

# Structural Diversity of *Aspergillus* (Section *Nigri*) Spores

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**Abstract:** The taxonomy of fungal species, similar to that of many other microorganisms, suffers frequent revisions due to the discovery of new species and to the development and gathering of characterization data and morphological information. Morpho-taxonomy helps in the identification of many species. This work presents the macro, micro-morphological, and spectral mass analyses for phenotypic characterization of 13 species of *Aspergillus* section *Nigri*, showing that the characterization of spores (conidia) by scanning electron microscopy can be used as a tool to discriminate key morphological characteristics and separate closely related fungi. These results were corroborated by colony plates, stereomicroscopy, light microscopy, and spectral mass data.

**Key words:** *Aspergillus* section *Nigri*, fungal taxonomy, morpho-taxonomy, spore characterization

## INTRODUCTION

Species of *Aspergillus* section *Nigri* (known as black aspergilli) have been extensively used for various biotechnological purposes and are among the best-studied fungi. Many are responsible for biodeterioration of diverse commodities, including food (Abarca et al., 2004). The *Nigri* section has evolved continuously, and the last taxonomy revision includes 26 different taxa (Varga et al., 2011). However, because of molecular problems detected on  $\beta$ -tubulin DNA primers by Hubka and Loralik (2012), *Aspergillus japonicus* should be treated as a synonym of *Aspergillus violaceofuscus*, reducing the number to 25 different taxa. Similarly, *Aspergillus fijiensis*, described by Varga et al. (2011) as a new species, was found to be the already known *Aspergillus brunneoviolaceus*. Meanwhile, two more species were described by Jurjević et al. (2012), which means that we currently accept 27 different taxa.

The identification of species is a fundamental goal in microbiology. Information about each microorganism, such as morphological description, physiological and biochemical properties, ecological roles, and societal risks or benefits, is a key element in this process.

The concept of a distinct species is clearly abstract; delimitations are very difficult and often not consensual. Identification of fungi, even for experts, can be time-consuming and is hampered by frequent revisions of the taxonomic schemes. In addition, each taxonomic group has specialized literature, terminology, and characters. Some practical solutions have been designed to overcome these limitations with very limited success (Paterson et al., 2004, 2006). This occurs to the extent that identifications can only be undertaken with a degree of reliability, by a small number of scientists skilled in the “art.”

The black aspergilli are considered a taxonomically challenging group of fungi. It is difficult to recognize some of these species. Several approaches have been developed to

this end, such as new taxonomic schemes using extensive molecular analysis (de Vries et al., 2005; Varga et al., 2011), and all available information is valuable in differentiating each strain. Therefore, a more complete and polyphasic approach allows better results in fungal taxonomy studies (Varga et al., 2011; Simões et al., 2013). The aim of this study was to contribute additional information on the existing strains of the section *Nigri* from the genus *Aspergillus*.

## MATERIALS AND METHODS

Thirteen strains belonging to *Aspergillus* section *Nigri* were selected and supplied by the Micoteca da Universidade do Minho (MUM) fungal culture collection (Table 1). For the macro-morphological analysis, the selected strains were grown on potato dextrose agar (PDA, OXOID CM0139, Basingstoke Hampshire, UK), malt extract agar (MEA, malt extract 2%, mycological peptone 0.1%, glucose 2%, and agar 2%), and Czapek yeast extract agar (CYA, sucrose 2%, yeast extract 0.5%, NaNO<sub>3</sub> 0.3%, KCl 0.05%, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.05%, FeSO<sub>4</sub>·7H<sub>2</sub>O 0.001%, K<sub>2</sub>HPO<sub>4</sub> 0.1%, and agar 2%). To guarantee the same medium depth in all of the plates and a standardized growth, 20 mL of medium was poured into standard (90 mm) sterile and disposable Petri dishes. Each plate was inoculated at three points, equidistant from the center, and incubated in the dark at 25°C for 7 days. To prevent stray colonies on the plates, inoculation was made from a spore suspension on a solution of 0.2% agar with 0.05% tween 80. For microscopy analysis the strains were grown for 3 or 4 days on MEA.

Digital images of colonies were obtained on a register recording system for fungal plates that consists of a dedicated macro-photo stand with a Canon EOS 5D Mark II camera. The camera was operated with a remote shutter release device equipped with a 150-mm interchangeable Sigma (St. Louis, MO, USA) lens APO MACRO F2.8EX DG HSM. The equipment consisted of a Polaroid MP4<sup>+</sup> land camera stand where the camera was held horizontally at bull's eye level and pointing down toward the culture plates

