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Aspergillus brasiliensis sp. nov., a biseriate black *Aspergillus* species with world-wide distribution

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A novel species, *Aspergillus brasiliensis* sp. nov., is described within *Aspergillus* section *Nigri*. This species can be distinguished from other black aspergilli based on intergenic transcribed region, β-tubulin and calmodulin gene sequences, by amplified fragment length polymorphism analysis and by extrolite profiles. *A. brasiliensis* isolates produced naphtho-γ-pyrones, tensidol A and B and pyrophen in common with *Aspergillus niger* and *Aspergillus tubingensis*, but also several unique compounds, justifying their treatment as representing a separate species. None of the isolates were found to produce ochratoxin A, kotanins, funalenone or pyranonigrins. The novel species was most closely related to *A. niger*, and was isolated from soil from Brazil, Australia, USA and The Netherlands, and from grape berries from Portugal. The type strain of *Aspergillus brasiliensis* sp. nov. is CBS 101740^T (=IMI 381727^T=IBT 21946^T).

INTRODUCTION

Black aspergilli (*Aspergillus* section *Nigri*; Gams *et al.*, 1985) have a significant impact on modern society. Many species cause food spoilage, and several are used in the fermentation industry to produce hydrolytic enzymes, such as amylases or lipases, and organic acids, such as citric acid and gluconic acid (Varga *et al.*, 2000). They are also candidates for genetic manipulation in the biotechnology industries as *Aspergillus niger* used under certain industrial conditions has been granted the GRAS (generally regarded as safe) status by the Food and Drug Administration of the USA government. Although the main source of black aspergilli is soil, members of this section have been isolated from various other sources (Kozakiewicz, 1989; Abarca *et al.*, 2004; Samson *et al.*, 2004). Besides their economical importance, black aspergilli are also important as ochratoxin-producing organisms that contaminate several agricultural products including grape-derived products, coffee and cocoa (Cabañes *et al.*, 2002; Samson *et al.*, 2004).

Black aspergilli are one of the more difficult groups regarding classification and identification. The taxonomy of *Aspergillus* section *Nigri* has been studied by many taxonomists and was recently reviewed by Abarca *et al.* (2004). Nuclear and mitochondrial DNA (mtDNA) polymorphisms and PCR-based techniques led to the recognition of at least two species within the *A. niger* species complex (*Aspergillus niger*, *Aspergillus tubingensis*) (Kusters-van Someren *et al.*, 1991; Varga *et al.*, 1994). Regarding other black *Aspergillus* species, phylogenetic analyses of sequences of the intergenic transcribed spacer and the 5.8S rRNA gene (ITS region) and the D1–D2 region of the 28S rRNA gene indicated that, apart from those mentioned earlier, at least five other species belong to section *Nigri*: *Aspergillus heteromorphus*, *Aspergillus ellipticus*, *Aspergillus carbonarius*, *Aspergillus japonicus* and *Aspergillus aculeatus* (Varga *et al.*, 2000; Parenicova *et al.*, 2001). Several other black *Aspergillus* species have been

Abbreviations: AFLP, amplified fragment length polymorphism; ITS, intergenic transcribed spacer; SEM, scanning electron microscopy.

The GenBank/EMBL/DDBJ accession numbers for the β-tubulin, ITS and calmodulin gene sequences determined in this study are shown in Table 1.

The Mycobank accession number for *Aspergillus brasiliensis* sp. nov. is MB510581 (http://www.mycobank.org).

Neighbour-joining trees based on ITS and calmodulin gene sequence data of *Aspergillus* section *Nigri*, a dendrogram based on cluster analysis of AFLP data and tables listing the *Aspergillus* section *Nigri* strains used in AFLP analysis and the extrolites produced by the *Aspergillus brasiliensis* isolates are available with the online version of this paper.

described recently, including *Aspergillus vadensis* (de Vries *et al.*, 2005), *Aspergillus costaricaensis*, *Aspergillus piperis*, *Aspergillus lacticoffeatus* and *Aspergillus sclerotioniger* (Samson *et al.*, 2004) and *Aspergillus ibericus* (Serra *et al.*, 2006).

