

Use of a polyphasic approach including MALDI-TOF MS for identification of *Aspergillus* section *Flavi* strains isolated from food commodities in Brazil

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Abstract Brazil is one the largest producers and exporters of food commodities in the world. The evaluation of fungi capable of spoilage and the production mycotoxins in these commodities is an important issue that can be of help in bioeconomic development. The present work aimed to identify fungi of the genus *Aspergillus* section *Flavi* isolated from different food commodities in Brazil. Thirty-five fungal isolates belonging to the section *Flavi* were identified and characterised. Different classic phenotypic and genotypic methodologies were used, as well as a novel approach based on proteomic profiles produced by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS). Type or reference strains for each taxonomic group were included in this study. Three isolates that presented discordant identification patterns were further analysed using the internal transcribed spacer (ITS) region and calmodulin gene sequences. The data obtained from the phenotypic and spectral analyses divide the isolates into three

groups, corresponding to taxa closely related to *Aspergillus flavus*, *Aspergillus parasiticus*, and *Aspergillus tamaritii*. Final polyphasic fungal identification was achieved by joining data from molecular analyses, classical morphology, and biochemical and proteomic profiles generated by MALDI-TOF MS.

Keywords *Aspergillus* section *Flavi* · Food mycology · MALDI-TOF MS · Mycotoxin · Polyphasic identification

Introduction

The genus *Aspergillus* is one of the most important microfungi in industry, agriculture, and public health. Since the last taxonomic revision, more than 250 species belonging to this genus have been accepted as being widely distributed in the world (Simões et al. 2013). In addition, some of the most important microorganisms used in food fermentation, enzyme production, and industrial bioprocesses are part of the genus *Aspergillus*.

The *Aspergillus* section *Flavi* comprises 25 species, from which six of them are of economic importance and are very closely related among themselves from a morphological and phylogenetic point of view. Based on their impact on food and/or human health, fungi belonging to the section *Flavi* are often separated into two groups (Godet and Munaut 2010). The first group includes *Aspergillus flavus*, *A. parasiticus* and *A. nomius*. Some strains of this group of species are reported to be producers of aflatoxins. The second group comprises *A. oryzae*, *A. sojae* and *A. tamaritii*. These species are described as non-producers of aflatoxins and are normally used in Asian countries for fermenting processes (Kumeda and Asao 2001; Hedayati et al. 2007).

The identification of *Aspergillus* section *Flavi* has traditionally been based on macro- and micro-morphological and biochemical traits (Raper and Fennel 1965; Samson et al.

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2000; Rodrigues et al. 2011). The macromorphological traits include conidial and colony colour, colony diameter, colony reverse colour, production of exudates and soluble pigments, and the presence of sclerotia and cleistothecia, while the micromorphology traits include seriation, shape, and size of the vesicle, conidia and stipe morphology, the presence of Hülle cells, and morphology of cleistothecia and ascospores (Klich 2002).

From a biochemical traits point of view, some strains of *A. flavus* have been considered to produce aflatoxins B (AFBs) and/or cyclopiazonic acid (CPA). However, *A. flavus* is not considered to produce aflatoxins G (AFGs). On the other hand, some *A. parasiticus* strains are able to produce AFB1, AFB2, AFG1 and AFG2. Nevertheless, this fungal species has not been described as a producer of CPA (Bennett and Klich 2003; Yu et al. 2004).

Other species comprising the *Aspergillus* section *Flavi*, namely *A. toxicarius*, *A. nomius*, *A. bombycis*, and *A. pseudotamarii*, have also been described as producers of AFBs and CPA. In contrast, *A. oryzae* has not been described as a producer of AFBs, although it can produce CPA and β -nitropropionic acid. Furthermore, the species *A. oryzae*, *A. parasiticus*, *A. sojae*, *A. nomius*, *A. bombycis*, *A. tamarii*, *A. caelatus*, and *A. pseudotamarii* may also produce kojic acid (Varga et al. 2003).

Over the past few years, molecular methods have been widely applied to the identification of different microbial taxa, including a large number of *Aspergillus* species (Batista et al. 2008; Rodrigues et al. 2009, 2011; Lima-Neto et al. 2014; Midorikawa et al. 2014; Nicolau et al. 2014). Molecular traits provide the greatest number of variable characters for fungal taxonomy. Molecular technology, when used together with a well-developed bioinformatics toll, allows the comparison of results. It produces data that are generally highly correlated with reproducible phenotypic differences and ensures that molecular characters have a primary role in recognising fungal taxa. Nonetheless, even with the high level of sensitivity and resolution associated with the molecular methods, some problems still arise when this technique is applied for the identification of species within the *Aspergillus* section *Flavi*. These difficulties are due to the high level of genetic similarity found in some of species of the *Aspergillus* section *Flavi* (Rodrigues et al. 2011).

Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) is a technique able to analyse organic compounds, including those of high-weight molecular mass. It works through the soft ionisation of the molecules, resulting in minimum fragmentation (Tanaka et al. 1988). MALDI-TOF MS can be applied to analyse the chemical cellular composition of microorganisms, providing rapid and discriminatory proteomic profiles for their identification and subtyping. The remarkable reproducibility of this technique is based on the measurement of constantly

expressed and highly abundant proteins. The usually observable molecular mass range is between 2,000 and 20,000 Da, where important ribosomal proteins appear. It is an advantage because the ribosomal proteins can easily be used as biomarkers (Santos et al. 2010). Recently, proteomic profiles by MALDI-TOF MS have been used as an important step in the polyphasic identification of filamentous fungi isolated from different fields (Santos et al. 2010, 2011; Dias et al. 2011; Rodrigues et al. 2011; Passarini et al. 2013; Pereira et al. 2014).