**Table 1.** List of Strains Selected for This Study.

Species	Isolate Number	Geographical Origin	Substrate
<i>A. aculeatus</i>	MUM 03.11 <sup>T</sup> (=NRRL 5094 <sup>T</sup> ) <sup>a</sup>	Unknown	Tropical soil
<i>A. brasiliensis</i>	MUM 06.179	Portugal	Grapes Cabernet Sauvignon
<i>A. carbonarius</i>	MUM 01.08	Unknown	Unknown
<i>A. ellipticus</i>	MUM 03.12 <sup>T</sup> (=NRRL 5120 <sup>T</sup> )	Costa Rica	Soil
<i>A. ibericus</i>	MUM 04.68	Portugal	Grapes Aragonês
<i>A. japonicus</i>	MUM 98.03 (=DSM 2345) <sup>b</sup>	Unknown	Unknown
<i>A. lacticoffeatus</i>	MUM 06.150 <sup>T</sup> (=CBS 101883 <sup>T</sup> ) <sup>c</sup>	Indonesia	Coffee robusta (Rubiaceae)
<i>A. niger</i>	MUM 05.13	Portugal	Grapes Aragonês
<i>A. phoenicis</i>	MUM 03.05 (=NRRL 365)	Unknown	Unknown
<i>A. sclerotioniger</i>	MUM 06.151 <sup>T</sup> (=CBS 115572 <sup>T</sup> )	India	Arabic coffee
<i>A. tubingensis</i>	MUM 06.152 <sup>T</sup> (=CBS 134.48 <sup>T</sup> )	Unknown	Unknown
<i>A. uvarum</i>	MUM 08.01	Portugal	Grapes Tinta Barroca
<i>A. vadensis</i>	MUM 06.153 <sup>T</sup> (=CBS 113365 <sup>T</sup> )	Unknown	Dead plant tissue

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using a constant distance of 89 cm. Two 45° oblique lighting arms, each holding a cool fluorescent LED-500 (Walimex-Pro, Burgheim, Germany) with 630 lx and 6200 K color temperature for reflected light and a white light panel (LP-400), were used as a baseboard for transmitted light. The aperture and depth of field were constants and the optimal focus was reached using an ad-hoc optimization. All photographs were acquired using EOS utility software (Canon, Reigate, Surrey, UK) for acquisition of images and were saved in an uncompressed file.

To have a clear image comparison of the size of the conidial head, a stereomicroscope (Leica MZ12.5, Heerburg, Switzerland) equipped with a digital camera (Leica DC 150, PAL) was used. Fungal characterization was continued on a light microscope [Leica DM R, equipped with a digital camera (Leica DC 150, PAL)] and a scanning electron microscope [NanoSEM-FEI Nova<sup>TM</sup> 200 (FEG/SEM); EDAX-Pegasus X4M (EDS/EBS)]. For both the stereomicroscope and light microscope, Leica LAS EZ V1.7.0 software was used for acquisition and analysis of images.

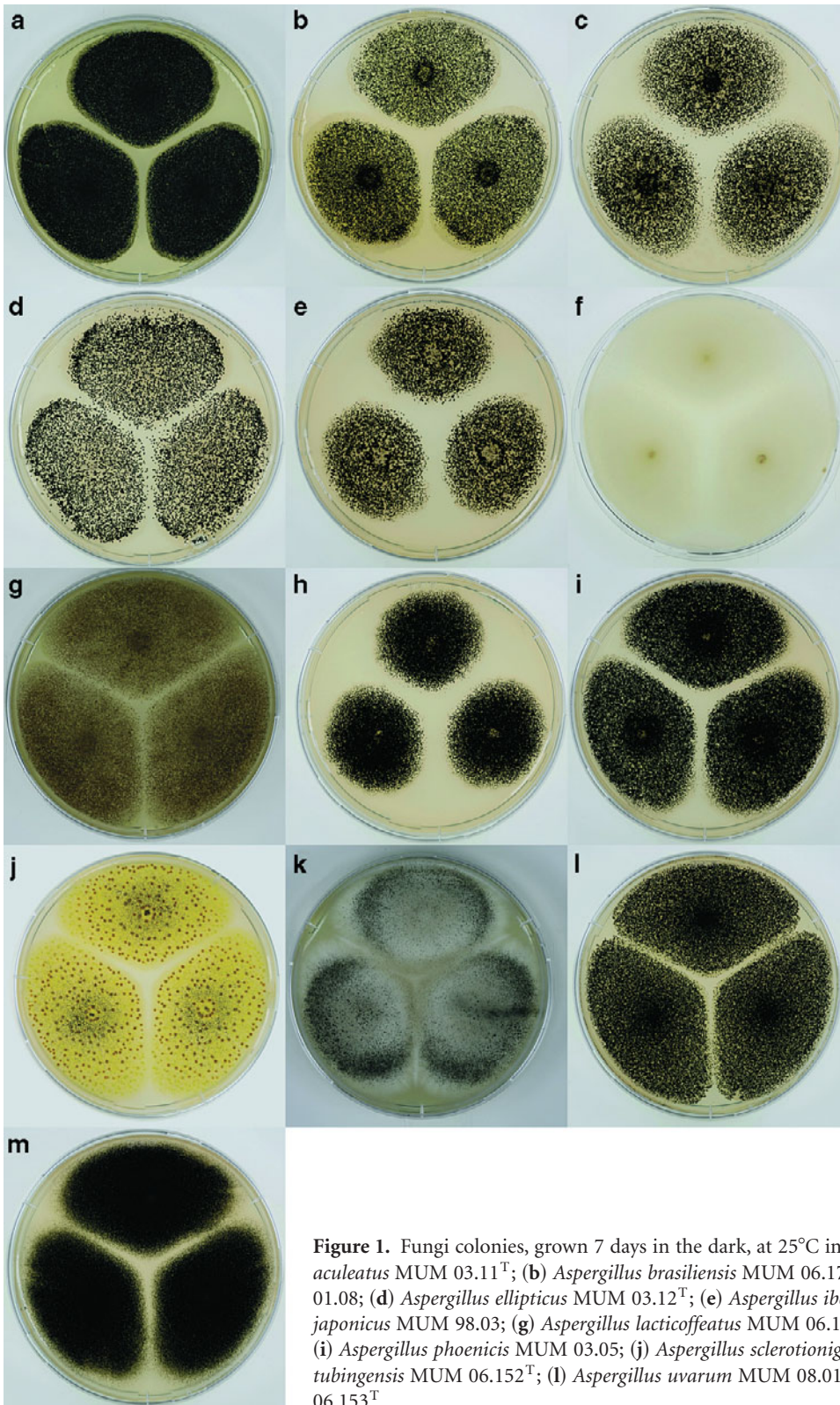
Samples for light microscopy were mounted on a slide, abundantly washed with alcohol (96%) to remove excessive spores, and stained with a lactophenol blue solution.

