Aspergillus saccharolyticus sp. nov., a black *Aspergillus* species isolated in Denmark

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A novel species, *Aspergillus saccharolyticus* sp. nov., belonging to the *Aspergillus* section *Nigri* group is described. This species was isolated in Denmark from treated hardwood. Its taxonomic status was determined using a polyphasic taxonomic approach including phenotypic (morphology and extrolite profiles) and molecular (β -tubulin, internal transcribed spacer and calmodulin gene sequences, and universally primed PCR fingerprinting) analysis. Phenotypic and molecular data enabled this novel species to be clearly distinguished from other black aspergilli. *A. saccharolyticus* is a uniseriate *Aspergillus* species that is morphologically similar to *Aspergillus japonicus* and *Aspergillus aculeatus*, but has a totally different extrolite profile compared to any known *Aspergillus* species. The type strain of *A. saccharolyticus* sp. nov. is CBS 127449^T (=IBT 28509^T).

The black aspergilli (*Aspergillus* section *Nigri*) (Gams *et al.*, 1985) are important industrial workhorses as they are frequently used in the biotechnological industry for production of hydrolytic enzymes and organic acids (Pel *et al.*, 2007; Goldberg *et al.*, 2006; Pariza & Foster, 1983).

Black aspergilli are among the most common fungi that are responsible for post-harvest decay of fruit (Pitt & Hocking, 2009). Of these, *Aspergillus carbonarius* is especially problematic due to its high production of the mycotoxin carcinogenic ochratoxin A found in wine and raisins (Abarca *et al.*, 2003). Recently, *Aspergillus niger* has also been shown to contaminate wine with the carcinogenic mycotoxin fumonisin B₂ (Mogensen *et al.*, 2010). In addition to these toxins, the black aspergilli are well known for their prolific production of many secondary metabolites. In the biserial species, a diverse array of polyketides and several alkaloids are known, whereas the uniseriate species, *Aspergillus aculeatinus*, *Aspergillus aculeatus*,

Aspergillus japonicus and *Aspergillus uvarum*, predominantly produce alkaloids and a few polyketides (Nielsen *et al.*, 2009).

Aspergillus section *Nigri* is one of the more taxonomically difficult groups, but the uniseriate subgroup differs significantly in morphology and physiology from the biserial subgroup (Samson & Varga, 2009; Samson *et al.*, 2007a; Perrone *et al.*, 2008; Noonim *et al.*, 2008). The species concept of black aspergilli has been discussed by several researchers within the *Aspergillus* research community and it is generally agreed that it is important to delimit species in the genus by combining molecular, morphological and physiological characteristics using a polyphasic approach (Samson & Varga, 2009; Samson *et al.*, 2007b).

During a broad screening of different fungal strains collected in Denmark for prominent β -glucosidase-producing fungi (Sørensen *et al.*, 2011), a uniseriate *Aspergillus* strain that had high β -glucosidase activity was discovered and a thorough characterization was carried out in order to identify the strain. The isolate was morphologically similar to *A. japonicus* but molecular data and the extrolite profile showed that this fungus differed significantly from black *Aspergillus* species described so far. In this paper, the relationship between this isolate and other black aspergilli was investigated using a polyphasic approach including internal transcribed spacer (ITS), calmodulin and β -tubulin sequence phylogenies, universally primed PCR (UP-PCR)

Abbreviations: ACN, acetonitrile; ESI, electrospray; ITS, internal transcribed spacer; UP-PCR, universally primed PCR.

The GenBank/EMBL/DDBJ accession numbers for the β -tubulin, ITS and calmodulin gene sequences of the strains examined in this study are shown on the phylogenetic trees.

The Mycobank accession number for *Aspergillus saccharolyticus* sp. nov. is MB 158695 (<http://www.mycobank.org>).

A supplementary table and four figures are available with the online version of this paper.

fingerprinting, macro- and micro-morphology, temperature tolerance and extrolite production.

The strain was isolated indoors from underneath a treated oak wood toilet seat in Denmark. The isolate was maintained on potato-dextrose agar (PDA) at room temperature. All reference strains and accession numbers used for comparison are listed in Supplementary Table S1 (available in IJSEM Online).