During a survey of black *Aspergillus* isolates collected worldwide, we discovered some strains that did not fit into any species of *Aspergillus* section *Nigri*. We have used a polyphasic taxonomic approach in order to determine the delimitation and variability of this novel species. For phenotypic analyses, macro- and micromorphologies of the isolates were examined, and secondary metabolite and enzyme profiles were studied. For genotypic studies, partial sequences of the β-tubulin and calmodulin genes and the ITS region of the rRNA gene cluster and amplified fragment length polymorphism (AFLP) profiles were analysed (also used by Geiser *et al.*, 1998; Varga *et al.*, 2000; Hong *et al.*, 2006; Perrone *et al.*, 2004, 2006).

METHODS

The strains examined are listed in Table 1 and were maintained on malt extract autolysate (MEA) agar slants.

Morphological analysis. For macromorphological observations, Czapek yeast autolysate (CYA), MEA agar, Czapek yeast autolysate with 5 % NaCl (CYAS) agar, yeast extract-sucrose (YES) agar, oatmeal agar (OA) and Czapek agar (CZA) were used (Samson *et al.*, 2004). The isolates were inoculated at three points on each plate of each medium and incubated at 25 °C in the dark for 7 days. For micromorphological observations, microscopic mounts were made in lactic acid from MEA colonies and a drop of alcohol was added to remove air bubbles and excess conidia. Scanning electron microscopy (SEM) was performed using a Hitachi S570 microscope. For SEM preparation, conidia were transferred to aluminium stubs using double-sided adhesive tape. A small drop of 10 mM ACES buffer containing 0.05 % Tween 80 was added to the conidiophores. The suspension was air-dried and coated with platinum. The strains were also inoculated on creatine-sucrose agar (CREA), CYA at 37 °C and on CYA with 5 % NaCl (Frisvad & Samson, 2004).

Extrolite analysis. Extrolites were analysed by HPLC using alkylphenone retention indices and diode array UV-VIS detection as described by Frisvad & Thrane (1987), with modifications as described by Smedsgaard (1997). Standards of ochratoxin A and B, aflavinines, asperazine, austdiol, kotanin and other extrolites from the collection at Biocentrum-DTU (Denmark) were used to compare the extrolites from the species under study. Pyranonigrin A, tensidol A and B and pyrophen were identified by comparison with literature UV and MS data (Hiort *et al.*, 2004; Fukuda *et al.*, 2006).

Table 1. Origin and GenBank accession numbers of sequences of the isolates examined in this study

GenBank accession numbers for ITS sequences of other black aspergilli determined in this study: *A. costaricensis* CBS 115574^T, DQ900602; *A. piperis* CBS 112811^T, DQ900603; *A. lacticoffeatus* CBS 101884, DQ900604; *A. carbonarius* CBS 111.26^T, DQ900605; *A. sclerotioniger* CBS 115572^T, DQ900606; *A. homomorphus* CBS 101889^T, EF166063. GenBank accession number for β-tubulin gene sequence of *A. ibericus* ITEM 4776, AM419748.

Isolate*	Origin	Accession no.		
		ITS	β-tubulin	Calmodulin
CBS 101740^T=IMI 381727^T=JHC 614^T	Soil, Pedreira, São Paulo, Brazil	AJ280010	AY820006 (AM295186)	AM295175
JHC 601	Soil, St Rosa do Viterbo, Brazil	DQ900599	DQ900609	–
JHC 603	Soil, St Rosa do Viterbo, Brazil	DQ900601	DQ900610	†
JHC 605	Soil, Araçatuba, São Paulo, Brazil	–	–	–
JHC 606	Soil, A. Nogueira, São Paulo, Brazil	–	–	–
JHC 607	Soil, Campinas, São Paulo, Brazil	DQ900600	DQ900611	†
CBS 246.65=IBT 28083	Soil, Sydney, New South Wales, Australia	DQ900597	DQ900607	–
CBS 733.88=IBT 28084	Soil, North Carolina, USA	DQ900598	DQ900612	–
CBS 116970=IBT 28085	Soil, The Netherlands	DQ900596	DQ900613	–
ITEM 4540	Grapes (Cabernet Sauvignon), Ribatejo Region, Portugal, 2001	AM295180	AM295183	AM295178
ITEM 4544	Grapes (Tinta Miùda), Ribatejo Region, Portugal, 2001	‡	AM295184	AM295177
ITEM 6139	Grapes (Vinhão), Vinhos Verdes Region, Portugal, 2001	AM295181	AM295185	AM295174
ITEM 4539	Grapes (Tinta Barroca), Douro Region, Portugal, 2002	‡	AM295182	AM295179

*CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; JHC, James H. Croft's culture collection, Birmingham, UK; IBT, BioCentrum-DTU, Kgs. Lyngby, Denmark; IMI, CABI Bioscience Genetic Resource Collection, Egham, UK; ITEM, Agri-Food Toxigenic Fungi Culture Collection, Institute of Sciences of Food Production, Bari, Italy.