For scanning electron microscopy (SEM), a drop of medium (MEA) was mounted on a SEM stub under aseptic conditions. The stub was inoculated and incubated in the dark at 25°C. After visible fungal growth, the sample was directly covered with a mixture of gold and platinum (80/20%) for posterior analysis. Each single image was digitally produced and registered at variable magnifications.

Samples for spectral analysis by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS, AXIMA LNR Shimadzu, Kratos Analytical, Manchester, UK) were grown in MEA and incubated in the dark at 25°C for 4 days. The procedures were followed as described by Rodrigues et al. (2011). Briefly, for the flex target plate preparation, ~1 µg of spores and young mycelium of each species was transferred directly from the culture plate to the 48-well MALDI-TOF plate. Immediately thereafter, 0.5 µL of matrix solution [7.5% 2,5-dihydroxybenzoic acid in ethanol/water/acetonitrile (1:1:1) with 0.03% trifluoroacetic acid] was added to the samples and mixed gently. The sample mixtures were air-dried at room temperature. Each sample was spotted in duplicate to test reproducibility. During the analyses, all solutions were prepared and stored at 4°C. Data were analyzed on the basis of a matrix of pairwise correlation values for spectra after smoothing baseline corrections and peak detections. The peak lists of the selected strains were directly transferred into SARAMIS® (AnagnosTec, Potsdam, Germany) software.

## RESULTS

Identification of macro-morphological features, observable with the naked eye (Fig. 1) or with a stereomicroscope (Fig. 2), is the first approach when identifying fungal species. All of the fungi analyzed in this study presented common colony features (Fig. 1): conidial color, mycelial color, and colony diameter. As expected, the common feature, sclerotia, of *Aspergillus sclerotioniger* MUM 06.151 was