Consistent identification and characterisation of filamentous fungi species have been developed by application of the so-called polyphasic approach, which involves the use of the various techniques discussed above (e.g., macro- and micro-morphology, biochemical, molecular biology, and proteomic profiles by MALDI-TOF MS) ensuring the systematisation of scientific knowledge (Simões et al. 2013). Information obtained from each technique is compared with that from the others in a step-by-step methodology, the molecular techniques of which have been recognised as the gold standard for the identification of *Aspergillus* species (Simões et al. 2013). The aim of this study was to apply a polyphasic approach for the identification of 35 fungal strains belonging to the section *Flavi* isolated from different food commodities in Brazil. Furthermore, five additional fungal strains obtained from the Portuguese Culture Collection *Micoteca da Universidade do Minho (MUM)* were used as reference strains. The polyphasic approach consisted of macro- and micro-morphology, biochemical analyses, proteomic profiling by MALDI-TOF MS, and molecular biology using the ITS region of the rRNA gene and partial calmodulin gene.

Material and methods

Fungal isolates and culture conditions

Thirty-five isolates of *Aspergillus* section *Flavi* were isolated from food commodities (coffee, maize, Brazil nuts, peanuts, rice, and cereal snack bars) acquired at a local market in the city of Lavras (MG), Brazil. After purification and identification, the isolates were deposited at the EcoCentro Mycological Collection of the Company of Agricultural Research of Minas Gerais (EPAMIG, <http://www.epamig.br/>), Lavras (MG), Brazil.

The code “CMEcoCentro 000nn” was attributed for each fungal isolate, where “nn” means isolate number. The reference strains *A. flavus* MUM 92.01 (NRRL6412), *A. parasiticus* MUM 92.02 (NRRL3386), *A. oryzae* MUM 98.04 (NRRL3484), *A. tamarii* MUM 92.03 (NRRL 427) and *A. sojae* MUM 10.241 (CBS 100928) were obtained from the Micoteca da Universidade do Minho (MUM, www.micoteca.deb.uminho.pt/) Culture Collection, Braga, Portugal.

Morphological characterisation

All 35 isolates were submitted to the observation of macro- and micro-morphological traits. For the macro-morphological analysis, all isolates were grown and maintained on malt extract agar (MEA: malt 20 g L⁻¹, glucose 20 g L⁻¹, peptone 1 g L⁻¹, agar 20 g L⁻¹), Czapek yeast autolysate agar (CYA: sucrose 30 g L⁻¹, powdered yeast extract 5 g L⁻¹, K₂HPO₄ 1 g L⁻¹, NaNO₃ 2 g L⁻¹, KCl 0.5 g L⁻¹, MgSO₄ 7H₂O 0.5 g L⁻¹, FeSO₄ 7H₂O 0.01 g L⁻¹, ZnSO₄ 7H₂O 0.01 g L⁻¹, CuSO₄ 5H₂O 0.005 g L⁻¹, agar 20 g L⁻¹), Czapek yeast extract agar with 20 % sucrose (CY20S: sucrose 200 g L⁻¹, yeast extract 5 g L⁻¹, K₂HPO₄ 1 g L⁻¹, Czapek concentrate 10 mL, trace metals solution 1 mL, agar 15 g L⁻¹) and Czapek medium (CZ: sucrose 30 g L⁻¹, K₂HPO₄ 1 g L⁻¹, NaNO₃ 2 g L⁻¹, KCl 0.5 g L⁻¹, MgSO₄ 7H₂O 0.5 g L⁻¹, FeSO₄ 7H₂O 0.01 g L⁻¹, ZnSO₄ 7H₂O 0.01 g L⁻¹, CuSO₄ 5H₂O 0.005 g L⁻¹, agar 20 g L⁻¹).

Spores of each isolate suspended in 1 mL of 0.2-% agar were used for three-point inoculations on 9-cm diameter Petri dishes containing 20 mL of each medium described above. Cultures were incubated for 7 days in the dark at 25 °C for culture media MEA, CYA and CY20S, at 37 °C for CYA medium, and at 42 °C for CZ medium. For the micro-morphological analysis, all isolates were grown on MEA and cultures were incubated for 7 days in the dark at 25 °C, as described above.

In order to confirm group identification by colony reverse colour, all isolates were cultured on *Aspergillus flavus* and *A. parasiticus* agar (AFPA, Oxoid, Basingstoke, United Kingdom) for 3 to 5 days at 25 °C in the dark. All cultures were then analysed for colony colour, presence of sclerotia, head seriation and conidia ornamentation. Fungal identification followed the taxonomic keys and guides available for the *Aspergillus* genus (Klich 2002; Samson et al. 2004; Rodrigues et al. 2009, 2011).

Biochemical characterisation

In order to evaluate the ability of a fungus to produce aflatoxin, all 35 isolates were grown on aflatoxin-inducing yeast extract sucrose medium (YES: yeast extract 20 g L⁻¹, sucrose 150 g L⁻¹, agar 15 g L⁻¹). Spores of each isolate suspended in 0.5 mL of 0.2 % agar were used for inoculation on a single point on 6-cm diameter Petri dishes and incubated at 25 °C in the dark for 7 days.

For aflatoxin extraction, the methodology described by Rodrigues et al. (2011) was applied. Briefly, three agar plugs were removed from the fungal colony and placed in a 4-mL vial, where 2 mL of methanol was added. After 60 min, the extract was filtered by 0.45-µm filters and analysed by high performance liquid chromatography (HPLC) equipped with a Jasco FP-920 fluorescence detector (365 nm excitation

wavelength, 435 nm emission wavelength), using a photochemical post-column derivatisation reactor (PHRED unit, Aura Industries, New York, NY, USA).

Chromatographic separations were performed on a reverse-phase C18 column (Waters Spherisorb ODS2, 4.6 mm × 250 mm, 5 µm) fitted with a pre-column with the same stationary phase. The mobile phase used was pumped at 1.0 mL min⁻¹ and consisted of an isocratic programme as follows: water- to-acetonitrile-to-methanol (3:1:1, v/v/v). The injection volume was 50 µL.

Aflatoxin standards were supplied by R-Biopharm Rhône Ltd. A mix of aflatoxins containing 1 µg mL⁻¹ of AFB1, AFB2, AFG1, and AFG2 was used. Samples were interpreted as positive for each one of the toxins when they yielded a peak at a retention time similar to each standard, with a height fivefold higher than the baseline noise.

