniseriate species in this section and they clearly show in the dendrogram a sister clade. *A. ellipticus* is an uncommon species and appears as neighbour of these two species in this dendrogram. However it is a clearly distant related species. *A. flavus* was used as an out-group species.

Conclusion

In conclusion, results of MALDI-TOF Mass Spectrometry analysis using mass range from 5000 – 20000 Da were similar to those of phylogenetic analysis giving a sound input for *A. ibericus* characterisation and showing the potentialities of the method for taxonomic purposes.

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References

ERHARD, M., VON DÖHREN, H., JUNGBLUT, P. Rapid typing and elucidation of new secondary metabolites of intact cyanobacteria using MALDI-TOF mass spectrometry. Nature Biotechnol 15, 906-909, 1997.

KALLOW, W., ERHARD, M., LIMA, N., SANTOS, I.M., SERRA, R., VENÂNCIO, A., FRIEDL, T., MÜLLER, J., DE HOOG, G.S., VERKLEY G.J.M. Microbial strain characterisation by MALDI-TOF MS - possibilities and limits. *In*: Proceedings of the Annual General Meeting of the European Culture Collections' Organisation. Budapest, NCAIM, Corvinus University of Budapest, pp. 146-159, 2006.

SERRA, R., CABAÑES, F.J., PERRONE, G., CASTELLA, G., VENÂNCIO, A., MULE, G., KOZAKIEWICKZ, Z. *Asgergillus ibericus*: a new species of section *Nigri* isolated from grapes. Mycologia 98, 295-306, 2006.