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# IDENTIFICATION OF FUNGI OF THE GENUS *ASPERGILLUS* SECTION *NIGRI* USING POLYPHASIC TAXONOMY

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## ABSTRACT

In spite of the taxonomy of the *Aspergillus* species of the *Nigri* Section being regarded as troublesome, a number of methods have been proposed to aid in the classification of this Section. This work aimed to distinguish *Aspergillus* species of the *Nigri* Section from foods, grains and caves on the basis in Polyphasic Taxonomy by utilizing morphologic and physiologic characters, and sequencing of ß-tubulin and calmodulin genes. The morphologic identification proved useful for some species, such as *A. carbonarius* and *Aspergillus* sp UFLA DCA 01, despite not having been totally effective in elucidating species related to *A. niger*. The isolation of the species of the *Nigri* Section on Creatine Sucrose Agar (CREA) enabled to distinguish the *Aspergillus* sp species, which was characterized by the lack of sporulation and by the production of sclerotia. Scanning Electron microscopy (SEM) allowed distinguishing the species into two distinct groups. The production of Ochratoxin A (OTA) was only found in the *A. carbonarius* and *A. niger* species. The sequencing of β-tubulin gene was efficient in differing most of the *Aspergillus* species from the *Nigri* Section with the exception of *Aspergillus* UFLA DCA 01, which could not be distinguished from A. *costaricaensis*. This species is morphologically similar to *A. costaricaencis* for its low sporulation capacity and high sclerotia production, but it differs morphologically from *A. costaricaensis* for its conidial ornamentation and size of vesicles. Equally, based on partial calmodulin gene sequence data *Aspergillus* UFLA DCA 01 differs from *A. costaricaensis.*

**Key words:** Polyphasic Taxonomy, β-tubulin gene, *Aspergillus* spp morphology.

## INTRODUCTION

Species of the genus *Aspergillus* Section *Nigri* or the Black *Aspergillus* are widely distributed around the world and have a capacity of developing in a vast variety of substrates. Many species are able to cause deterioration of food although some of them are used in fermentation industries to produce organic acids, such as citric and gluconic acids, as well as

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hydrolytic enzymes like lipases and amylases (1, 26). *A. niger* is one of the species that is widely used in biotechnological processes and it is the only one that has the "GRAS status" (Generally Regarded As Safe) by the "Food and Drug Administration". However, some species of the Section *Nigri* distinguish themselves by producing mycotoxins.

The taxonomy of fungi belonging to the Section *Nigri* comprises one of the most confusing and complex due to the subtle differences between the species. For a long time, classification and identification of these species were studied through morphologic criteria (19, 22). In this manner, some species, such as *A. carbonarius* and the uniseriate species (*A. japonicus* e *A. aculeatus*), can be easily recognized through identification manuals; while species related to the *A. niger* aggregate complex have been difficult to distinguish using morphologic criteria Samson et al. (22). Polyphasic taxonomy has been used for identification, as well as description of new species of the genus *Aspergillus* (16, 18, 27). Recently, the taxonomy of the Section *Nigri* is undergoing reinvestigation using polyphasic taxonomy, which uses different methods (morphologic, physiologic, metabolite production and important molecular data) with the aim of simplifying and elucidating this section's confusing taxonomy.

The objective of this study was to use Polyphasic Taxonomy to identify species belonging to the Section *Nigri* isolated from different sources, such as foods, grains and caves.

## MATERIAL AND METHODS

### Morphologic analysis

One hundred and ten fungi strains belonging to the Section *Nigri* were used in this study. All of them were obtained from the Fungi Collection of the Mycology and Mycotoxins Laboratory of the Department of Food Sciences, Federal University of Lavras - Lavras – MG, and were isolated from different products and environments as presented in Table 1.

After pure culture, the strains were inoculated into Petri dishes containing the culture medium CYA - Czapeck Yeast Agar ($K_2HPO_4$ 1.0 g; Czapek concentrate 10.0 mL; Yeast extract, 5.0 g, Agar 15.0 g, Distilled water 1 Liter; Czapek concentrate $NaNO_3$ 30.0g, KCl 5.0g, $MgSO_4.7H_2O$, 5.0g, $FeSO_4.7H_2O$ 0.1g, $ZnSO_4.7H2O$ 0.1g, $CuSO_4.5H_2O$ 0.05g, Distilled water 100 mL) and MEA (Malt Extract Agar 20.0 g, Peptone 1.0 g, Glucose 30.0 g, Agar 20.0 g, Distilled water 1 Liter) at 25 ºC and CYA at 37 ºC; in OA (Oatmeal Agar CBS – 30.0 g of oats, 15.0 g of Agar, Distilled water 1 Liter) at 25 ºC; CY20S (Czapeck Yeast Extract Agar with 20% of Sucrose, $K_2HPO_4$ 1 g, Concentrated Czapeck 10 mL, metal solution 1 mL ($ZnSO_4.7H_2O$ 1%, $CuSO_4.5H_2O$ 0,5%), Yeast extract 5.0 g, Sucrose 30.0 g, Agar 15.0 g, Distilled water 1 Liter) at 25 ºC. After 7 days of incubation, the microscopic and macroscopic characteristics were observed (14, 22, 23).