In order to evaluate the fungal capacity for producing CPA, all isolates were grown on a single point on 6-cm diameter plates containing CYA and incubated at 25 °C in the dark for 14 days. For the CPA extraction, the methodology described by Rodrigues et al. (2011) was applied.

Samples were analysed using an HPLC equipped with a Varian 2050 UV detector (285 nm). Chromatographic separations were performed on a Eurospher 100 NH2 column (Knauer, 4.6 mm × 250 mm, 5 µm), fitted with a pre-column with the same stationary phase. The mobile phase used was pumped at 1.0 mL min⁻¹, and consisted of an isocratic programme as follows: acetonitrile:50 mM ammonium acetate (3:1, v/v) with a pH of 5.0. The injection volume was 50 µL. CPA standard was supplied by Sigma-Aldrich (St. Louis, MO, USA). Samples were interpreted as positive when they yielded a peak at a retention time similar to the CPA standard, with a height fivefold that of the baseline noise.

Phenotypic data analysis

A dendrogram of phenotypic relatedness was constructed. The characters analysed were both (a) the macro- and micro-morphological traits, namely, the head seriation, colony colour on CYA, conidia ornamentation, reverse colony colour on AFPA; and (b) the biochemical traits, namely the production of AFB1 and AFB2, AFG1 and AFG2, and CPA. Statistical analysis was performed based on a hierarchical clustering with the average linkage method (between groups), and was achieved using the statistical package SPSS version 16.0 (IBM/SPSS Inc. Chicago, IL, USA).

Proteomic profile by MALDI-TOF MS

Seven-day-old spores of each fungal isolate suspended in 0.5 mL of 0.2 % agar were used for inoculation on a single point on 6-cm diameter Petri dishes with MEA. All isolates were incubated for 3 days at 25 °C. Approximately 1 µg of

mycelium or a mycelium/spore mixture from the growing edge colony was transferred directly from the culture plate to the 48-well MALDI flex target plate (FlexiMass™, Shimadzu Biotech, Buckinghamshire, UK). Immediately, 0.5 µL matrix solution (75 mg mL⁻¹ 2,5-dihydroxybenzoic acid [DHB] in ethanol/water/acetonitrile [1:1:1] with 0.03 % trifluoroacetic acid [TFA]) was added and mixed gently. *Escherichia coli* strain DH5α was obtained from the MUM Culture Collection, and its cells were used for in situ extraction of proteins, which were used as the standard for the MALDI-TOF MS external calibration. Cells of *E. coli* DH5α were grown on Luria-Bertani medium agar (LB; 10 g L⁻¹ bacto-tryptone, 5 g L⁻¹ bactoyeast extract, 10 g L⁻¹ NaCl) at 30 °C for 20 h. About 1 µg of cellular material from a single *E. coli* colony was transferred from the Petri dish to the MALDI flex plate and the matrix solution was applied as described above for the fungal analysis. All sample plates were air dried at room temperature. Each sample was spotted in duplicate to test reproducibility. During the analyses, all solutions were prepared daily and stored at +5 °C.

Analyses were performed on an Axima LNR MALDI-TOF MS system (Kratos Analytical, Shimadzu, UK) equipped with a nitrogen laser (337 nm), where the laser intensity was set just above the threshold for ion production. Twelve defined ribosomal proteins of intact *E. coli* DH5α cells (4,365.4, 5,096.8, 5,381.4, 6,241.4, 6,255.4, 6,316.2, 6,411.6, 6,856.1, 7,158.8, 7,274.5, 7,872.1, 9,742 and 12,227.3 Da) were used as external calibrants. The mass spectra based on the mass range from 2 to 20 kDa were recorded using the linear mode with a delay of 104 ns and an acceleration voltage of +20 kV. The final spectra were generated by summing 20 laser shots accumulated per profile and 50 profiles produced per sample, which led to 1,000 laser shots per summed spectrum.

The resulting peak lists were exported to the SARAMIS™ software package (Spectral Archiving and Microbial Identification System; AnagnosTec, Zossen, Germany), where the final microbial identification was achieved. In SARAMIS™, peak lists of individual samples were compared to a database, generating a ranked list of matching spectra. This software uses a point system based on the peak list with mass signals weighted according to their specificity. The similarity between individual spectra is expressed as the relative or absolute number of matching mass signals after subjecting the data to a single link agglomerative clustering algorithm. Microbial identifications by the SARAMIS™ package are based on the presence or absence of each peak in the spectra. A dendrogram of proteomic profile proximity among isolates was created using the SARAMIS™ package.

Molecular identification and phylogenetic analysis

The isolates CMecoCentro 00043, CMecoCentro 00055, and CMecoCentro 00095 were subjected to molecular analysis of

the ITS region of the rRNA gene and partial calmodulin gene. In both cases, DNA extraction followed the protocol described by Rodrigues et al. (2009). The ITS region (spanning part of the 18S rRNA gene, the internal transcribed spacer 1, the 5.8S rRNA gene, the internal transcribed spacer 2 and part of the 28S rRNA gene; *c.a.* 600 bp) was amplified using the primer pair ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), as described by White et al. (1990).

Amplification of a portion of the calmodulin gene (comprising part of exon 2, exons 3–5, part of exon 6, and introns 2–5; *c.a.* 730 bp) was set up with the primers CL1 (5'-GARTWCAAGGAGGCCTTCTC-3') and CL2A (5'-TTTTGCATCATGAGTTGGAC-3'), as described by O'Donnell et al. (2000). Polymerase chain reaction (PCR) amplifications were performed on 25 µL of a reaction mixture containing MgCl₂-free reaction buffer, 1.5-mM MgCl₂, 1.25 U of Taq polymerase, 200 µM of each dNTP, 0.2 µM of each primer, and 1 ng µL⁻¹ of template DNA. PCR was carried out as follows: (1) one step at 95 °C for 5 min; (2) 30 cycles of the following three steps: 30 s at 95 °C, 1 min at 62 °C, 2 min 72 °C; and (3) one final 5-min step at 72 °C.