Fungal biomass for DNA extraction was obtained by scraping the surface of a PDA plate of a 7-day-old colony. Cells were disrupted by bead beating and DNA extraction was carried out as described by Yu & Mohn (1999). The two fungal primers Bt2a (5'-GGTAACCAAATCGGT-GCTGCTTTC) and Bt2b (5'-ACCCTCAGTGTAGTG-ACCCTTGGC) were used to amplify a fragment of the β -tubulin gene (Glass & Donaldson, 1995), while primers Cmd5 (5'-CCGAGTACAAGGAGGCCTTC) and Cmd6 (5'-CCGATAGAGGTCATAACGTGG) were used to amplify a segment of the calmodulin gene (Hong *et al.*, 2006), and primers ITS1 (5'-TCCGTAGGTGAACCTGCGG) and ITS4 (5'-TCCTCCGCTTATTGATATG) were used to amplify rRNA gene spacers, ITS1 and ITS2, as well as the 5.8S rRNA gene between the spacers (White *et al.*, 1990).

Phylogenetic analysis of the β -tubulin, calmodulin, and rRNA ITS1 and ITS2 sequences of the novel isolate was carried out as described by Varga *et al.* (2007) using the β -tubulin, calmodulin and ITS region sequences of the aspergilli presented in the article by Samson *et al.* (2007a). CLUSTAL W multiple alignment was used for sequence alignment and manual improvement of the alignment was performed using BioEdit (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>). The PHYLIP program package version 3.69 was used for preparation of phylogenetic trees (Felsenstein, 2004). The distance matrix of the dataset was calculated based on the Kimura method (Kimura, 1983) using the program DNADIST. The phylogenetic tree was prepared by running the program NEIGHBOR using the neighbour-joining method (Saitou & Nei, 1987) to obtain unrooted trees. *Aspergillus flavus* was defined as the outgroup in the program RETREE and the tree was visualized using the program TreeView (win32) (Page, 1996). Bootstrap values (Felsenstein, 1985) were calculated by running the program SEQBOOT to produce 1000 bootstrapped datasets from the original dataset. Again, DNADIST with the Kimura method was used to prepare distance matrices of the multiple datasets and NEIGHBOR was used with the neighbour-joining method to obtain unrooted trees of the multiple datasets. Finally, bootstrap values were obtained from the consensus tree which was identified by the majority-rule consensus method by running the program CONSENSE.

UP-PCR fingerprinting was carried out using two different UP primers, L45 (5'-GTAAAACGACGGCCAGT) and L15/AS19 (5'-GAGGGTGGCGGCTAG) (Lübeck *et al.*, 1999), for DNA amplification in separate reactions. Amplification was performed as described by Lübeck *et al.* (1999) except

that reactions were carried out in a 25 μ l volume containing 50 mM Tris pH 8, 0.23 mg BSA ml^{-1} , 0.5 % Ficoll, 2.5 mM MgCl_2 , 0.2 mM dNTP, 0.4 μ M primer and 1 U RUN polymerase (A&A Biotechnology).

For microscopic analysis, microscopic mounts were made in lactophenol from colonies grown on malt extract autolysate (MEA) and oatmeal agar (OA).

For investigation of morphological characteristics, a dense spore suspension of the novel isolate was three-point inoculated on the following media and incubated for 7 days in the dark at 25 °C: CREA (creatine-sucrose), CYA (Czapek yeast autolysate), CY20S (CYA with 20 % sucrose), CY40S (CYA with 40 % sucrose), CYAS (CYA with 50 g NaCl l^{-1}), MEA, OA and yeast extract-sucrose (YES) agar (Samson *et al.*, 2004a). Additionally, a three-point inoculation was made on CYA and incubated for 7 days in the dark at 37 °C. For temperature tolerance analysis, three-point inoculation was performed on CYA and plates were incubated for 7 days in the dark at different temperatures (22, 30, 33, 36 and 40 °C).

For extrolite analysis, three 6 mm diameter plugs were taken from each strain grown as three-point inoculations in the dark at 25 °C for 7 and 14 days on YES, CYA20, CYA40, PDA, CYA media (Nielsen *et al.*, 2009; Samson *et al.*, 2004a). The plugs were transferred to a 2 ml vial and 1.4 ml ethyl acetate containing 1 % formic acid was added. The plugs were placed in an ultrasonication bath for 60 min. The ethyl acetate was transferred to a new vial in which the organic phase was evaporated to dryness by applying nitrogen airflow at 30 °C. The residues were redissolved by ultrasonication for 10 min in a 150 μ l acetonitrile (ACN)/ H_2O (1:1, v/v) mixture.