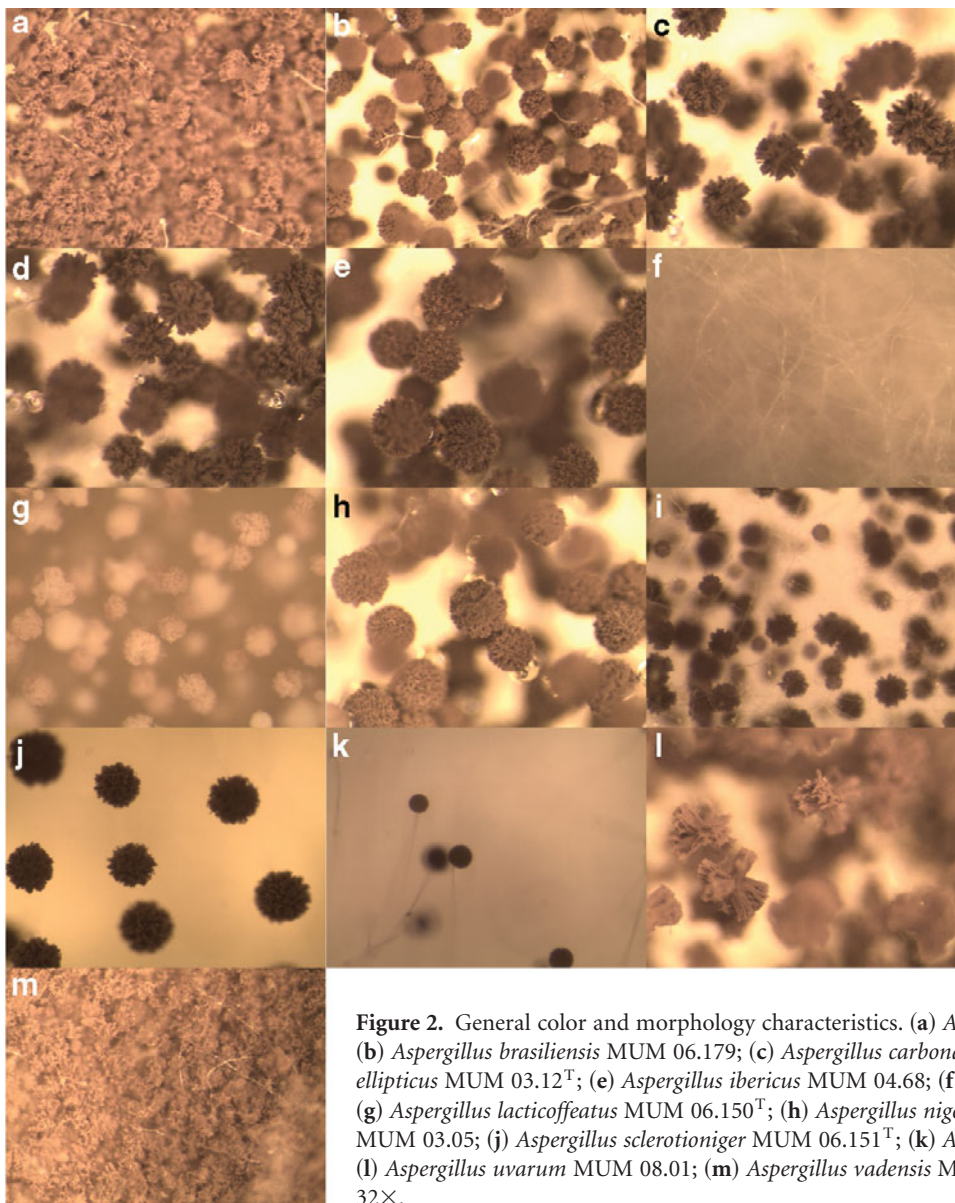
**Figure 1.** Fungi colonies, grown 7 days in the dark, at 25°C in malt extract agar. (a) *Aspergillus aculeatus* MUM 03.11<sup>T</sup>; (b) *Aspergillus brasiliensis* MUM 06.179; (c) *Aspergillus carbonarius* MUM 01.08; (d) *Aspergillus ellipticus* MUM 03.12<sup>T</sup>; (e) *Aspergillus ibericus* MUM 04.68; (f) *Aspergillus japonicus* MUM 98.03; (g) *Aspergillus lacticoffeatus* MUM 06.150<sup>T</sup>; (h) *Aspergillus niger* MUM 05.13; (i) *Aspergillus phoenicis* MUM 03.05; (j) *Aspergillus sclerotioniger* MUM 06.151<sup>T</sup>; (k) *Aspergillus tubingensis* MUM 06.152<sup>T</sup>; (l) *Aspergillus uvarum* MUM 08.01; (m) *Aspergillus vadensis* MUM 06.153<sup>T</sup>.

also observed (Fig. 1j) (representative data shown for MEA only).

*Aspergillus japonicus* MUM 98.03 presented poor sporulation (Fig. 2f) when compared with the other strains on both media on which it was grown, but showed its common white-to-cream-colored mycelia with abundant yellow-to-

brown soluble pigments on the media more noticeable on the colony reverse (data not shown).

On MEA, conidiophores are largely produced allowing easy retrieval of spores for analysis and characterization. *Aspergillus aculeatus*, *A. japonicus*, and *Aspergillus uvarum* were the only uniseriate species presenting only phialides



**Figure 2.** General color and morphology characteristics. (a) *Aspergillus aculeatus* MUM 03.11<sup>T</sup>; (b) *Aspergillus brasiliensis* MUM 06.179; (c) *Aspergillus carbonarius* MUM 01.08; (d) *Aspergillus ellipticus* MUM 03.12<sup>T</sup>; (e) *Aspergillus ibericus* MUM 04.68; (f) *Aspergillus japonicus* MUM 98.03; (g) *Aspergillus lacticoffeatus* MUM 06.150<sup>T</sup>; (h) *Aspergillus niger* MUM 05.13; (i) *Aspergillus phoenicis* MUM 03.05; (j) *Aspergillus sclerotioniger* MUM 06.151<sup>T</sup>; (k) *Aspergillus tubingensis* MUM 06.152<sup>T</sup>; (l) *Aspergillus uvarum* MUM 08.01; (m) *Aspergillus vadensis* MUM 06.153<sup>T</sup>. Total magnification: 32 $\times$ .

(Figs. 3a, 3f, 3l); all the other strains presented metulae and phialides (biseriate). Moreover, each strain presented spores with different characteristics (Fig. 4), either in shape or in size of ornamentation features.