For the calmodulin gene, the initial denaturation was performed at 95 °C for 2 min. Amplified products were purified using the commercial kit PCR Product Purification JetQuick (Genomed, Lisbon, Portugal) according to the manufacturer instructions. The amplified products were then quantified and subjected to sequencing using the BigDye Terminator Cycle Sequencing Standard Kit for an automated ABI 3730xl DNA Analyser (Applied Biosystems, Foster City, CA, USA). Here, the sets of primers used for sequencing were the same as described above.

Sequences were compared with ITS-rRNA sequence data from strains available at the public databases Genbank (<http://www.ncbi.nlm.nih.gov>) and CBS Fungal Biodiversity Centre (<http://www.cbs.knaw.nl/fungi/BioloMICSSequences.aspx>) using the BLAST N sequence match routines. Sequences were aligned using the CLUSTALX program, and phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4.0 (Tamura et al. 2007). To estimate the evolutionary distance, the Kimura two-parameter mode (Kimura 1980) was used. Phylogenetic reconstruction was done using the neighbor-joining (NJ) algorithm, with bootstrap values calculated from 1,000 replicate runs, using the software routines included in the MEGA software.

Results

Phenotypic characterisation

The results obtained by phenotypic characterisation of all 35 isolates evaluated in this study are summarised in Table 1.

Table 1 Morphological and chemical characterisation of isolates of *Aspergillus* section *Flavi*

Isolate code	Origin	Morphology			Colony diameter (cm)							Reverse colour	Mycotoxins				Identification		
		Sclerotia	Head seriation	Conidia ornamentation	Colony colour	Colony diameter (cm)							AFB1	AFB2	AFG1	AFG2		CPA	
						CYA 25 °C	CYA 37 °C	MEA 25 °C	MEA 37 °C	CY20S 25 °C	CZ 25 °C								CZ 42 °C
CMEcoCentro 00041	Coffee	+	u	R	d	6.2	6.9	6.7	7.0	4.3	y/o	+	+	+	-	-	-	-	<i>A. parasiticus</i>
CMEcoCentro 00095	Coffee	+	u	R	d	6.3	6.9	6.2	7.0	4.0	y/o	+	+	+	-	-	-	-	<i>A. parasiticus</i>
CMEcoCentro 00040	Coffee	+	u	R	d	6.2	6.2	6.6	7.1	3.5	y/o	+	+	+	-	-	-	-	<i>A. parasiticus</i>
CMEcoCentro 00097	Brazilian nut	+	u	R	y/d	5.9	6.9	5.1	6.7	3.0	y/o	+	+	+	-	-	-	-	<i>A. parasiticus</i>
CMEcoCentro 00042	Coffee	+	u	S	d	6.6	6.7	6.1	7.0	2.6	o	-	-	-	-	-	-	-	<i>A. flavus</i>
CMEcoCentro 00043	Coffee	+	u	Fr	d	6.6	6.5	5.6	7.0	2.3	o	-	-	-	-	-	-	-	<i>A. flavus</i>
CMEcoCentro 00044	Coffee	-	u	Fr	y	6.7	6.5	6.5	7.2	3.6	o	-	-	-	-	-	-	-	<i>A. flavus</i>
CMEcoCentro 00045	Coffee	+	u	S	y	6.9	6.5	6.3	6.9	1.4	o	+	+	+	-	-	-	-	<i>A. flavus</i>
CMEcoCentro 00047	Coffee	+	u	S	d	6.3	6.5	5.3	7.0	2.3	o	-	-	-	-	-	-	-	<i>A. flavus</i>
CMEcoCentro 00048	Coffee	-	u	S	d	5.5	6.4	5.9	7.0	3.3	o	-	-	-	-	-	-	-	<i>A. flavus</i>
CMEcoCentro 00049	Coffee	+	u	Fr	y/d	6.6	6.5	6.4	6.9	2.1	o	-	-	-	-	-	-	-	<i>A. flavus</i>
CMEcoCentro 00050	Coffee	-	u	S	y/d	5.4	6.6	6.4	7.0	5.9	o	-	-	-	-	-	-	-	<i>A. flavus</i>
CMEcoCentro 00051	Coffee	+	b	S	y/d	5.9	5.2	6.5	7.0	3.0	o	-	-	-	-	-	-	-	<i>A. flavus</i>
CMEcoCentro 00052	Coffee	-	u	S	y/d	6.4	6.1	6.2	6.9	3.9	o	+	+	+	-	-	-	-	<i>A. flavus</i>
CMEcoCentro 00053	Coffee	+	u	Fr	y/d	6.3	6.9	5.7	7.0	3.4	o	-	-	-	-	-	-	-	<i>A. flavus</i>
CMEcoCentro 00054	Coffee	+	u	Fr	y/d	6.4	6.4	6.0	7.0	2.9	o	-	-	-	-	-	-	-	<i>A. flavus</i>
CMEcoCentro 00055	Coffee	-	u	Fr	d	6.8	6.3	6.2	7.0	3.7	o	-	-	-	-	-	-	-	<i>A. flavus</i>
CMEcoCentro 00056	Coffee	+	u	Fr	d	6.5	6.7	6.2	7.1	4.1	o	-	-	-	-	-	-	-	<i>A. flavus</i>
CMEcoCentro 00057	Maize	+	b	S	d	6.2	5.4	6.2	7.1	4.3	o	-	-	-	-	-	-	-	<i>A. flavus</i>
CMEcoCentro 00058	Maize	+	b	S	y/d	6.0	6.2	6.5	7.0	4.4	o	-	-	-	-	-	-	-	<i>A. flavus</i>
CMEcoCentro 00059	Maize	+	b	Fr	y/d	6.6	5.0	6.6	7.0	4.7	o	-	-	-	-	-	-	-	<i>A. flavus</i>
CMEcoCentro 00061	Maize	+	b	Fr	y/d	6.6	5.7	6.7	6.7	4.7	o	-	-	-	-	-	-	-	<i>A. flavus</i>
CMEcoCentro 00062	Peanut	-	b	S	y/d	5.8	5.1	5.4	6.9	4.6	o	+	+	+	-	-	-	-	<i>A. flavus</i>
CMEcoCentro 00084	Rice	+	b	S	y/d	5.7	4.1	6.3	7.0	5.1	o	-	-	-	-	-	-	-	<i>A. flavus</i>
CMEcoCentro 00085	Peanut	-	u	S	y/d	6.5	6.3	6.4	6.8	5.0	o	-	-	-	-	-	-	-	<i>A. flavus</i>
CMEcoCentro 00086	Maize	+	b	S	y/d	6.2	6.6	6.1	7.0	1.5	o	-	-	-	-	-	-	-	<i>A. flavus</i>
CMEcoCentro 00089	Cereal bar	-	u	S	d	5.5	6.6	6.0	6.8	3.9	o	-	-	-	-	-	-	-	<i>A. flavus</i>
CMEcoCentro 00090	Cereal bar	+	b	S	y/d	5.7	6.5	6.4	7.0	2.4	o	-	-	-	-	-	-	-	<i>A. flavus</i>
CMEcoCentro 00091	Cereal bar	+	b/u	S	y/d	6.7	6.8	6.3	6.8	2.8	o	-	-	-	-	-	-	-	<i>A. flavus</i>
CMEcoCentro 00093	Cereal bar	-	b/u	S	y/d	5.9	6.7	5.5	6.9	2.4	o	-	-	-	-	-	-	-	<i>A. flavus</i>
CMEcoCentro 00094	Maize	+	b	S	y/d	6.3	6.5	6.4	7.0	3.1	o	+	+	+	-	-	-	-	<i>A. flavus</i>