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

| Species | Origin | Species | Origin |
|---|---|---|---|
| *A.aculeatus* (0128) | Cave | *A.niger* (01270) | Pistachio nut |
| *A.aculeatus* (01201) | Raisin | *A.niger* (01272) | Pistachio nut |
| *A.aculeatus* (0113) | Cave | *A.niger* (0191) | Raisin |
| *A.aculeatus* (01111) | Raisin | *A.niger* (01122) | Raisin |
| *A.aculeatus* (01114) | Raisin | *A.niger* (01129) | Raisin |
| *A.aculeatus* (01151) | Cave | *A.niger* (01171) | Raisin |
| *A carbonarius* (01130) | Cave | *A.niger* (01202) | Cave |
| *A.carbonarius* (01218) | Raisin | *A.niger* (0122) | Raisin |
| *A.carbonarius* (01244) | Pepper | *A.niger* (0123) | Raisin |
| *A.carbonarius* (0118) | Raisin | *A.niger* (01210) | Cocoa |
| *A.carbonarius* (0121) | Raisin | *A.niger* (01197) | Raisin |
| *A.carbonarius* (01238) | Raisin | *A.niger* (01198) | Raisin |

| | | | |
|---|---|---|---|
| *A.carbonarius* (0131) | Guarana | *A.niger* (01278) | Cave |
| *A.carbonarius* (0184) | Raisin | *A.niger* (0124) | Raisin |
| *A.carbonarius* (0187) | Raisin | *A.niger* (0175) | Raisin |
| *Aspergillus* sp DCA UFLA (01162) | Cave | *A.niger* (01209) | Cashew nut |
| *A.foetidus* (01236) | Guarana | *A.niger* (0115) | Raisin |
| *A.foetidus* (01132) | Raisin | *A.niger* (0105) | Raisin |
| *A.foetidus* (01133) | Raisin | *A.niger* (0166) | Raisin |
| *A.foetidus* (01134) | Raisin | *A.niger* (0116) | Raisin |
| *A.foetidus* (01135) | Raisin | *A.niger* (0117) | Raisin |
| *A.foetidus* (01158) | Raisin | *A.niger* (0183) | Raisin |
| *A.foetidus* (0143) | Raisin | *A.niger* (01115) | Raisin |
| *A.foetidus* (01119) | Raisin | *A.niger* (01121) | Raisin |
| *A.foetidus* (01124) | Raisin | *A. niger* (01345) | Raisin |
| *A.foetidus* (01125) | Raisin | *A.niger* (01224) | Guarana |
| *A.foetidus* (0168) | Raisin | *A.niger* (01343) | Raisin |
| *A.foetidus* (01254) | Bean | *A.niger* (81) | Coffee |
| *A.foetidus* (01204) | Cave | *A.niger* (84) | Coffee |
| *A.foetidus* (01340) | Hazelnut | *A.niger* (78) | Coffee |
| *A.foetidus* (01123) | Raisin | *A.niger* (75) | Raisin |
| *A.foetidus* (01159) | Cave | *A .niger* (72) | Raisin |
| *A.foetidus* (01213) | Cashew nut | *A.niger* (01208) | Almond |
| *A.foetidus* (01296) | Cashew nut | *A.niger* Aggregate (0176) | Coffee |
| *A.foetidus* (01205) | Cave | *A.niger* Aggregate (01235) | Guarana |
| *A.foetidus* (01140) | Raisin | *A.niger* Aggregate (01239) | Raisin |
| *A.foetidus* (01206) | Cave | *A.niger* Aggregate (01172) | Raisin |
| *A.foetidus* (01168) | Raisin | *A.niger* Aggregate (01147) | Guarana |
| *A.foetidus* (01380) | Guarana | *A.niger* Aggregate (0119) | Raisin |
| *A.foetidus* (01284) | Cashew nut | *A.niger* Aggregate (01137) | Raisin |
| *A.foetidus* (01286) | Coffee | *A.niger* Aggregate (01175) | Raisin |
| *A.foetidus* (01242) | Guarana | *A.niger* Aggregate (01289) | Cocoa |
| *A.foetidus* (01269) | Hazelnut | *A.niger* Aggregate (01257) | Bean |
| *A.foetidus* (01282) | Cocoa | *A.niger* Aggregate (01336) | Hazelnut |
| *A.japonicus* (01184) | Cave | *A.niger* Aggregate (0192) | Raisin |
| *A.japonicus* (01148) | Cave | *A.niger* Aggregate (01215) | Pistachio nut |
| *A.japonicus* (0125) | Cave | *A.niger* Aggregate (01191) | Cave |
| *A.japonicus* (01182) | Cave | *A.tubingensis* (01248) | Pepper |
| *A.japonicus* (01161) | Cave | *A.tubingensis* (01196) | Raisin |
| *A.niger* (01278) | Almond | *A.tubingensis* (01176) | Raisin |
| *A niger* (01207) | Cave | *A.tubingensis* (01200) | Raisin |
| *A niger* (01216) | Raisin | *A.tubingensis* (0102) | Raisin |
| *A.niger* (0165) | Raisin | *A.tubingensis* (01144) | Raisin |
| *A niger* (01292) | Cashew nut | *A.tubingensis*(01260) | Raisin |
| *A niger* (01217) | Rice | *A.tubingensis* (01233) | Raisin |

**Growth and acid production in CREA (Creatine Sucrose Agar) culture medium**

The capabilities of growth and production of acid by the cultures were tested in CREA medium (Creatine Sucrose Agar - Creatine 3.0 g, Sucrose 30 g, KCl 0.5 g, $MgSO_4.7H_2O$ 0.5 g, $FeSO_4.7H_2O$ 0.5 g, $K_2HPO_4.3H_2O$ 1.3 g, Bromocresol purple 0.05 g, Agar 15.0 g, Distilled water 1 Liter) according to Frisvad and Samson (7, 22).

**Determining the ochratoxigenic potential of the identified species**

In order to determine the toxigenic potential of the species, the Plug Agar methodology, described by Filtenborg & Frisvad (6), was used.