From the analysis of the images presented in Figures 3 and 4, the data were composed as shown in Table 2.

Mass spectral analysis allowed for the construction of a dendrogram of relatedness presented in Figure 5.

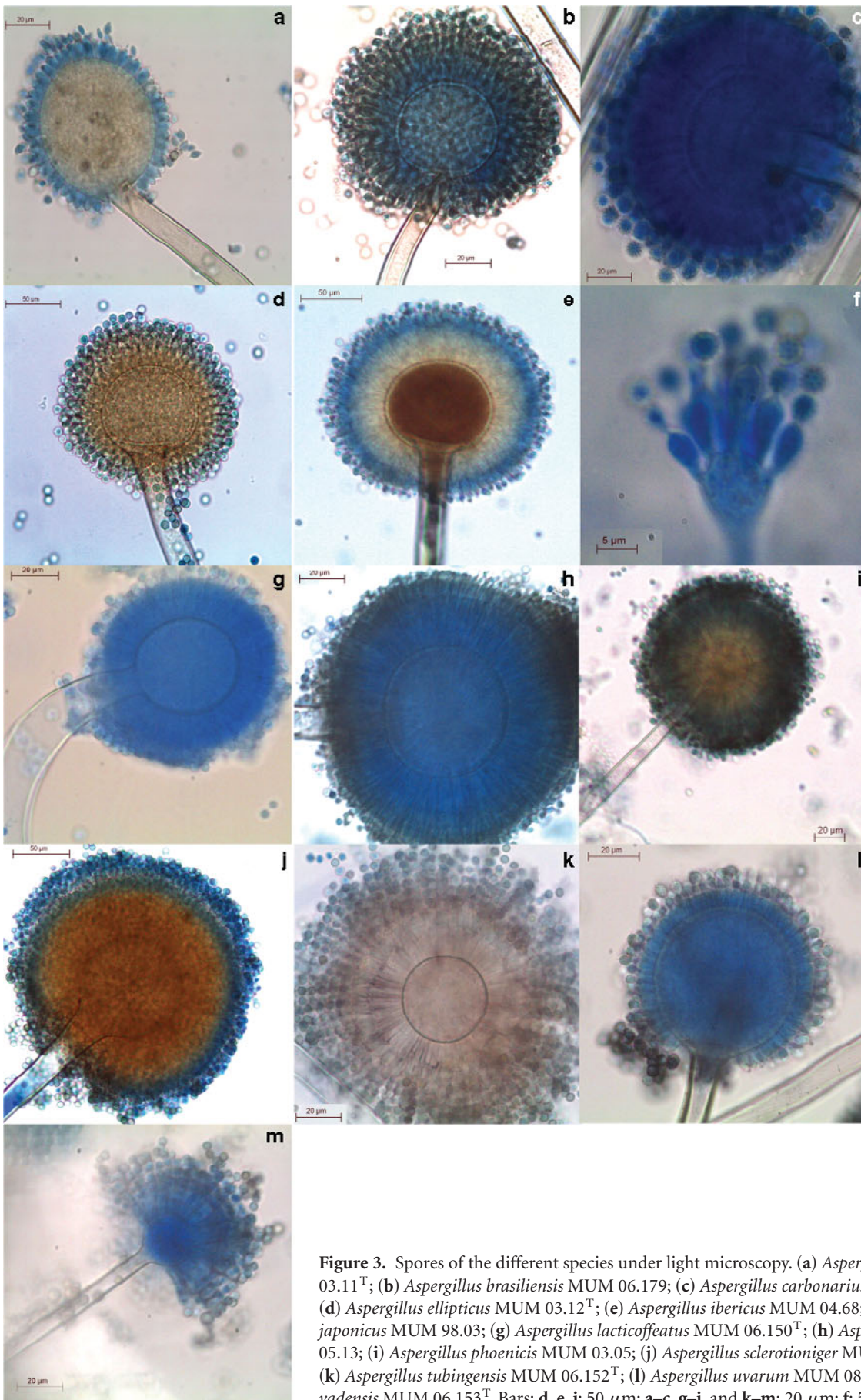
## DISCUSSION

Phenotypic data are essential for taxonomy. All of the characterization data hereby presented contribute to the increase in associated information of strains belonging to *Aspergillus* section *Nigri*. Species belonging to *Aspergillus* section *Nigri* usually present dark-brown-to-black spores, with uniseriate or biseriate conidiophores, spherical vesicles, and hyaline or lightly pigmented hyphae