†100 % identity with the deposited sequence AM295175.

‡100 % identity with the deposited sequence AM295180.

Isolation and analysis of nucleic acids. Total nucleic acids were isolated according to the literature (Leach *et al.*, 1986). Fragments containing the region encoding the intergenic transcribed spacer 1 (ITS-1), 5.8S rDNA and intergenic transcribed spacer 2 (ITS-2) were amplified using primers ITS1 and ITS4, as described previously (Varga *et al.*, 2000) and by White *et al.* (1990). Amplification of part of the β-tubulin gene was performed using the primers Bt2a and Bt2b (Glass & Donaldson, 1995; Samson *et al.*, 2004). Amplifications of the partial calmodulin gene were set up as described previously (Serra *et al.*, 2006). Sequence analysis was performed with a Big Dye Terminator Cycle Sequencing Ready Reaction kit for both strands, and the sequences were aligned with the MT Navigator software (Applied Biosystems). All the sequencing reactions were purified by gel filtration through Sephadex G-50 (Amersham Pharmacia Biotech) equilibrated in double-distilled water and analysed on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The resulting sequences of all the isolates were aligned by using the CLUSTAL method with the program DNAMAN (Lynnon Corporation). The unique ITS, β-tubulin and calmodulin sequences were deposited in the GenBank nucleotide sequence database (Table 1).

AFLP analysis. Twenty-three strains belonging to *Aspergillus* section *Nigri* were analysed by AFLP analysis (see Supplementary Table S1 available in IJSEM Online). Fungal strains were grown in shake cultures (150 r.p.m., 25 °C, 2 days) in Wickerham's medium (40 g glucose, 5 g peptone, 3 g yeast extract, 3 g malt extract and distilled water to 1 l). Genomic DNA was extracted using an E.Z.N.A. Fungal DNA Miniprep kit (Omega Bio-tek), according to the manufacturer's protocol. The DNA was dissolved in sterile water, diluted to 20 ng μl^{-1} and stored at −20 °C.

We used an AFLP Microbial Fingerprinting kit (Applied Biosystems-Perkin-Elmer Corporation) according to the manufacturer's instructions using primer combinations according to Perrone *et al.* (2006). Peak height thresholds were set at 200. Genotyper software (Applied Biosystems) was set to medium smoothing. Bands of the same size in different individuals were assumed to be identical and to represent the same allele. Bands of different sizes were treated as independent loci with two alleles (present and absent). Data were analysed with an AFLP manager database developed by ACGT BioInformatica S.r.l. and were exported in a binary format with '1' for the presence of a band/peak and '0' for its absence. For clustering two different analyses were performed, fragments between 100 and 500 bp and between 200 and 500 bp were analysed with NTSYS software by using the Dice similarity coefficient and clustered by using the unweighted pair group method (UPGMA) (Nei & Li, 1979).

Analysis of sequence data. Sequence alignments were performed by using CLUSTAL_X (Thompson *et al.*, 1997) and improved manually. Evolutionary distances between the sequences were calculated using Kimura's formula (Kimura, 1980) with the program DNADIST of the PHYLIP program package (Felsenstein, 1995). Phylogenetic trees were prepared by using the neighbour-joining method (Saitou & Nei, 1987) with the program NEIGHBOR of the PHYLIP package. Bootstrap values were calculated from 1000 replications of the bootstrap procedure using programs SEQBOOT, DNADIST, NEIGHBOR and CONSENSE of the package (Felsenstein, 1985, 1995). For parsimony analysis, the PAUP* version 4.0 software was used (Swofford, 2000). *Aspergillus flavus* CBS 100927^T was used as an outgroup in these experiments.