**Extraction of Genomic DNA**

Conidia of the *Aspergillus* strains were inoculated in a complete liquid medium ($NaNO_3$ 6.0 g; $KH_2PO_4$ 1.5 g; $MgSO_4.7H_2O$ 0.5 g; KCl 0.5 g; $FeSO_4$ 0.001 g; $ZnSO_4$ 0.001 g; glucose 10.0 g; Yeast extract 0.5 g; Peptone 2.0 g; Hydrolyzed casein 1.5 g; Vitamin solution 1 mL; Distilled water 1 L) and incubated at 28 ºC, for 24 hours, at 180 rpm (20). The genomic DNA was extracted according to Azevedo (4) and measured using the fluorimetric method (Dyna Quant, Pharmacia).

**DNA amplification and sequencing**

Primers used to amplify a region of the β-tubulin and calmodulin genes were obtained from Glass and Donaldson (9) and Hong et al (10), respectively. The 50 µL PCR reaction mixtures contained 20 ng of genomic DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.0 mM $MgCl_2$, 0.2 mM of dNTP, 0.4 µM of each primer and 2.0 U of Taq DNA polymerase (Invitrogen). The mixtures was subjected to the following amplification program: initial denaturation at 94ºC for 5 min, followed by 35 cycles of denaturation (94ºC, 1 min), primer annealing (64ºC, 30 s) and elongation (72ºC, 1 min), and a final elongation for 5 min at 72ºC. DNA fragments were purified with the CONCERT™ Rapid PCR Purification System (GIBCOBRL, UK). The sequencing reaction was performed by using DYEnamic™ ET dye Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech, Inc.) on MegaBACE 1000 (Amersham Biosciences).

**Sequence analysis**

The quality of the sequences was analyzed using the Phre/Phrap/Consel package. For identification of the strains, the obtained nucleotide sequences were compared to those already stored in the National Center for Biotechnology and Information (NCBI) sequence database, using a research tool, BLAST (3).

**Sample preparation for analysis using a Scanning Electron Microscope**

Seven significant strains of each species belonging to the Section *Nigri* (Table 1) and initially identified using traditional methods analyzed in this work were inoculated in CYA 25 ºC for 5 days. After the incubation period, the sample discs were immersed in a fixative solution (Modified Karnovsky`s fixative 2.5% glutaraldehyde – 2.5% paraformaldehyde, 0.05M cacodilate buffer, $CaCl_2$ 0.001 M) at pH 7.2. The discs were then washed in cacodilate buffer (three times, for 10 min each wash), post-fixed in 1% osmium tetroxide solution and water for 1 hour and washed three times in distilled water, followed by dehydration in increasingly more concentrated acetone solutions (25, 50, 75, 90 and 100%, once for concentrations up to 90% and thrice for the 100% concentration). Afterwards, the samples were transferred to a desiccator containing silica to complete the drying process. The specimens obtained were assembled in aluminum supports known as stubs, with a double-faced carbon tapes put on a film of aluminum foil, covered with gold in a sputter (BALZERS SCD 050) and observed in a scanning electron microscope LEO EVO 40XVP. A number of images for each sample were digitally produced and registered at variable magnifications.

## RESULTS AND DISCUSSION

**Morphology of the colonies**

The strains belonging to the genus *Aspergillus* Section *Nigri* characteristically present dark-brown to black conidia, with uniseriate or biseriate conidiophores, spherical vesicles and hyaline or lightly pigmented hyphae near the apex (12).

Figure 1 presents the growth characteristics of the species *Aspergillus* Section *Nigri* studied in CYA and MEA 25 ºC after 7 days in culture. *Aspergillus* sp UFLA DCA 01 could be distinguished due to its low capacity of sporulation and its abundant production of oval shaped sclerotia with a yellow-orange color with gray tones. This strain is morphologically similar to the species *A. costaricaensis*. However, *Aspergillus* sp DCA 01 can be macroscopically distinguished from *A. costaricaensis* by the color of the mycelium. *Aspergillus* sp has a white mycelium, while *A. costaricaensis* has a yellow mycelium. Other differences between these two species are: the reverse color in MEA 25 ºC (Table 2) and the sclerotia colors, that of *A. costaricaensis* varies from pink to yellow with gray tones, while that of *Aspergillus* UFLA DCA 01 is light brown.

The species *A. tubingensis* is morphologically very similar to *A. niger*, what makes it difficult to distinguish them based only on morphological information. Nevertheless, in this study *A. tubingensis* could be macroscopically distinguished by its production of sclerotia, which present a characteristic white to pink color. Although Samson et al. (22) reported that the sclerotia production by species of *A. tubingensis* is not always observed. Studies demonstrated that the other species have a capacity to produce these structures, including *A. carbonarius*, *A. ellipticus*, *A. aculeatus*, *A. costaricaensis*, *A. piperis*, *A. sclerotioniger*, *A. aculeatinus* and *A. sclerotiicarbonarius* (22, 23). However, these structures were never observed in the species of *A. ibericus* (24).

The results also describe a morphologic similarity between *Aspergillus niger* Aggregate and *A. niger*, *A. tubingiensis* and *A. foetidus*. Morphologically, the differences are subtle as already observed by other authors (22). In relation to the uniseriate species, including *A. japonicus* and *A. aculeatus,* these could not be distinguished based only on the macroscopic observation of their morphological characteristics.