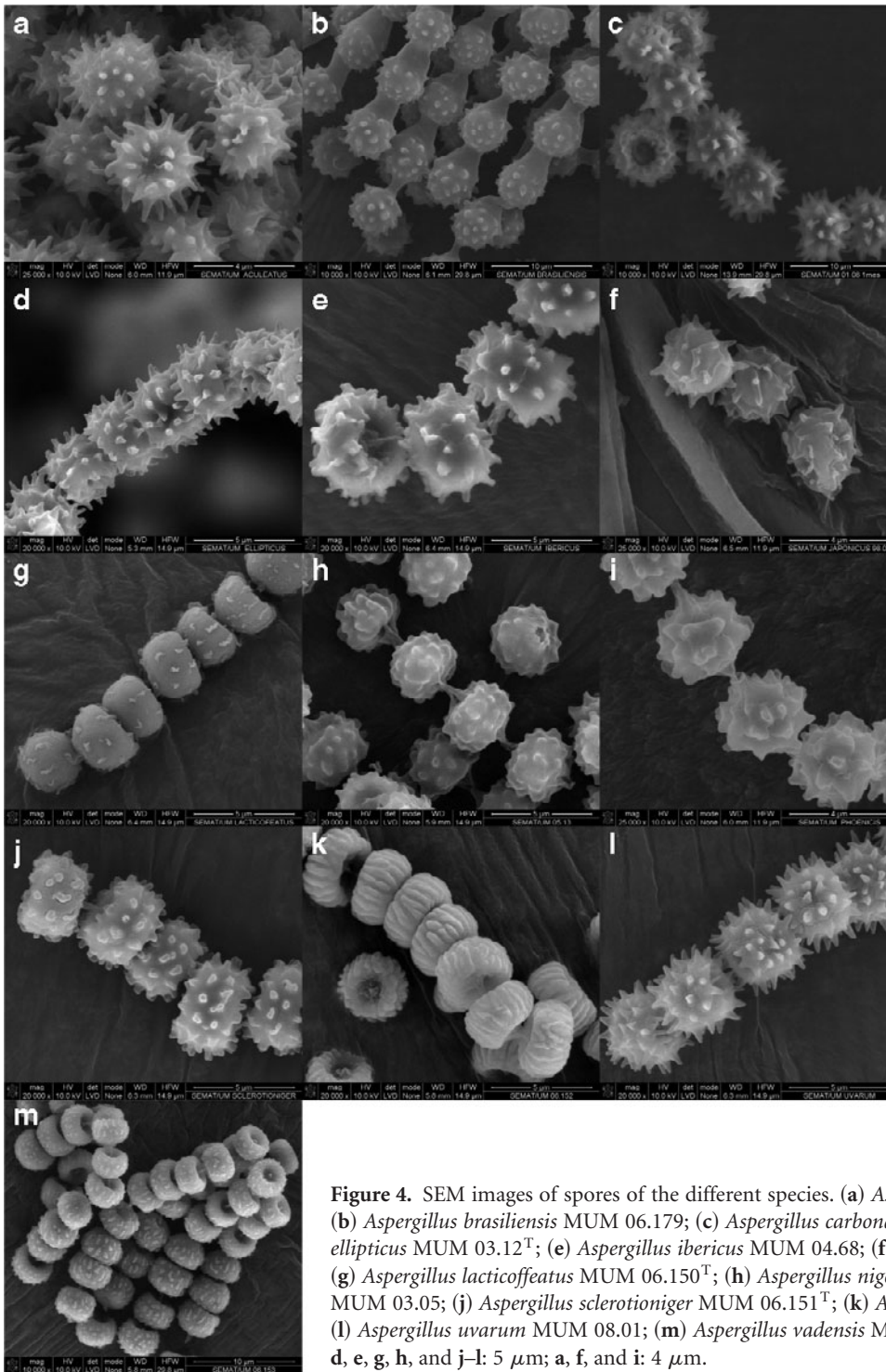
(Klich, 2002). Although the strains evaluated presented dimensional, morphological, and structural diversity (Fig. 2), common or typical features were attributed and related to each taxon, similar to those represented in Figures 3 and 4.

Sclerotia were only detected in *A. sclerotioniger* (Fig. 1), despite previous reports on the presence of such structures in some of the other strains included in our study (Samson et al., 2004, 2007). This is likely because of variability among cultures of the same isolate as described for some species (Geiser et al., 2007).

It was noticeable that *A. aculeatus*, *A. japonicus*, and *A. vadensis* present the shortest conidiophores (Figs. 2a, 2f, 2m). Most of the analyzed fungi present globose conidial heads, but *A. japonicus* and *A. vadensis* have radiate conidial heads (Figs. 3f, 3m). *Aspergillus ellipticus*, *Aspergillus ibericus*, *A. sclerotioniger*, and *A. uvarum* present conidial heads with the largest vesicle (Figs. 3d, 3e, 3j, 3l). Even though



**Figure 3.** Spores of the different species under light microscopy. (a) *Aspergillus aculeatus* MUM 03.11<sup>T</sup>; (b) *Aspergillus brasiliensis* MUM 06.179; (c) *Aspergillus carbonarius* MUM 01.08; (d) *Aspergillus ellipticus* MUM 03.12<sup>T</sup>; (e) *Aspergillus ibericus* MUM 04.68; (f) *Aspergillus japonicus* MUM 98.03; (g) *Aspergillus lacticoffeatus* MUM 06.150<sup>T</sup>; (h) *Aspergillus niger* MUM 05.13; (i) *Aspergillus phoenicis* MUM 03.05; (j) *Aspergillus sclerotioniger* MUM 06.151<sup>T</sup>; (k) *Aspergillus tubingensis* MUM 06.152<sup>T</sup>; (l) *Aspergillus uvarum* MUM 08.01; (m) *Aspergillus vadensis* MUM 06.153<sup>T</sup>. Bars: d, e, j: 50  $\mu\text{m}$ ; a–c, g–i, and k–m: 20  $\mu\text{m}$ ; f: 5  $\mu\text{m}$ .