Table 1 (continued)

Isolate code	Origin	Morphology		Colony diameter (cm)			Reverse colour		Mycotoxins				Identification										
		Sclerotia	Head seriation	Conidia ornamentation	Colony colour			AFPA	AFB1	AFB2	AFG1	AFG2		CPA									
					CYA 25 °C	CYA 37 °C	MEA 25 °C								CY20S 25 °C	CZ 42 °C							
CMEcoCentro 00096	Brazilian nut	-	b	Fr	d	6.2	6.9	5.9	6.9	2.8	o	-	-	-	-	-	-	-	-	-	-	-	<i>A. flavus</i>
CMEcoCentro 00098	Brazilian nut	-	u	R	b	7.0	6.2	6.9	5.1	nd	nd	-	-	-	-	-	-	-	-	-	-	-	<i>A. tamaritii</i>
CMEcoCentro 00099	Brazilian nut	+	u	Fr	b	6.0	6.9	5.4	5.8	2.1	nd	-	-	-	-	-	-	-	-	-	-	-	<i>A. oryzae</i>
CMEcoCentro 00100	Brazilian nut	-	u	R	b	6.1	4.8	5.4	7.0	7	nd	-	-	-	-	-	-	-	-	-	-	-	<i>A. sojae</i>
MUM 92.01	Reference strain	-	b/u	S	y	6.4	6.8	6.0	7.0	1.8	nd	-	-	-	-	-	-	-	-	-	-	-	<i>A. flavus</i>
MUM 92.02	Type strain	-	u	R	d	5.9	6.9	6.6	6.1	nd	nd	+	+	+	+	+	+	+	+	+	+	+	<i>A. parasiticus</i>
MUM 98.04	Type strain	-	u	Fr	y	5.9	6.7	6.4	5.1	1.9	nd	-	-	-	-	-	-	-	-	-	-	-	<i>A. oryzae</i>
MUM 92.03	Reference strain	-	u	R	b	6.7	7.1	6.5	5.5	nd	nd	-	-	-	-	-	-	-	-	-	-	-	<i>A. tamaritii</i>
MUM 10.241	Type strain	-	u	R	d	6.5	6.6	6.3	6.2	nd	nd	-	-	-	-	-	-	-	-	-	-	-	<i>A. sojae</i>

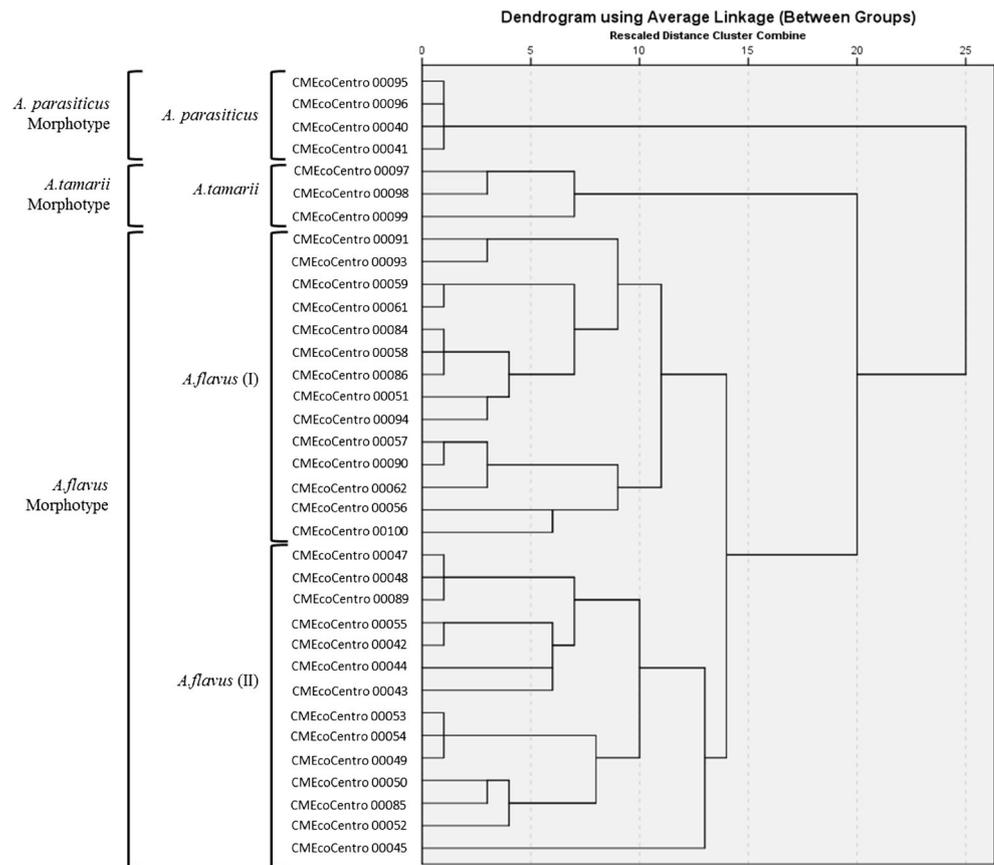
^a sclerotia; + detected; - not detected; ^b u Uniseriate; b/u Predominantly biseriate; ^s Smooth; r Rough; fr Finely rough; ^d y Yellow-green; ^d Dark-green; ^{y/d} In between y and d; b Brown; ^e Average of three colonies in cm; ^s y/o Yellow-orange; o Orange; c Cream; ^h nd Not determined