HPLC-UV/VIS-high resolution MS analysis was performed with an Agilent 1100 system equipped with a diode array detector and coupled to a Micromass LCT equipped with an electrospray (ESI) (Nielsen *et al.*, 2009; Nielsen & Smedsgaard, 2003). Separation of 2 μ l samples was performed on a 50 $\times$ 2 mm inner diameter, 3 μ m Luna C₁₈ II column (Phenomenex) using a linear water/ACN gradient at a flow of 0.300 ml min^{-1} with 15–100 % ACN in 20 min followed by a plateau at 100 % ACN for 3 min (Nielsen *et al.*, 2009). Both solvents contained 20 mM formic acid. Samples were analysed in both ESI[−] and ESI⁺ mode.

For compound identification, each peak was matched against an internal reference standard database (~800 compounds) (Nielsen *et al.*, 2009; Nielsen & Smedsgaard, 2003). Other peaks were tentatively identified by matching data from previous studies in our laboratory and by searching for the accurate mass in the ~13 500 fungal metabolites reported in Antibase 2010 (Laatsch, 2010).

Morphological data showed that the novel strain was related to *A. japonicus* or *A. aculeatus*, but extrolite profiles and DNA sequencing data showed that the isolate was clearly different from all known species. The genetic

relatedness of the isolate to other black aspergilli was investigated by comparing sequence data of parts of the β -tubulin and calmodulin genes as well as the ITS region, using *A. flavus* as the outgroup. The black aspergilli chosen for comparison are the same as the ones presented by Samson *et al.* (2007a). Phylogenetic trees were prepared for the novel isolate based on these sequence data and data obtained in this work, with the ITS and calmodulin sequence trees in particular showing similar topologies (Fig. 1; Supplementary Figs S1 and S2, available in IJSEM Online). It should be noted that strain CBS 114.80^T has recently been defined as a representative of a novel species: *Aspergillus indologenus* (Varga *et al.*, 2011). Based on phylogenetic analysis of the ITS and calmodulin gene sequence data, the novel isolate was found to belong to the clade with *Aspergillus homomorphus*, *A. aculeatinus*, *A. indologenus*, *A. uvarum* and *A. japonicus* with high bootstrap values, whereas using the β -tubulin gene sequence data, the novel isolate clustered with *A. homomorphus*, *A. aculeatinus*, *A. uvarum* and *A. aculeatus* CBS 114.80. The separate grouping in the β -tubulin tree of *A. japonicus* and *A. aculeatus* has consistently been shown in other publications that have used sequences of the same strains (Noonim *et al.*, 2008; Samson *et al.*, 2007a; Varga *et al.*, 2007; de Vries *et al.*, 2005; Samson *et al.*, 2004b). As described by Peterson

(2008), and also found in our alignment, this separate grouping is due to the two aspergilli having one less intron in the β -tubulin gene compared to the other *Nigri* species. For all three loci, the novel isolate was placed on its own branch far from other species in the clade supported by the majority-rule consensus analysis for all three loci and high bootstrap values for the β -tubulin and calmodulin loci, but low bootstrap value (51 %) for the ITS locus. Sequence alignment revealed that amongst the species from series *Aculeata* and *Homomorpha* (Frisvad *et al.*, 2007) that are phylogenetically closely related to the novel isolate, interspecific sequence divergences are ≤ 0.7 , 7.1 and 5.7 % for the ITS, calmodulin and β -tubulin regions, respectively. However, the interspecific sequence divergences in the ITS, calmodulin and β -tubulin regions between the novel isolate and the other species in the clade are, on average, 12.9 ± 0.6 , 20 ± 0.5 and 15.4 ± 1.2 %, respectively. The variation in sequence data observed between the isolate and *A. homomorphus* is the same as the variation between *A. homomorphus* and the smaller clade(s) of *A. aculeatinus*, *A. uvarum*, *A. japonicus*, *A. indologenus* and *A. aculeatus* strains. Searching the NCBI database does not give any closer genetic match. Based on this, there is a clear genetic foundation for proposing a novel species to accommodate the isolate.

Fig. 1. Neighbour-joining phylogenetic tree based on partial calmodulin gene sequence data for *Aspergillus* section *Nigri*. Numbers above the branches are bootstrap values. Only values ≥ 70 % are indicated. Bar, 0.02 substitutions per nucleotide.

Furthermore, the novel strain could readily be distinguished from other black aspergilli by UP-PCR analysis using each of the two UP primers L45 and L15/AS19 (Supplementary Fig. S3, available in IJSEM Online). UP-PCR is a PCR fingerprinting method that has demonstrated its applicability in different aspects of mycology. These applications include analysis of genome structures, identification of species, analysis of population and species diversity, identification of genetic relatedness at infra- and inter-species levels, and identification of UP-PCR markers at different taxonomic levels (strain, group and/or species) (Lübeck & Lübeck, 2005). Each of the analysed aspergilli (the novel isolate, *A. aculeatinus*, *A. ellipticus*, *A. homomorphus*, *A. niger*, *A. uvarum*, *A. aculeatus* and *A. japonicus*) produced a unique banding profile and did not share any bands (Supplementary Fig. S3). This is an indication of clearly separated species, as strains within a species should at least have some similarities in their banding profiles (Lübeck & Lübeck, 2005).