**Figure 4.** SEM images of spores of the different species. (a) *Aspergillus aculeatus* MUM 03.11<sup>T</sup>; (b) *Aspergillus brasiliensis* MUM 06.179; (c) *Aspergillus carbonarius* MUM 01.08; (d) *Aspergillus ellipticus* MUM 03.12<sup>T</sup>; (e) *Aspergillus ibericus* MUM 04.68; (f) *Aspergillus japonicus* MUM 98.03; (g) *Aspergillus laticoffeatus* MUM 06.150<sup>T</sup>; (h) *Aspergillus niger* MUM 05.13; (i) *Aspergillus phoenicis* MUM 03.05; (j) *Aspergillus sclerotiumniger* MUM 06.151<sup>T</sup>; (k) *Aspergillus tubingensis* MUM 06.152<sup>T</sup>; (l) *Aspergillus uvarum* MUM 08.01; (m) *Aspergillus vadensis* MUM 06.153<sup>T</sup>. Bars: b, c, m: 10  $\mu$ m; d, e, g, h, and j–l: 5  $\mu$ m; a, f, and i: 4  $\mu$ m.

most vesicles present a globose shape, *A. aculeatus* presents ellipsoidal-shaped vesicles (Fig. 3a).

Within the *Aspergillus* genus, vesicle ornamentation is used as a primary separation parameter for several sections. However, some species are variable in seriation. It has already been shown by Rodrigues et al. (2007) that this is no longer a mainstay characteristic to differentiate species. *Aspergillus parasiticus*, for instance, which belongs to a different section but within the same genus, can present itself as

uniseriate and in some cases biseriate. Also, most *Aspergillus flavus* isolates are biseriate but this is not consistent. This is where spore characteristics become a primary taxonomic trait of decision when differentiating morpho-species. When observing Figure 4 it is clear that each strain has a particular spore with different ornamentations, sizes, and shapes.

Perrone et al. (2008), on the basis of morphology, described *A. japonicus* and *A. uvarum* as being closely related when compared with the other uniseriates of this

**Table 2.** Morphological Characteristics of Each Taxon.

Species	Vesicle Ornamentation	Basic Spore Shape	Spore Diameter ( $\mu\text{m}$ )
<i>A. aculeatus</i> MUM 03.11 <sup>T</sup>	Uniseriate	Equinulate	4.527 ( $\pm 0.215$ )
<i>A. brasiliensis</i> MUM 06.179	Biseriate	Equinulate	4.421 ( $\pm 0.182$ )
<i>A. carbonarius</i> MUM 01.08	Biseriate	Equinulate	7.658 ( $\pm 0.259$ )
<i>A. ellipticus</i> MUM 03.12 <sup>T</sup>	Biseriate	Equinulate	6.997 ( $\pm 0.252$ )
<i>A. ibericus</i> MUM 04.68	Biseriate	Equinulate	6.205 ( $\pm 0.367$ )
<i>A. japonicus</i> MUM 98.03	Uniseriate	Equinulate	3.878 ( $\pm 0.245$ )
<i>A. lacticoffeatus</i> MUM 06.150 <sup>T</sup>	Biseriate	Punctate	3.861 ( $\pm 0.315$ )
<i>A. niger</i> MUM 05.13	Biseriate	Equinulate	3.340 ( $\pm 0.209$ )
<i>A. phoenicis</i> MUM 03.05	Biseriate	Equinulate	4.044 ( $\pm 0.162$ )
<i>A. sclerotiumniger</i> MUM 06.151 <sup>T</sup>	Biseriate	Equinulate	5.553 ( $\pm 0.280$ )
<i>A. tubingensis</i> MUM 06.152 <sup>T</sup>	Biseriate	Striate with low ridges	3.972 ( $\pm 0.252$ )
<i>A. uvarum</i> MUM 08.01	Uniseriate	Equinulate	4.865 ( $\pm 0.223$ )
<i>A. vadensis</i> MUM 06.153 <sup>T</sup>	Biseriate	Verruculose	4.180 ( $\pm 0.431$ )

section. However, for the isolates evaluated in this study there are significant differences between both species: *A. japonicus* has smaller conidial heads (Figs. 3f, 3l), with spores with smaller diameter and far less pronounced echines (Figs. 4f, 4l), compared with *A. uvarum*.