Based on the bright orange or yellow-orange colour of the reverse of the colony on AFPA, all isolates evaluated were classified as belonging to the *Aspergillus* section *Flavi*. According to the data obtained from the macro- and micro-morphological traits—primarily colony colour on CYA and conidia ornamentation—five different fungal species were found among the whole population under study. The first group of species was *A. parasiticus* represented by four isolates (c.a. 11 %) that presented dark-green colonies and rough conidia. The second group of species was *A. flavus* represented by 28 isolates (80 %) presenting dark-green to yellow-green colonies, smooth, and from smooth to finely rough conidia. One isolate (c.a. 3 %) of *A. oryzae*, *A. sojae* and *A. tamaritii*, was further morphologically identified. The isolate of the species *A. tamaritii* presented a brown colony and rough conidia, whereas the isolate belonging to the species *A. oryzae* showed a brown colony and smooth to finely rough conidia. The isolate of the species *A. sojae* presented a light brown colony and rough conidia (Table 1).

Eighteen isolates (c.a. 51 %) belonging to *A. flavus* and 4 isolates (c.a. 11 %) belonging to *A. parasiticus* were producers of sclerotia. In addition, 4 isolates (c.a. 11 %) and 1 isolate (c.a. 3 %) of *A. flavus* were producers of AFB1 and AFB2, respectively, while 16 isolates (c.a. 46 %) of this species were producers of CPA. The production of AFG1 and AFG2 was not detected for the isolates of *A. flavus*. All the 4 isolates belonging to *A. parasiticus* (c.a. 11 %) were producers of the mycotoxins AFB1, AFB2 and AFG1. On the other hand, the production of AFG2 and CPA were not detected by these fungal isolates. As expected, the isolates of the species *A. oryzae*, *A. sojae* and *A. tamaritii* were not detected producing AFB1, AFB2, AFG1 and AFG2. On the other hand, the isolates of *A. oryzae* and *A. tamaritii* were producers of CPA (Table 1).

By associating morphotypes to chemotypes of both field isolates and reference strains, all isolates were statistically grouped into four phenotypes (Fig. 1). The phenotype *A. parasiticus* showed the distinct characteristics described above and clustered forming a single clade in the phenotypic dendrogram. However, the group *A. flavus* was subdivided into two phenotypes [Fig. 1, *A. flavus* phenotype (I) and (II)]. From the 14 *A. flavus* isolates that were grouped as phenotype (I), the following characteristics were observed: 11 isolates (c.a. 79 %) were biseriate; 10 isolates (c.a. 71 %) showed smooth conidia; 11 isolates (c.a. 79 %) presented from yellow-green to dark-green colonies; 4 isolates (c.a. 29 %) of the whole fungal population of this phenotype were producers of mycotoxins. Furthermore, 9 isolates (c.a. 64 %) were isolated from maize and its derivatives. On the other hand, from the 14 isolates of *A. flavus* grouped as phenotype (II), the following characteristics were observed: 13 isolates

Fig. 1 Dendrogram of relatedness among isolates of *Aspergillus* section *Flavi* based on the analysis of seriation, colony colour on CYA, conidia ornamentation, reverse on AFPA, AFB production, AFG production, CPA production, and origin of the isolate



(93 %) were producers of mycotoxins; all of them (14 isolates, 100 %) were uniseriate; 8 isolates (*c.a.* 57 %) presented smooth conidia and 6 isolates (*c.a.* 43 %) presented finely rough conidia; 6 isolates (*c.a.* 43 %) presented dark green colony colour; 12 of them (*c.a.* 86 %) were isolated from coffee. The phenotype *A. tamarii* comprises the isolates of the non-mycotoxigenic strains, namely, *A. oryzae*, *A. sojae*, and *A. tamarii*.

Proteomic profiles by MALDI-TOF MS

Figure 2 shows the dendrogram of relatedness based on proteomic profiles by MALDI-TOF MS, including all 35 isolates evaluated in this study and the reference strains *A. flavus* MUM 92.01 and *A. tamarii* MUM 09.03 (NRRL 427), and the type strains *A. oryzae* MUM 98.04 (NRRL3484) and *A. sojae* MUM 10.241 (CBS 100928). Also, the reference strain *Trichophyton rubrum* MUM 08.05 was used as an outgroup and was included in the dendrogram. In this analysis, the following distribution for the field isolates was obtained: 14 isolates of *A. flavus* (I) (*c.a.* 52 % of all *A. flavus* isolates); 10 isolates of *A. flavus* (II) (*c.a.* 37 % of all *A. flavus* isolates); 4 isolates of *A. flavus* (III) (*c.a.* 15 % of all *A. flavus* isolates); and 4 isolates of *A. parasiticus* (100 % of *A. parasiticus* isolates).