RESULTS AND DISCUSSION

Isolates of the novel species described below have an unique echinulate conidium surface ornamentation, in contrast to all other members of the *Aspergillus niger* complex or the *A. niger* clade in Samson *et al.* (2004). The conidia of the novel species were more similar to those of *A. aculeatus* and *A. carbonarius*, but differed from those of the former species in being biseriate and from those of *A. carbonarius* in being much smaller. Physiological features such as very good growth and sporulation at 37 °C, poor growth but strong acid production on CREA agar, and very good growth and sporulation on CYA agar with 5 % NaCl were indicative of a close relationship with *A. niger* and *A. tubingensis*. The strong acid production on CREA agar indicates that the novel species can produce citric acid. Like *A. niger*, the novel species also produces xylanases and thermostable β-xylosidases (Pedersen *et al.*, 2007). Regarding extrolite profiles, the isolates of the novel species produced naphtho-γ-pyrones (including aurasperone B), pyrophen (Barnes *et al.*, 1990) and tensidol A and B (Fukuda *et al.*, 2006), in common with *A. niger* and *A. tubingensis*, but also produced several unique compounds (see Supplementary Table S2 in IJSEM Online), justifying the treatment of the isolates as representing a separate species. None of the isolates were found to produce ochratoxin A, kotanins, funalenone, antafumicins, asperazine or pyranonigrins, common to other species in the *A. niger* complex (Table 2). Two isolates produced large white sclerotia (ITEM 4544 and ITEM 6139). These strains contained the same sclerotial indol alkaloids as *A. piperis* and *A. costaricaensis* (Samson *et al.*, 2004). From a chemotaxonomic point of view, we regard isolates to be members of a novel species if they produce unique combinations of extrolites not seen in any other species. In this case some species-specific, but not yet structure-elucidated, extrolites were present in the novel species, whereas certain extrolites regarded as being species specific for other species in *Aspergillus* section *Nigri* were not present in the novel species.

In previous studies, a wide-ranging variation in mtDNA restriction fragment length polymorphism profiles was observed both among collection strains and in natural populations of the *A. niger* species complex (Varga *et al.*, 1993, 1994). Most isolates were classified as *A. niger* or *A. tubingensis* according to their *Hae*III–*Bgl*II digested mtDNA patterns. *A. niger* and *A. tubingensis* were grouped into five and six mtDNA types, respectively. However, six of the 73 Brazilian isolates examined exhibited unique mtDNA profiles using *Hae*III and *Bgl*II restriction enzymes (Varga *et al.*, 1994, 2000). These isolates also differ from other isolates belonging to *Aspergillus* section *Nigri* in their *Sma*I-digested nuclear DNA hybridization patterns using rRNA probes (Varga *et al.*, 1994, 2000). Later, a survey of isolates held in the culture collection of the Centraalbureau voor Schimmelcultures (CBS) assigned to either the *A. niger* or *A. tubingensis* species, and another survey of black aspergilli isolated from grapes in Portugal using sequence analysis of the ITS, β-tubulin and calmodulin genes, identified another three and four isolates, respectively, which were found to be related to the Brazilian strains. These strains were isolated from soil from Brazil, Australia, USA and

Table 2. Morphological characteristics and extrolite production of species belonging to the *A. niger* species complex in *Aspergillus* section *Nigri*

Species	Conidial size (μm)	Vesicle size (μm)	Colour and size of sclerotia (mm)	Extrolites produced
A. brasiliensis sp. nov.	3.5–4.5	30–45	Found only in some strains, white, 1–1.5	Naphtho-γ-pyrones (including aurasperone B), pyrophen, tensidol A and B, (dihydrocarolic acid, 14-epi-14-hydroxy-10,23-dihydro-24,25-dehydro-aflavinine, 10,23-dihydro-24,25-dehydro-aflavinine)*
A. costaricaensis	3.1–4.5	40–80	Pink to yellow, 1.2–1.8	Aflavinines (see above), corymbiferan lactones, funalenone, naphtho-γ-pyrones
A. foetidus	3.5–4.5	50–80	–	Antafumicins, asperazine, funalenone, naphtho-γ-pyrones, pyranonigrin A
A. lacticoffeatus	3.4–4.1	40–65	–	Kotanins, ochratoxin A, pyranonigrin A, tensidol B
A. niger	3.5–5	45–80	–	Funalenone, (kotanins), naphtho-γ-pyrones, (ochratoxin A), pyranonigrin A, pyrophen, tensidol A and B
A. piperis	2.8–3.6	40–55	Yellow to pink–brown, 1.0–1.7	Aflavinines (see above), naphtho-γ-pyrones, pyranonigrin A
A. tubingensis	3–5	40–80	White to pink, 0.5–0.8	Asperazine, funalenone, naphtho-γ-pyrones, pyranonigrin A, tensidol A and B
A. vadensis	3–4	25–35	–	Asperazine, funalenone, naphtho-γ-pyrones, nigragillin, polar kotanin-like compound

*These extrolites were only produced by isolates producing sclerotia.