The extrolite profiles further showed that the novel isolate produced the largest chemical diversity on YES agar (25 °C), whereas CYA (25 and 30 °C), and CYAS, CY20S, CY20, CY40S and PDA (all at 25 °C) yielded fewer peaks. Results further showed that the isolate probably represents a novel species as it does not share any metabolites with other species in the *Nigri* section where, for example, the naphtho- γ -pyrones are consistently produced (Nielsen *et al.*, 2009) and only two compounds, ACU-1 and ACU-2, are shared with the series *Aculeata* (Table 1; Supplementary Fig. S4, available in IJSEM Online), whereas the well-known compounds from the series (neoxaline, secalononic acids, cycloclavine and aculeasins) were not detected (Parenicová *et al.*, 2001). In addition, none of the 12 detected peaks matched with the approximately 13 500 fungal extrolites in Antibase 2010 (Laatsch, 2010) indicating that the species has not been investigated by natural product chemists.

Morphologically, the novel isolate was most closely related to *A. japonicus* (Fig. 2), but with larger conidia of 5–6 μ m and vesicle size in the upper end of the range for *A. japonicus*. Differences were found in physiological features of the novel isolate and other uniseriate species in the *Nigri* section. Growth on CREA resembled that of *A. aculeatinus*, as poor growth and good acid production was observed. Growth on CYA generally resembled that of *A. aculeatus*; however, the reverse side of cultures of the novel isolate was olive-green/brownish with sulcate structure, whereas that of *A. aculeatus* is curry-yellowish/brown [Fig. 2 compared with Samson *et al.* (2007a)]. On MEA, colony size was clearly different, with colonies of the isolate being smaller than those of other uniseriate aspergilli. The novel isolate grew better on CYA than *A. aculeatus* and *A. japonicus*, but growth was limited compared to *A. aculeatinus* and *A. uvarum*. Growth diameter of the novel isolate on CYA at 37 °C was approximately the same as for *A. uvarum*; however, *A. aculeatus* and *A. japonicus* were less inhibited, and *A. aculeatinus* was even less inhibited, thus having the largest diameter of all uniseriate species at this elevated temperature (Table 1). Furthermore, the novel isolate differed from the other species by having a different colony morphology, especially an uneven colony margin.

With regard to temperature tolerance, growth was examined on CYA at 22, 30, 33, 36 and 40 °C. Maximum growth temperature of the isolate was 36 °C, but growth at this temperature was restricted compared to that at lower temperatures, which is also generally the case for the other uniseriate black aspergilli (Samson *et al.*, 2007a). The isolate showed a distinct change in morphology, especially an uneven colony margin and a more floccose colony texture, when grown on CYA at 30 and 33 °C, but maintained good growth at both temperatures (Fig. 3). The same tendency has been observed for *A. aculeatinus*.

Table 1. Physiological features and extrolite production by strains of uniseriate species in *Aspergillus* section *Nigri*

Extrolite production as described by Parenicová *et al.* (2001) and Noonim *et al.* (2008) and updated here.

Species	Growth on CYAS (diameter, mm)	Growth at 37 °C on CYA (diameter, mm)	Extrolites
<i>A. saccharolyticus</i> sp. nov. CBS 127449 ^T	11–14	7–14	12 compounds not described in the literature* including ACU-1† and ACU-2†
<i>A. aculeatinus</i> (CBS 121060 ^T , CBS 121875, IBT 29275)	37–54	18–52	Aculeasins, neoxaline, secalononic acid D and F
<i>A. aculeatus</i> CBS 172.66 ^T	0–4	15–26	Secalononic acid D and F, ACU-1† and ACU-2†
<i>A. japonicus</i> (CBS 114.51 ^T , IBT 29329, IBT 26338, ITEM 4497)	0	8–25	Cycloclavine, festuclavine
<i>A. uvarum</i> [CBS 121591 ^T (=ITEM 4834 ^T), ITEM 4856, ITEM 5024]	54–73	11–14	Asterric acid, dihydrogeodin, erdin, geodin, secalononic acid D and F

*No matches found among the 13 500 fungal metabolites listed in Antibase 2010.