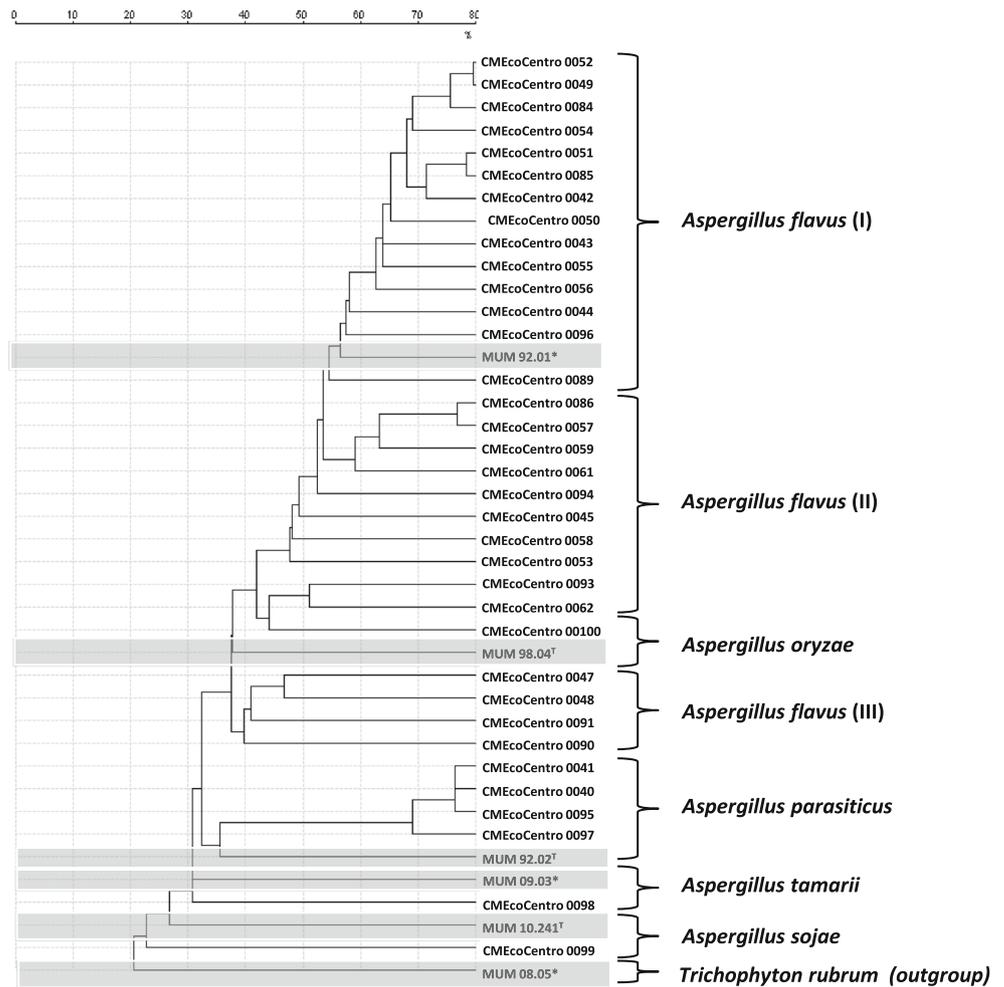
Molecular identification

Data derived from phylogenetic analyses of ITS regions for the three strains *A. flavus* CMecoCentro 00043, CMecoCentro 00055, and CMecoCentro 00095 was not resolved well enough in this study. Based on the analysis of the ITS region, a cluster containing these three *A. flavus* strains separated from the sequences of other strains such as *A. flavus* MUM 10.209 (HQ340107), *A. flavus* NRRL 32354 (HQ288050), and *A. oryzae* NRRL 35191 (EF591304) was obtained (data not shown). For the molecular analysis of the calmodulin gene, it can be observed that both samples CMecoCentro 00043 and CMecoCentro 00055 clustered with the sequence of *A. flavus* MUM 10.209 (HQ340087) with 99 % BLAST similarity (Table 2). Moreover, the isolate CMecoCentro 000100 clustered with sequences of *A. oryzae* NRR 447 (EF661506), *A. oryzae* CBS 466.91 (FN594585), and *A. flavus* NRRL 4822 (EF661513), also with 99 % BLAST similarity (Table 2).

Discussion

In the present study, 35 isolates of *Aspergillus* section *Flavi* were evaluated using a polyphasic approach. Morphological

Fig. 2 Dendrogram of proteomic relatedness among isolates of *Aspergillus* section *Flavi* based on MALDI-TOF MS. Distances are measured as a percentage of spectral similarity; * reference strains; ^T type strains



differentiation of species belonging to this group is difficult due to both interspecific similarities and intraspecific variability. In this analysis, colony colour on CYA, conidia ornamentation, and production of the mycotoxins AFB1, AFB2, AFG1, and AFG2 retained their high association with the species. However, in some cases, this specific association is found at lower levels than those expected for phenotypic identification.

Aspergillus flavus and *A. parasiticus* are morphologically distinguished by colony colour and texture of the wall of conidia (Klich and Pitt 1988). According to Rodrigues et al. (2009, 2011), this differentiation can be improved by the use of a mycotoxigenic profile of each species. *Aspergillus parasiticus* produces AFBs and AFGs but not CPA, and *A. flavus* produces AFBs and CPA, but not AFGs. Aflatoxin production by *A. tamaraii* has been reported in the literature (Goto et al. 1996; Ito et al. 2001). On the other hand, *A. oryzae* and *A. sojae* are considered non-mycotoxigenic variants of *A. flavus* and *A. parasiticus*, respectively (Kurtzman et al. 1987).