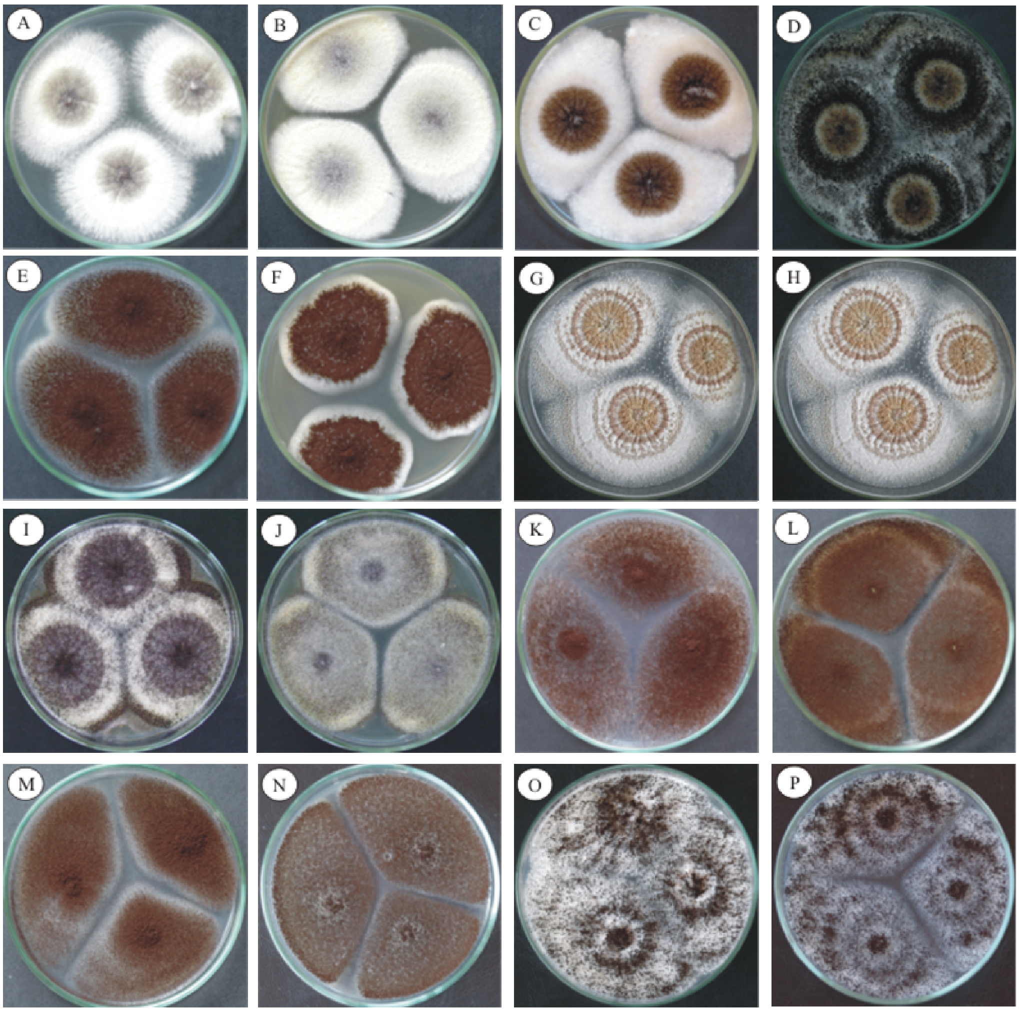


**Figure 1.** Photographs of the colonies of *Aspergillus* Section *Nigri* in CYA and MEA 25 ºC after 7 days showed morphologic differences. *A. aculeatus* (A-B); *A. carbonarius* (C-D); *A. foetidus* (E-F); *Aspergillus* sp UFLA DCA 01 (G-H); *A. japonicus* (I-J); *A. niger* (K-L); *A. niger Aggregate* (M-N); *A. tubingensis* (O-P).

**Table 2.** Macroscopic characteristics of the species of *Aspergillus* Section *Nigri*

| | Colony CYA 25ºC | | | Colony MEA 25ºC | | | | |
|---|---|---|---|---|---|---|---|---|
| **Species** | **Diameter of colony** | **Color** | **Reverse color** | **Diameter of colony** | **Color** | **Reverse color** | **Production of sclerotia** | **Production of OTA** |
| *A. aculeatus* | 73-76 | Dark brown/ gray tones | Pale to yellow | 74-79 | Dark brown/ gray tones | Straw-colored | Absent | _ |
| *A. carbonarius* | 65-67 | Black | Colorless | 51-57 | Black | Colorless | Absent | + |
| *A. foetidus* | 62-65 | Dark brown to black | Tones of gray to brown center | 62-66 | Black | Colorless | Absent | _ |
| *A. japonicus* | 67-73 | Dark brown/ gray tones | Pale to yellow | 64-70 | Dark brown/ to black | Colorless | Absent | _ |
| *A. niger* | 67-70 | Black to dark brown | Colorless to light yellow | 53-69 | Black | Colorless | Absent | + |
| *A. niger* Aggregate | 65-69 | Dark brown/ to black | Straw-colored | 64-68 | Dark brown/ to black | Light yellow | Absent | _ |
| *A. tubingensis* | 65-72 | Black | Pale | 56-57 | Black | Colorless | Present | _ |
| *Aspergillus* sp UFLA DCA 01 | 75-76 | Black | Cream | 65-71 | Black | Colorless | Present (abundant) | _ |
| **A. costaricaensis* | 63-78 | Black | Straw-colored | 26-62 | Black | Yellow | Present (abundant) | _ |

**A. costaricaensis* - listed in the table for comparison of the characteristics of *Aspergillus* sp UFLA DCA 01.

**Conidial ornamentation**

Among the biseriate species, *A. carbonarius* could be easily distinguished from the other species based on size and conidial ornamentation, whose diameter varied from 7 to 9 µm, although some reached 10 µm. Other species that produce large conidia include *A. homomorphus*, *A. sclerotiicarbonarius, A. sclerotioniger* (22, 23) and *A. ibericus* (22, 23, 24). The rest of the species studied presented conidia with varying sizes, between 3 to 5 µm. *Aspergillus foetidus*, *A. niger* and *A. tubingensis* are species that are difficult to distinguish based on morphology (22); however, *A. foetidus* could be distinguished from these species by its conidial ornamentation, which when formed present themselves as delicately spiny and, when mature, as smooth conidia. The uniseriate species *A. aculeatus* and *A. japonicus* could not be distinguished by their conidial ornamentation as both present spiny conidia. Although these two species are morphologically similar, some differences were observed. *A. aculeatus* presents larger vesicles compared to those of *A. japonicus*. Another characteristic that was observed and which helped distinguish these two species was the shape of the conidia; the species *A. aculeatus* presents predominantly ellipsoidal conidia while *A. japonicus,* presents globular and subglobular conidia (Figure 2), as was noted by Klich (12).