The Netherlands, and from grape berries from Portugal (Table 1).

We examined the genetic relatedness of these isolates to other black aspergilli using sequence analysis of the ITS region of the rRNA gene cluster and parts of the calmodulin and β-tubulin genes. The isolates were found to form a monophyletic clade supported by high bootstrap values on phylogenetic trees based on β-tubulin, ITS and calmodulin sequence data (Fig. 1 and Supplementary Figs S1 and S2 in IJSEM Online). During analysis of part of the β-tubulin gene, 392 characters were analysed. Among the 193 polymorphic sites, 160 were found to be phylogenetically informative. The neighbour-joining tree based on partial β-tubulin gene sequences is shown in Fig. 1. The topology of the tree is the same as that of a parsimony tree constructed by using the program PAUP (length, 414 steps; consistency index, 0.6836; retention index, 0.8039). The calmodulin dataset included 671 characters, with 283 parsimony informative characters. The topologies of the neighbour-joining tree (Supplementary Fig. S1) and the parsimony tree were the same (tree length, 718; consistency index, 0.6964; retention index, 0.8546). The ITS dataset included 479 characters with 70 parsimony informative characters. The neighbour-joining tree shown in Supplementary Fig. S2 has the same topology as the parsimony tree (tree length, 139; consistency index, 0.8849; retention index, 0.9342).

These isolates also formed a well-defined cluster on an UPGMA tree based on AFLP data (see Supplementary Fig. S3 in IJSEM Online). AFLP data have the special advantage of containing both individual (fingerprinting) and species-specific information. Our data indicate that these isolates are well separated from other black aspergilli based on all molecular approaches used. During AFLP analysis, clear polymorphisms both within and between species were obtained for each of the four primer pairs. Each primer combination consistently distinguished the nine different species of black aspergilli analysed by AFLP with similarity among the different species of less than 20 % (Supplementary Fig. S3). The 11 strains that were grouped by sequence analysis as *A. brasiliensis* formed a main AFLP cluster that was clearly differentiated from all the other species of section *Nigri*, but they showed a great degree of genetic variability among each other with a similarity of 25 % (Dice similarity index). In particular, two main clusters were formed within the *A. brasiliensis* group; the first grouped the four Portuguese strains from grapes at a similarity of 52 %, and the second one grouped at a similarity of 42 % all other strains isolated from soil from Brazil, Australia, USA and The Netherlands.

Our *Aspergillus* species concept is based on a polyphasic approach (Frisvad & Samson, 2004): A novel species is different from any other species in a diagnostic sense in both phenotypic and genotypic features. Here we have used morphological, physiological and chemotaxonomical features to characterize the phenotype and sequencing of three genes combined with AFLP results to characterize the isolates genotypically. Because the isolates were unique with regard to morphology, extrolite profiles and genotypic features, we propose the name *Aspergillus brasiliensis* sp. nov. for these isolates.

The name *brasiliensis* refers to the locality where the culture was isolated. Since its discovery the species has also been