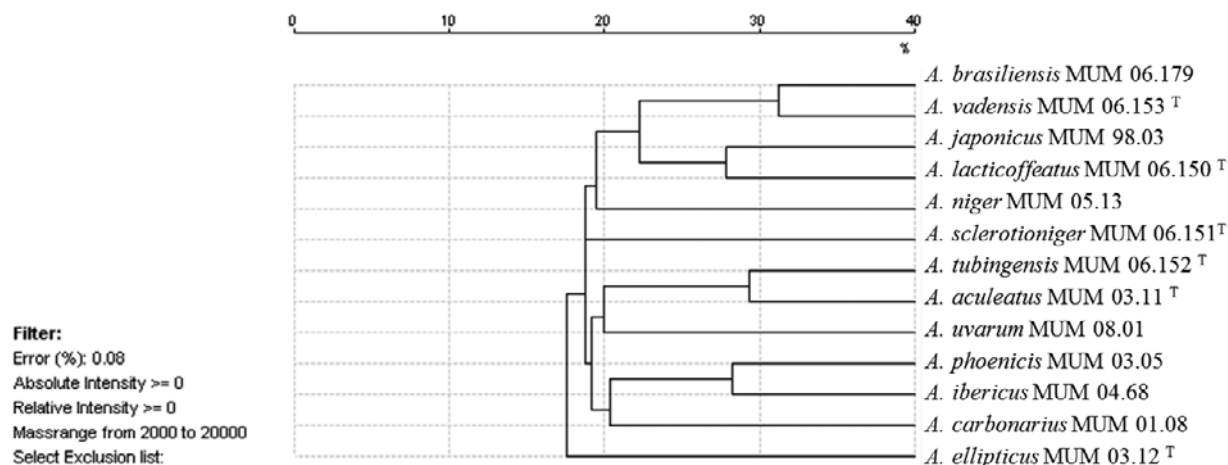
Varga et al. (2007) described *Aspergillus brasiliensis* as having a unique echinulate spore surface ornamentation. We confirmed this uniqueness (Fig. 4b); however, contrary to the description by these authors, resemblance in shape or size between the spores of *A. brasiliensis* and the spores of *A. aculeatus* and *Aspergillus carbonarius* was not found. The latter two strains do present a similar shape in spores but they have different diameter sizes (Figs. 4b, 4c), and both are very different from *A. brasiliensis* spores.

The identification of species belonging to the section *Nigri* has been successfully performed by molecular biology on the basis of multilocus sequence typing, as recently described by Jurjević et al. (2012). Another current technique used for identification of microorganisms is MALDI-TOF MS (Santos et al., 2010; Rodrigues et al., 2011). The previously published molecular identification strongly agrees

with the MALDI-TOF MS results from our study. The individual phenotypic characteristics obtained by SEM analysis of the spores for each species evaluated in the present study were supported by macro-morphological and micro-morphological analyses and were concordant with the MALDI-TOF MS results (Fig. 5).

## CONCLUSIONS

To develop the macro-morphological, micro-morphological, and mass spectral analyses for phenotypic characterization, 13 strains of *Aspergillus* section *Nigri* were selected from a fungal culture collection and evaluated as representative samples for each species. This work presents morpho-taxonomy, especially SEM-obtained data, as an attempt to resolve some of the difficulties encountered when identifying the referred fungal section. All of the strains chosen present the same characteristics as described for each type of strain on their first taxonomic description. It is noteworthy that elaborate preparation of SEM samples is unnecessary for the analyzed fungi as already suggested by



**Figure 5.** Dendrogram of relatedness between the selected *Aspergillus* strains of section *Nigri* based on MALDI-TOF MS analysis.

Kozakiewicz (1989). From our experience we can say that using complex protocols for this matter deteriorates and collapses the samples.

The size and shape of the fungal spores, as well as their ornamentation, are highly differentiated (Table 2). Although the species investigated presented dimensional, morphological, and structural diversity, common or typical features were inferred and related to each taxon (Figs. 2–4). It is possible to conclude from the phenotypic analysis that within this section the spore wall ornamentation and its size and shape continue to present themselves as important primary diagnostic traits for species differentiation. This can be clearly observed in Figure 4, where spore ornamentation differences are clearly seen. As referred by Serra et al. (2006) and shown in the developed work of Samson et al. (2007), we confirmed that *A. carbonarius* presents the largest equinulate spores. The smallest spores were observed for *Aspergillus niger*, *Aspergillus lacticoffeatus*, and *A. japonicus*. *Aspergillus tubingensis* presented the most differentiable conidia with striate spores with low ridges, being the easiest to identify when observing only the spore characteristics. All species differentiation was confirmed and supported by MALDI-TOF MS data (Fig. 5). In conclusion, SEM analysis of fungal spores is a key method to provide information for fungal identification based on morphology.

## ACKNOWLEDGMENTS

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