*Aspergillus* sp UFLA DCA 01 presented conidia with a spiny ornamentation to a finely wrinkled one, what differs from the ornamentation presented by *A. costaricaensis,* smooth conidia to distinctly wrinkled (Table 3).

The spore ornamentation as observed in MEV permitted the distinction of two groups of the analyzed species of *Aspergillus* Section *Nigri*: those that presented warty conidia and those that presented echinulated conidia (Figure 2). The species that present warty conidia are: *A. niger*, *A. niger* aggregate, *A.carbonarius* and *A. tubingensis*. The spores of *A. japonicus* and *A. aculeatus* are distinctly echinulated.

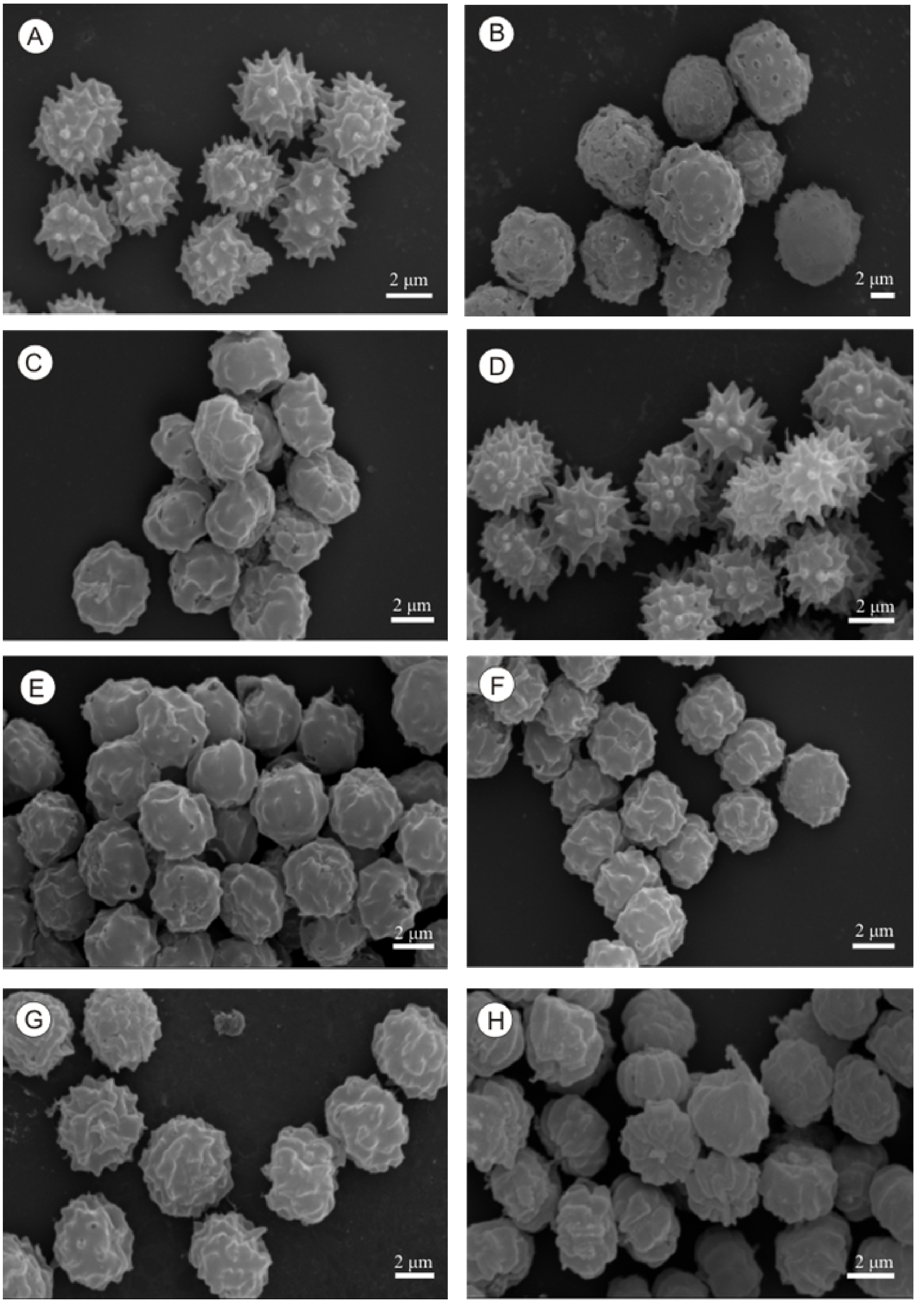


**Figure 2.** Scanning electron micrographs of the conidia of *Aspergillus* Section *Nigri*. *A.aculeatus* (A); *A. carbonarius* (B); *A.foetidus* (C); *A. japonicus* (D); *A.niger Aggregate* (E); *A niger* (F), *A. tubingensis* (G), *Aspergillus* sp UFLA DCA 01 (H).