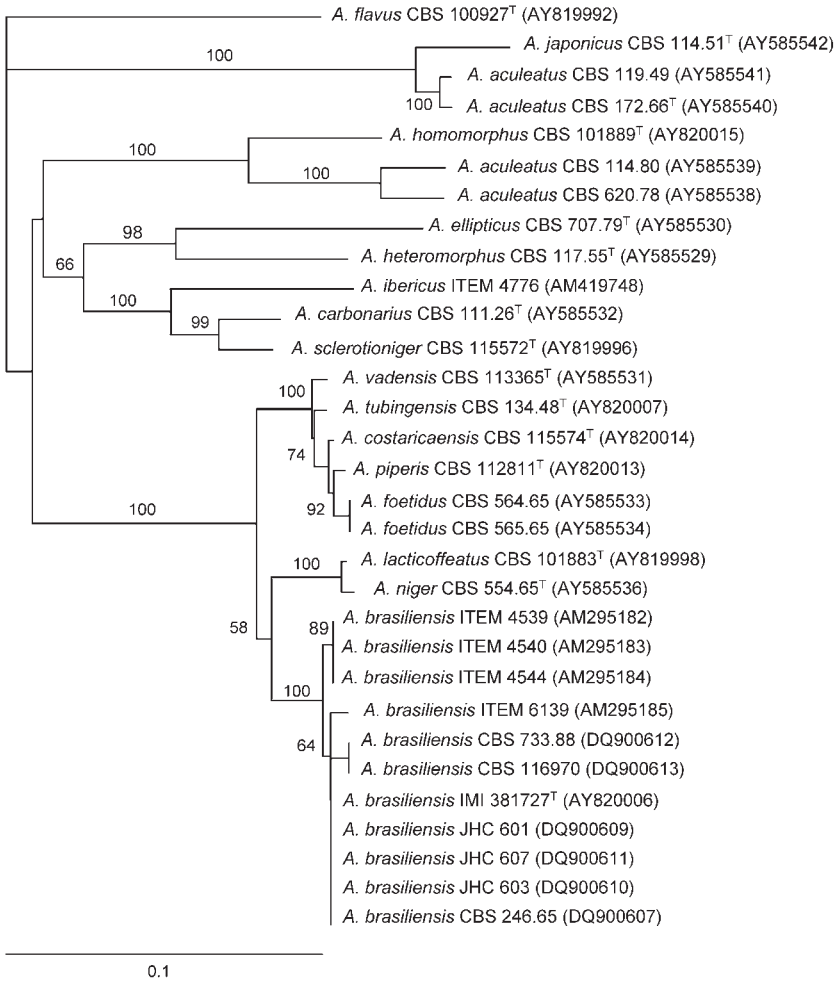

Fig. 1. Neighbour-joining tree based on β-tubulin sequence data of *Aspergillus* section *Nigri*. Numbers above branches are bootstrap values. Only values above 50 % are indicated. Bar, genetic distance.

found in other localities, but we propose to maintain the name to avoid confusion because the epithet has been used and cited in various publications.

Latin diagnosis of *Aspergillus brasiliensis* Varga, Frisvad *et* Samson sp. nov.

Coloniae post 7 *dies* 71–76 mm *diam in agaro* CYA *et* CYAS *dicto*, *in* MEA 52–70 mm, *in* YES 75–80 mm, *in agaro farina avenacea confecto* 32–36 mm, *in* CREA 32–44 mm. *Coloniae primum albae*, *deinde obscure brunneae vel atrae*, *reverseum cremeum vel dilute brunneum*. *Conidiorum capitula primum globosa*, *deinde radiantia*, *nonnumquam in nonnullas columnas divisa*; *stipes* 700–1700 × 8–13 μm, *crassitunicatus*, *levis*, *dilute brunneus*; *vesiculae* 30–45 μm *diam*, *fere globosae*; *capitula biseriata*; *metulae quasi totam superficiem vesiculae obtegentes*, 22–30 × 3–6 μm; *phialides lageniformes*, 7–9 × 3–4 μm; *conidia subglobosa*, 3.5–4.8 μm *diam*, *echinulata*. *Sclerotia haud visa*.

Typus siccus in herb. CBS 101740^{T} (=IMI 381727^{T}=IBT 21946^{T}) *et ex-typus vivus, isolatus e solo in* Pedreira, São Paulo, Brasilia.

Description of *Aspergillus brasiliensis* Varga, Frisvad & Samson sp. nov.

Aspergillus brasiliensis (bra.si′li.en′sis. N.L. masc. adj. *brasiliensis* from Brazil, the place of isolation).

Colony diameters at 7 days: CYA at 25 and 37 °C, and CYAS at 25 °C: 71–76 mm; MEA 52–70 mm; YES 75–80 mm; OA 32–36 mm; CREA 32–44 mm, poor growth, strong acid production. Colony first white then dark brown to black (Fig. 2). Exudates absent, reverse cream-coloured to light brown. Conidial heads globose at first and later radiate occasionally developing into several conidial columns; stipes 700–1700 × 8–13 μm, walls thick, smooth, pale brown; vesicles 30–45 μm wide, nearly globose; biseriate; metulae covering virtually the entire surface of the vesicle, measuring 22–30 × 3–6 μm; phialides flask-shaped, 7–9 × 3–4 μm; conidia subglobose, 3.5–4.8 μm in diameter, echinulate. No sclerotia observed in the culture ex type. All isolates produced several naphtho-γ-pyrones (including aurasperone B), tensidol A and B and pyrophen.