In the present study, 18 (c.a. 51 %) isolates of *A. flavus* and 4 isolates (c.a. 11 %) of *A. parasiticus* were producers of

sclerotia. However, the production of sclerotia was not directly related to the production of aflatoxins for these isolates of *A. flavus* and *A. parasiticus*. Kozakiewicz (1989) reported that the production of sclerotia was a rare characteristic of *A. flavus* strains. According to Klich (2007), the presence of sclerotia per se does not seem to be related to the production of aflatoxins. According to this author, however, the presence of small sclerotia appears to correlate with high aflatoxin production. Different authors have tried to establish a correlation between the ability to produce sclerotia and aflatoxigenicity (Chang et al. 2001; Pildain et al. 2004; Razzaghi-Abyaneh et al. 2006; Giomi et al. 2007). However, data published in the literature are contradictory. Some studies refer to a positive correlation between high production of aflatoxin and the presence of small sclerotia (Novas and Cabral 2002; Pildain et al. 2004), whereas other studies report no correlation between production/size of sclerotia and aflatoxigenicity (Razzaghi-Abyaneh et al. 2006; Giomi et al. 2007).

The genomic similarity between *A. flavus* and *A. oryzae* was shown by the molecular analysis of the calmodulin gene (Table 2). Previous phylogenetic studies indicated that *A. oryzae* may have originated from either an ancestral non-

Table 2 *Aspergillus* section *Flavi* identification and data from sampling, isolation and accession numbers

Strain	Source	Molecular identification BLAST similarity	Molecular and morphological identification
CMEcoCentro 00045	Brazil coffee	99 % <i>Aspergillus flavus</i> MUM 10.209 (HQ340087) 98 % <i>Aspergillus oryzae</i> NRRL 447 (EF661506) 98 % <i>Aspergillus effusus</i> NRRL 506 (JN185447) 96 % <i>Aspergillus parasiticus</i> NRRL 4123 (EF661518)	<i>A. flavus</i>
CMEcoCentro 00043	Brazil coffee	99 % <i>Aspergillus flavus</i> NRRL 3357 (AY974340) 99 % <i>Aspergillus flavus</i> MUM 10.209 (HQ340087) 99 % <i>Aspergillus oryzae</i> NRRL 447 (EF661506) 99 % <i>Aspergillus effusus</i> NRRL 506 (JN185447) 97 % <i>Aspergillus parasiticus</i> MUM 10.210 (HQ340088)	<i>A. flavus</i>
CMEcoCentro 00100	Brazil nut	99 % <i>Aspergillus oryzae</i> NRRL 447 (EF661506) 99 % <i>Aspergillus flavus</i> NRRL 4822 (EF661513) 99 % <i>Aspergillus effusus</i> NRRL 506 (JN185447) 99 % <i>Aspergillus oryzae</i> CBS 466.91 (FN594585) 97 % <i>Aspergillus parasiticus</i> MUM 10.210 (HQ340088)	<i>A. flavus</i>

aflatoxigenic variant of *A. flavus* (Chang et al. 2006), or may have lost the ability to produce aflatoxins during the domestication process (Samson et al. 2000). Rokas et al. (2007) reported that *A. flavus*, *A. parasiticus*, *A. oryzae* and *A. sojae* have been shown to possess high degrees of DNA relatedness. *Aspergillus flavus* and *A. oryzae* have been considered virtually impossible to discriminate. This is also the case with *A. parasiticus* and *A. sojae* (Hedayati et al. 2007).

The close relatedness among these four species of the section *Flavi* (*A. flavus*, *A. parasiticus*, *A. oryzae* and *A. sojae*), paralleled with their striking difference in terms of aflatoxigenicity and industrial applications, is the foremost example of the challenge posed to the accurate classification of section *Flavi* species (Rodrigues et al. 2011).

The proteomic profiles obtained via MALDI-TOF MS generated clades (Fig. 2) similar to those found on the phenotypic dendrogram (Fig. 1). The proteomic analysis was not able to separate the aflatoxigenic and non-aflatoxigenic isolates of *A. flavus*. Also, based on the proteomic clustering, *A. oryzae* (CMEcoCentro 000100) was not clearly differentiated from *A. flavus*. For that reason, from a proteomic perspective, it cannot be undoubtedly stated that *A. flavus* and *A. oryzae* constitute different species. Studies by Rodrigues et al. (2011) identifying *Aspergillus* section *Flavi* isolated from almonds using the polyphasic approach found the same technical limitation. Li et al. (2000) analysed intact spores of isolates of *A. flavus*, *A. parasiticus*, *A. oryzae* and *A. sojae* by MALDI-TOF MS and found that aflatoxigenic strains and non-aflatoxigenic strains have different mass peak profiles. However, only a few non-informative MALDI-TOF MS spectra were presented in this study. No statistical analysis was performed and no dendrogram of relatedness was presented to prove that statement. Furthermore, the aflatoxigenic *A. flavus*

strains used in that study produced both AFBs and AFGs, which, is an atypical feature of *A. flavus*.

Overall, the classical phenotypic approaches, including macro- and micromorphology and mycotoxigenic profiles, were time-consuming, not completely straight forward, and dubious results made accurate identification difficult to attain. However, this classical methodology is still of great value for species identification when integrated within a polyphasic approach (Simões et al. 2013). In addition, the use of the proteomic profile by MALDI-TOF MS increased the reliability of the results obtained in this study. This technique shows potential for discriminating different taxa, which is otherwise feasible only with molecular biology techniques. Moreover, its low cost and the answer time renders the proteomic approach by MALDI-TOF MS a useful toll in the microbial classification/identification of species of *Aspergillus* section *Flavi*.

In this study, a molecular technique providing a large number of variables for the taxonomy of fungi was used as the gold standard in the polyphasic methodology described herein. According to the results obtained, the ITS gene was not powerful enough for discrimination of the isolates evaluated molecularly in this study. On the other hand, the calmodulin gene was more informative for the identification analysis of the isolates under investigation. Molecular analyses of both ITS and calmodulin genes were unable to distinguish the isolate *A. flavus* (CMEcoCentro 00100). However, this isolate was identified as *A. flavus* using classical techniques and by the proteomic profiles. This result demonstrates that in a polyphasic approach, none of the methods should be underestimated, and the identification of a species of the *Aspergillus* section *Flavi* should consider all characteristics analysed.

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