**Table 3.** Microscopic characteristics of the species of *Aspergillus* Section *Nigri*

| Species | Diameter of Conidia (µm) | Texture of Conidia | Shape of Conidia | Diameter of Vesicles (mm) | Conidial Ornamentation (MEV) |
|---|---|---|---|---|---|
| Uniseriate | | | | | |
| *A. aculeatus* | 4-5 | spiny | Ellipsoidal | 31-60 | echinulated |
| *A. japonicus* | 4-5 | spiny | subglobular/globular | 16-33 | echinulated |
| Biseriate | | | | | |
| *A. carbonarius* | 7-10 | Wrinkled | Globular | 49-85 | warty |
| *A. foetidus* | 4-5 | delicately spiny/smooth | Globular | 34-69 | - |
| *A. niger* | 3-5 | finely wrinkled/wrinkled | globular/ ellipsoidal | 20-73 | warty |
| *A. niger Aggregate* | 4-5 | smooth/finely wrinkled | Globular | 18-54 | warty |
| *A. tubingensis* | 4-5 | finely wrinkled/wrinkled | globular/ subglobular | 45-69 | echinulated / warty |
| *Aspergillus* sp DCA 01 | 4-5 | spiny/ finely wrinkled | globular/ subglobular | 10-14 | - |
| *A. costaricaensis* | 3.1-4.5 | smooth/distinctly wrinkled | globular/subglobular | 45-90 | echinulated |

**A. costaricaensis* – listed in the table for comparison of the characteristics of *Aspergillus* sp UFLA DCA 01

**Growth and acid production in CREA (Creatine Sucrose Agar) culture medium**

This selective medium is widely used for the classification of a number of fungal cultures, especially in species of the genus *Penicillium* (7, 22). Recently, this medium was used to divide the species of *Aspergillus* Section *Nigri* into groups according to their acid production (23). All the tested species presented a capacity to grow in CREA, forming a yellow halo around the colonies. The biseriate species *A. carbonarius* and *A. niger* aggregate presented the greatest capacity of growth in this medium compared to the other tested species, as well as good acid production. *Aspergillus foetidus*, *A. niger*, *A. tubingensis* and *Aspergillus* sp UFLA DCA 01 presented moderate growth and good acid production. According to Samson et al. (23), some species like *A. sclerotiicarbonarius* manifest incapacity to grow in CREA, one of the characteristics that allow the distinction of this species from *A. carbonarius*, *A. sclerotioniger* and *A. ibericus,* which belong to the Section *Nigri*.

In relation to *A. aculeatus* and *A. japonicus*, these uniseriate species also present moderate growth and limited acid production compared to the biseriate species. Samson et al. (23) also observed limited acid production by the uniseriate species *A. aculeatus*, *A. japonicus* and *A. uvarum* in CREA.

**Evaluation of the ochratoxigenic potential**

Two species of the strains listed in Table 1 presented themselves to be potentially capable of producing OTA. Out of 39 *A. niger* strains, 6 species were capable of producing OTA. Some studies confirmed *A. niger* to be an OTA producer although the OTA production by these species is rarely reported (5, 11, 25). In relation to the species of *A. carbonarius*, 6 out of 9 tested species were potentially capable of producing OTA. This specie is considered to be a major OTA producer in grapes and grape derivatives (21).

The rest of the species listed in Table 1 did not produce

OTA. However, other studies reported OTA production by species of *A. foetidus* (15) and, recently, the species of *A. tubingensis* and *A. japonicus* were reported to be species capable of producing OTA (17). To Samson et al. (22), the species of *A. tubingensis* were never capable of producing OTA. The same authors also reported OTA production by the species of *A. carbonarius*, *A. sclerotioniger*, *A. niger* and *A. lacticoffeatus,* belonging to the Section *Nigri*.

**Molecular characterization to distinguish species of *Aspergillus* Section *Nigri***

The cladogram indicates the presence of two clades of the phylogenetic tree based on sequencing of the ß-tubulin gene. The smaller clade comprises the uniseriate species *A. japonicus* and *A. aculeatus*, while the larger clade comprises species of the *A. niger* complex and is subdivided into subclades (Figure 3). Subclade I is represented by the uniseriate species *A. homomorphus*, *A. aculeatinus* and *A. uvarum*.

Subclade II is represented by the species *A. heteromorphus* and subclade III by the species *A. ellipticus*.

Morphologically identified isolates like *A. carbonarius* 01218 and 01238 are grouped together with the species *A. carbonarius* CBS 11126 present in subclade IV. The species *A. ibericus*, *A. sclerotiicarbonarius* and *A. sclerotioniger,* also present in subclade IV, form a distinct group because they share some characteristics, such as OTA production, sclerotia production and larger conidia, when compared to the rest of the species that belong to the Section *Nigri* (22, 23).

Subclade V comprises a larger group, including the species *A. brasiliensis*, *A. vadensis*, *A. tubingensis*, *A. costaricaensis*, *A. piperis* and *A. foetidus*, *A. niger* and *A. lacticoffeatus*, related to the *A. niger* complex (2). This subclade also includes *Aspergillus* sp UFLA DCA 01 (01162), which is grouped together with the species of *A. costaricaensis*. These two species could be morphologically distinguished by growth and reverse pigmentation in MEA 25 ºC, as well as the color of their sclerotia. The conidial morphology is also different since *Aspergillus* sp UFLA DCA 01 presents spiny to finely wrinkled conidia while *A. costaricaensis* presents smooth to distinctly wrinkled conidia. The vesicle size in *A. costaricaensis* (40-90) is larger than that of *Aspergillus* sp UFLA DCA 01. The ß-tubulin gene was not efficient in the distinction of these two species. As had already been noted by Samson et al. (22), in *Aspergillus* Section *Nigri* all species can be distinguished from each other using calmodulin sequence data, with is not true by using β-tubulin sequence data. Based on this observation, we amplified and sequenced a portion of calmodulin gene by using DNA from the *Aspergillus* sp UFLA DCA 01. The alignment of 445 nucleotide positions from *Aspergillus* sp UFLA DCA 01 with those from *A. costaricaensis* strains revealed eight (1.8%) single nucleotide polymorphisms (Figure 4). This level of variation is high enough to suggest that *Aspergillus* sp UFLA DCA 01 is in fact a new species of Section *Nigri.*