†ACU-1 and ACU-2 are unidentified compounds with UV max 242 nm (100%) and 346 (88%) with mono isotopic masses of 315.1799 and 218.1268 Da, respectively.

Fig. 2. *A. saccharolyticus* sp. nov. CBS 127449^T. (a) Conidia, (b) small conidial head, (c) typical conidial head; (d–h) three-point inoculation on CREA, CYA, CYA 37 °C, MEA and CYAS, respectively, incubated for 7 days. Bars (a–c), 10 µm.

grown on MEA, but *A. japonicus* and *A. aculeatus* have shown no change in morphology at these temperatures, whereas growth of *A. uvarum* was inhibited at 33 °C (Samson *et al.*, 2007a).

It is concluded that the isolate represents a novel species. This conclusion is based on a polyphasic approach in which genotype characterization (phylogenetic analysis of three genes and UP-PCR analysis) has been combined with phenotypic analysis (morphological, physiological and chemotaxonomic characteristics). Because the strain was unique in its genetic phylogeny, UP-PCR profile, extrolite profile, and morphological and physiological characteristics, it represents a novel species for which the name *Aspergillus saccharolyticus* sp. nov. is proposed. Although the species is currently only represented by a single isolate, this isolate is entirely unique in its combination of features and the hypothesis that it represents a novel species is strongly supported. The inclusion of more strains in the study may have given more data regarding the interspecific variation in

individual features, but not regarding features at the species level. Such differential features and gene sequences have been shown to be rather constant in species where many isolates are available at the time of description. The novel species is an efficient producer of β -glucosidases (Sørensen *et al.*, 2011) and the name refers to its great ability to hydrolyse cellobiose and cellodextrins.

Latin diagnosis of *Aspergillus saccharolyticus* Sørensen, Lübeck et Frisvad sp. nov.

Coloniae post 7 dies 58–62 mm diam in agar CYA; in CYA, 37 °C, 7–14 mm; in MEA, 35–37 mm; in YES, 75–80 mm; in agar farina avenacea confecto, 39–42 mm; in CREA, 30–34 mm. *Coloniae* primum albae, deinde obscure brunneae vel atrae, reversum cremeum vel dilute brunneum. *Conidiorum* capitula primum globosa, stipes 200–850 × 5–7 µm, crassitunicatus, levis, vesiculae 25–40 µm diam, fere globosae; capitula uniseriata; phialides lageniformes, collulis brevis, 5.5–7.0 µm; conidia globosa vel subglobosa, 5.0–6.2 µm, echinulata. *Sclerotia* haud visa.

Fig. 3. *A. saccharolyticus* sp. nov. CBS 127449^T three-point inoculation on CYA incubated at various temperatures (from left to right: 22, 30, 33, 36 and 40 °C); plates show growth observed after incubation for 7 days.

Typus CBS 127449^T (=IBT 28509^T), *isolatus e lignore Quercetorum* in Gentofte, Dania, Philip J. Teller.

Description of *Aspergillus saccharolyticus* Sørensen, Lübeck & Frisvad sp. nov.

Aspergillus saccharolyticus [sac.cha.ro.ly'ti.cus. Gr n. *sakchar* sugar; N.L. masc. adj. *lyticus* (from Gr. masc. adj. *lutikos*) able to loose, able to dissolve; N.L. masc. adj. *sacchar-olyticus* able to degrade sugars].

Mycobank number MB 158695.

Colony diameter at 7 days: CYA at 25 °C, 58–62 mm, colony first white, then dark brown to black; CYA at 37 °C, 7–14 mm; CYAS, 11–14 mm; YES, 75–80 mm; OA, 39–42 mm; CY20S, 42–54 mm; CY40S, 43–54 mm; MEA, 35–37 mm; CREA, 30–34 mm, poor growth, good acid production (Fig. 2). Exudates are absent, reverse cream-coloured to light greyish olive brown on CYA and light brown on YES. Conidial heads are globose; stipes are 200–850 × 5–7 µm, walls are thick and smooth; vesicles are 25–40 µm diameter, globose; uniseriate, phialides are flask-shaped with a short broad collulum, 5.5–7.0 µm; conidia are mostly globose, but some are subglobose, 5.0–6.2 µm, distinctly echinulate, with long, sharp, discrete, 0.6–0.8 µm spines. Sclerotia have not been observed.

The type strain is CBS 127449^T, isolated by Philip J. Teller from under a toilet seat made of treated oak wood, Gentofte, Denmark. The type strain is available for the scientific community as IBT 28509^T (=IBT 30881^T) from the IBT culture collection, Technical University, Denmark.

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