The type strain, CBS 101740^{T} (=IMI 381727^{T}=IBT 21946^{T}), was isolated from soil, Pedreira, São Paulo,

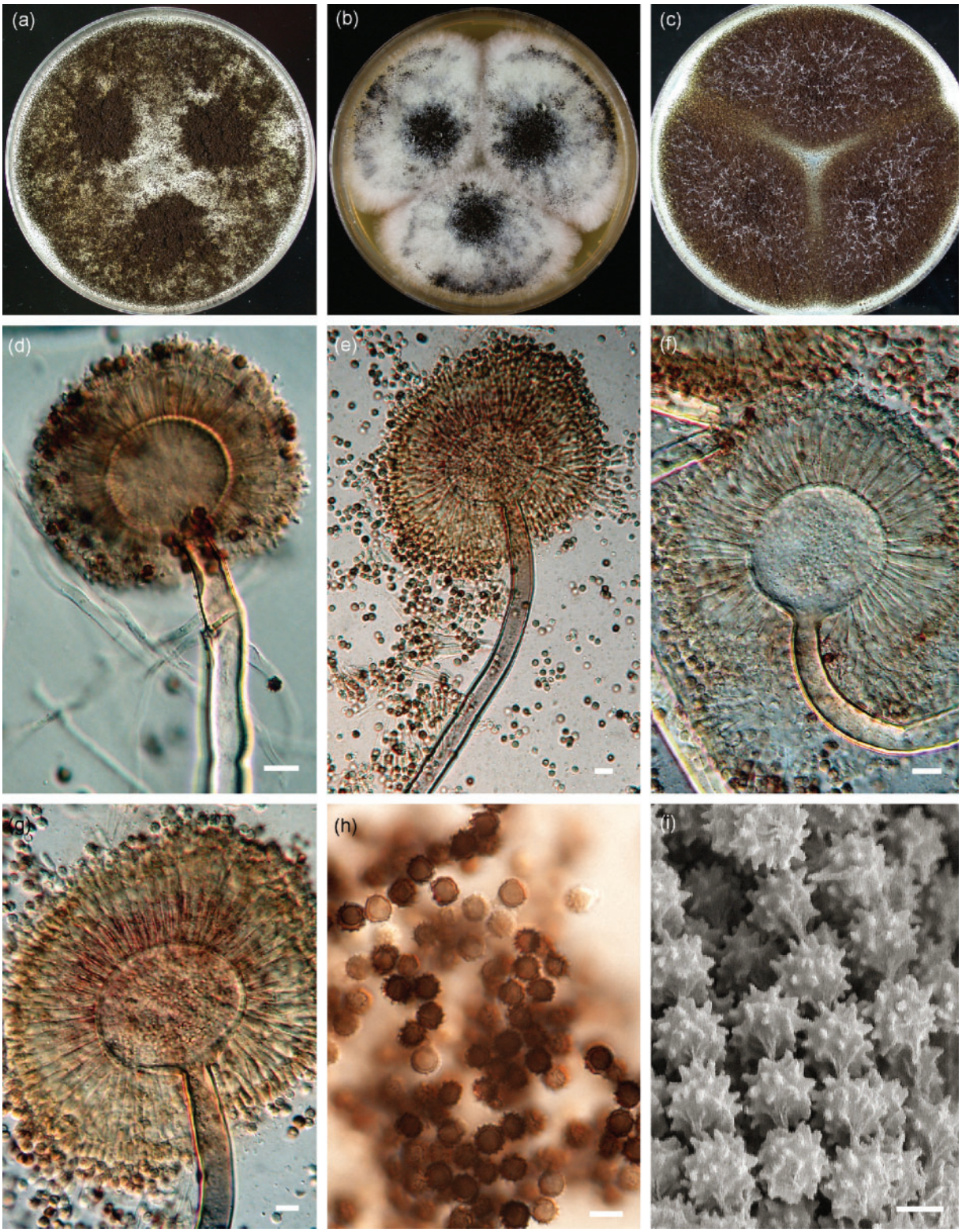

Fig. 2. *Aspergillus brasiliensis* sp. nov. CBS 101740^{T}. (a) Colonies on CYA; (b) colonies on OA; (c) colonies on MEA; (d–g) conidiophores; (h) conidia under light microscope; (i) conidia as seen using SEM. Bars, 10 μm (d–h) and 5 μm (i).

Brasil. Isolates ITEM 4544 and ITEM 6139 produce large white sclerotia.

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