Fungi morphologically identified as *A. tubingensis* (01176, 01233, 01248, 01260), also present in the subclade V were grouped together with the species *A. tubingensis*. *A. tubingensis* is a species which is morphologically very similar to *A. niger*. However, *A. tubingensis* could be distinguished by production of white to pink colored sclerotia, a characteristic of this species; this structure is rarely observed in the species of *A. niger* (22). Despite the difficulty to differentiate between *A. tubingensis* and *A. niger* using phenotypic methods, these species can be distinguished through sequencing of the ß-tubulin gene (26).

Based on morphologic characters, the fungi (01224, 01343, 78, 81, 84 e 01345) were classified as *A. niger*. The phylogenetic analysis revealed that these were strains to *A. lacticoffeatus*, thus they were characterized as *A. lacticoffeatus*. *A. lacticoffeatus* is a species that is morphologically very similar to *A. niger.* According to Samson et al. (22), based on the ß-tubulin gene sequences (Bt2a and Bt2b), these two species cannot be separated since they present identical gene sequences (22, 25), although, Geiser et al. (8) had reported that these two species could be distinguished using the ß-tubulin gene. To Samson et al. (22), *A. lacticoffeatus* can morphologically be distinguished from *A. niger* through the ornamentation and color of the conidia, by pigmentation in medium culture and by secondary metabolite profile (extrolytes). In this study, strains with characteristics similar to that of *A. lacticoffeatus* were grouped in this clade.

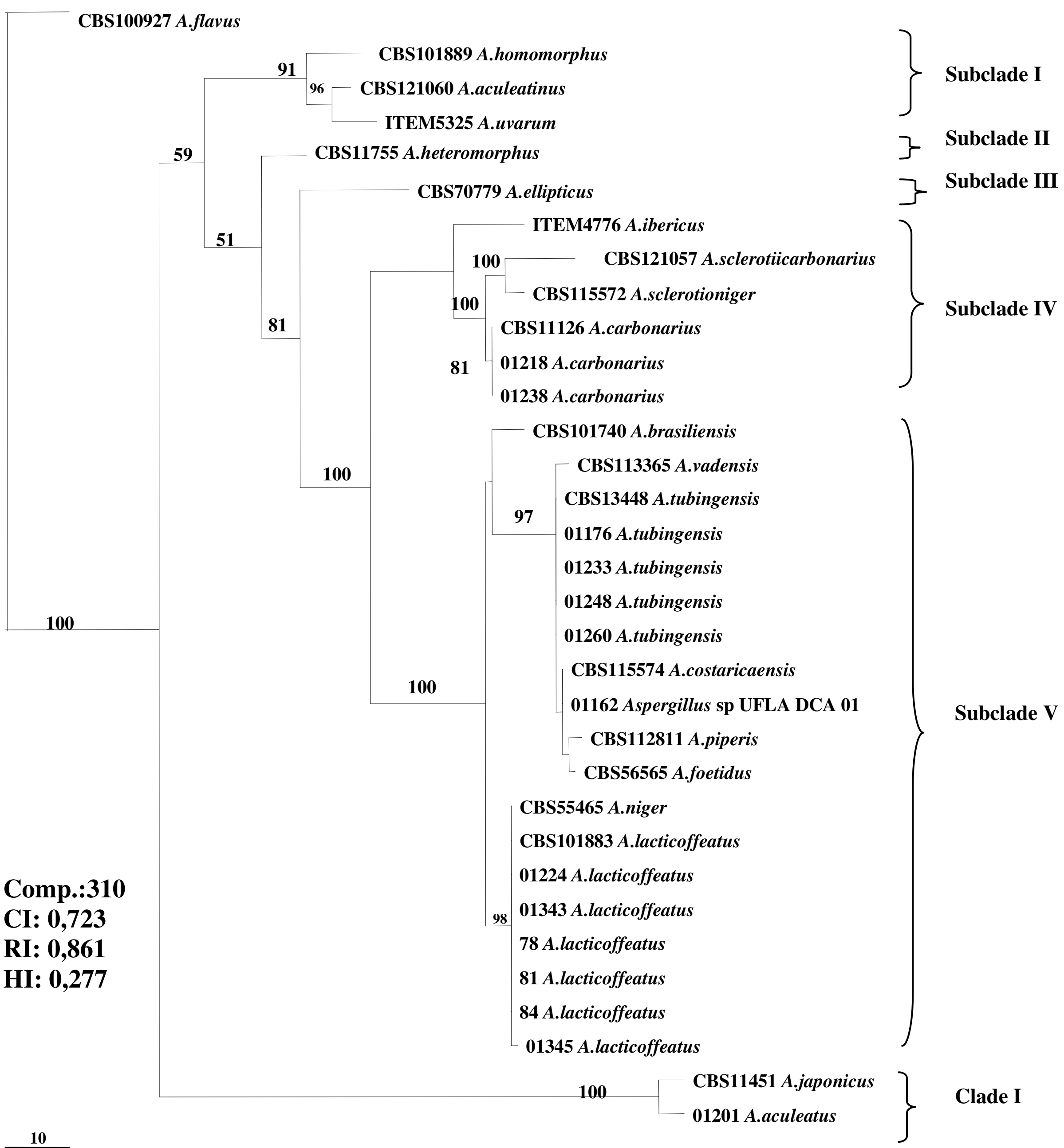


**Figure 3.** Maximum Parsimony Phylogenetic Tree based on the ß-tubulin gene of species belonging to the Section *Nigri*. The length of the branches is indicated by scale at the tree base and the bootstrap values (1000 repetitions) are shown as a percentage at the internodes.

Clade I is represented by uniseriate species. The strain morphologically identified as *A. aculeatus* 01201 was grouped in the same clade as *A. japonicus,* with 100% difference between these species. Although these two species are morphologically similar, some differences, such as conidial and vesicle morphology permit distinction (12).

The clades generated in the chromatogram reveal formation of groups with related morphologic and physiologic characteristics, permitting the manual identification of some species. The usage of ß-tubulin gene sequencing allows comparison with other species in the GenBank, although *Aspergillus* sp UFLA DCA 01 presents remarkable morphologic characteristics and can be characterized as a new species, despite the fact that it belongs to the same clade as *A. costaricaensis*. The morphologic differences can be an important tool for characterization of a new species even in members of the same clade. Thus, Polyphasic Taxonomy not only generates large amounts of information about the strain, but also permitted description, from fungal groups and of a new species of the genus *Aspergillus* Section *Nigri.*

From the results obtained in this study, it can be concluded that Polyphasic Taxonomy proved to be the most precise method for identification of species of *Aspergillus* Section *Nigri*.

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                    5          15         25         35         45
 55
EU163268.1   TCAATAGGAC AAGGATGGCG ATGGTGGGTG GAATTCTGTC CCCTTCACGT TTTACCTGTA
FN594545.1   TCAATAGGAC AAGGATGGCG ATGGTGGGTG GAATTCTGTC CCCTTCACGT TTTACCTGTA
UFLADCA01    TCAATAGGAC AAGGATGGCG ATGGTGGGTG GAATTCTGTC CCCTTCACGT TTTACCTGTA

                   65         75         85         95        105        115
EU163268.1   GCGCTCGATC CGACCGCGGG ATTTCGACAG CCATTCCCCC ATCGATCTCA ATCATTATAC
FN594545.1   GCGCTCGATC CGACCGCGGG ATTTCGACAG CCATTCCCCC ATCGATCTCA ATCATTATAC
UFLADCA01    GCGCTCCATC CGACCGCGGG ATTTCGACAG CCATTCCCCC ATCGATCTTA ATAATTATAC

                  125        135        145        155        165        175
EU163268.1   TGATGTAATC CGGAAATAGG CCAGATCACC ACCAAGGAGC TCGGCACTGT GATGCGCTCC
FN594545.1   TGATGTAATC CGGAAATAGG CCAGATCACC ACCAAGGAGC TCGGCACTGT GATGCGCTCC
UFLADCA01    TGATGTAATC TGGAAATAGG CCAGATCACC ACCAAGGAGC TCGGCACTGT GATGCGCTCC

                  185        195        205        215        225        235
EU163268.1   CTCGGCCAGA ACCCCTCCGA GTCTGAGCTT CAGGACATGA TCAACGAGGT TGACGCTGAC
FN594545.1   CTCGGCCAGA ACCCCTCCGA GTCTGAGCTT CAGGACATGA TCAACGAGGT TGACGCTGAC
UFLADCA01    CTCGGCCAGA ACCCCTCCGA GTCTGAGCTT CAGGACATGA TCAACGAGGT TGACGCTGAC

                  245        255        265        275        285        295
EU163268.1   AACAACGGAA CGATCGACTT CCCCGGTATG TGATAGATCT ACGCCTGTAA GGCGGGAATG
FN594545.1   AACAACGGAA CGATCGACTT CCCCGGTATG TGATAGATCT ACGCCTGTAA GGCGGGAATG
UFLADCA01    AACAACGGAA CGATCGACTT CCCCGGTATG TGATAGATCT ATGCCTATAA GGCGGGAATG

                  305        315        325        335        345        355
EU163268.1   CCGTATGGGT TGTGATTGAC TTTTGCCGCC AGAATTCCTC ACCATGATGG CTCGTAAGAT
FN594545.1   CCGTATGGGT TGTGATTGAC TTTTGCCGCC AGAATTCCTC ACCATGATGG CTCGTAAGAT
UFLADCA01    CCGTATGGGT TGTGATTGAC TTTTGCCGCC AGAATTCCTT ACCATGATGG CTCGTAAGAT

                  365        375        385        395        405        415
EU163268.1   GAAGGACACC GACTCCGAGG AGGAAATCCG CGAGGCTTTC AAGGTCTTCG ACCGCGACAA
FN594545.1   GAAGGACACC GACTCCGAGG AGGAAATCCG CGAGGCTTTC AAGGTCTTCG ACCGCGACAA
UFLADCA01    GAAGGACACC GACTCCGAGG AGGAAATCCG CGAGGCTTTC AAGGTCTTCG ACCGCGACAA

                  425        435        445
EU163268.1   CAATGGTTTC ATCTCCGCCG CGGAGTT
FN594545.1   CAATGGTTTC ATCTCCGCCG CGGAGTT
UFLADCA01    CAATGGTTTC ATCTCCGACG CGGAGTT
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**Figure 4.** Nucleotide sequence alignment of a portion from the calmodulin gene of *A.costaricaensis* (EU163268.1 and FN594545.1) and *Aspergillus* sp UFLA DCA 01. The gray markers indicate nucleotide substitutions.

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