Two novel species of *Aspergillus* section *Nigri* from Thai coffee beans

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Two novel species of *Aspergillus* section *Nigri* from Thai coffee beans are described as *Aspergillus aculeatinus* sp. nov. and *Aspergillus sclerotiicarbonarius* sp. nov. Their taxonomic status was determined using a polyphasic taxonomic approach with phenotypic (morphology and extrolite profiles) and molecular (β -tubulin, internal transcribed spacer and calmodulin gene sequences) characteristics. *A. aculeatinus* sp. nov. is a uniseriate species with a similar morphology to *Aspergillus aculeatus* and *Aspergillus japonicus*, but producing smaller conidia (2–5 μ m). *A. aculeatinus* sp. nov. produced neoxaline, secalonic acid D and F, and aculeacins. *A. sclerotiicarbonarius* sp. nov. is a biserial species similar to *Aspergillus carbonarius* and *Aspergillus ibericus*, but produces abundant sclerotia and some unique indol-alkaloids. The type strain of *Aspergillus sclerotiicarbonarius* sp. nov. is CBS 121057^T (=IBT 28362^T) and the type strain of *Aspergillus aculeatinus* sp. nov. is CBS 121060^T (=IBT 29077^T).

INTRODUCTION

Black aspergilli (*Aspergillus* section *Nigri*; Gams *et al.*, 1985) are common species in the environment. Although the main habitat of black aspergilli is soil, members of this section have also been isolated from various other sources (Kozakiewicz, 1989). They are not only responsible for food spoilage and biodeterioration, but are also frequently used in biotechnological processes for the production of various enzymes and organic acids. In addition, some black aspergilli have been reported as ochratoxin A-producing

species in various food commodities (Abarca *et al.*, 1994; Cabañes *et al.*, 2002; Schuster *et al.*, 2002; Abarca *et al.*, 2003; Taniwaki *et al.*, 2003).

During our surveys of the mycobiota of coffee beans from Thailand, black aspergilli were predominant. Several of these isolates were found that differed significantly from the known species assigned to section *Nigri*. We analysed the relationship of these isolates to other black aspergilli using a polyphasic taxonomic approach. For the phenotypic analyses, macro- and micro-morphology, extrolite production and growth temperatures were studied. For the phylogenetic analyses, calmodulin, β -tubulin and internal transcribed spacer region of rRNA (ITS) gene sequences were used.

METHODS

Isolates. The strains used in this study are listed in Table 1 and represent two groups of atypical isolates from Thai coffee beans (*Coffea arabica* and *Coffea canephora* var. *robusta*) and strains from the CBS Fungal Biodiversity Centre (Utrecht, the Netherlands). Other isolates came from the IMI (CABI Bioscience Genetic Resource

Abbreviations: ITS, internal transcribed spacer region of the rRNA gene cluster; NJ, neighbour-joining; SEM, scanning electron microscopy.

The GenBank/EMBL/DDBJ accession numbers for the β -tubulin, ITS and calmodulin gene sequences of strains examined in this study are shown in Fig. 1 and Supplementary Figs S1 and S2 (available with the online version of this paper).

The Mycobank (<http://www.mycobank.org>) accession numbers for *A. sclerotiicarbonarius* sp. nov. and *A. aculeatinus* sp. nov. are MB 504407 and MB 505075, respectively.

Neighbour-joining phylogenetic trees based on β -tubulin and ITS gene sequences are available as supplementary figures with the online version of this paper.

Table 1. Origin and strain numbers for the isolates examined in this study

Strain number	Origin
<i>A. aculeatinus</i>	
CBS 121060 ^T	<i>Arabica</i> coffee, Thailand
CBS 121061	<i>Robusta</i> coffee, Thailand
CBS 121062	<i>Robusta</i> coffee, Thailand
CBS 121871	<i>Robusta</i> coffee, Thailand
CBS 121872	<i>Robusta</i> coffee, Thailand
CBS 121873	<i>Arabica</i> coffee, Thailand
CBS 121874	<i>Arabica</i> coffee, Thailand
CBS 121875	<i>Arabica</i> coffee, Thailand
CBS 121876	<i>Arabica</i> coffee, Thailand
CBS 121877	<i>Arabica</i> coffee, Thailand
CBS 115570	Soil, Japan
' <i>A. aculeatus</i> '	
CBS 114.80	Soil, India
CBS 620.78 (=NRRL 2053)	Unknown
<i>A. aculeatus</i> CBS 172.66 ^T	Tropical soil, unknown
<i>A. brasiliensis</i> IMI 381727 ^T	Soil, Brazil
<i>A. carbonarius</i> CBS 111.26 ^T (=IMI 16136 ^T)	Paper, unknown
<i>A. costaricensis</i> CBS 115574 ^T	Soil, Costa Rica
<i>A. ellipticus</i> CBS 707.79 ^T (IMI 172283 ^T)	Soil, Costa Rica
<i>A. foetidus</i>	
CBS 564.65	Unknown, Japan
CBS 565.65	Unknown, Japan
<i>A. heteromorphus</i> CBS 117.55 ^T	Culture contaminant, Brazil
<i>A. homomorphus</i> CBS 101889 ^T	Soil of Dead Sea, Israel
<i>A. ibericus</i> ITEM 4776 ^T (=IMI 391429 ^T)	Grapes, Portugal.
<i>A. japonicus</i> CBS 114.51 ^T	Unknown
<i>A. lacticoffeatus</i> CBS 101883 ^T	Coffee bean, South Sumatra
<i>A. niger</i> CBS 554.65 ^T (=IMI 050566 ^T)	Unknown
<i>A. piperis</i> CBS 112811 ^T	Ground black pepper, Denmark
<i>A. sclerotiiicarbonarius</i>	
CBS 121056	<i>Robusta</i> coffee, Thailand
CBS 121057 ^T	<i>Robusta</i> coffee, Thailand
CBS 121851	<i>Robusta</i> coffee, Thailand
CBS 121852	<i>Robusta</i> coffee, Thailand
CBS 121853	<i>Robusta</i> coffee, Thailand
CBS 121854	<i>Robusta</i> coffee, Thailand
<i>A. sclerotioniger</i> CBS 115572 ^T	Green <i>Arabica</i> coffee, India
<i>A. tubingensis</i> CBS 134.48 ^T (=IMI 172283 ^T)	Unknown
<i>A. uvarum</i> CBS 121591 ^T	Grapes, Brindisi, Italy
<i>A. vadensis</i> CBS 113365 ^T	Dead plant tissue, unknown

Collection, Egham, England), ITEM (Agri-Food Toxigenic Fungi Culture Collection, Bari, Italy) or IBT collections (BioCentrum-DTU, Kgs. Lyngby, Denmark).

Morphological analysis. For macromorphological observations, Czapek yeast autolysate agar (CYA), malt extract autolysate agar (MEA), yeast extract sucrose agar (YES), creatine agar (CREA), oatmeal agar (OAT) and Czapek agar (CZA) were used (Samson *et al.*, 2004a). 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, mounts were made in lactic acid from colonies grown in MEA and a drop of alcohol was added to remove air bubbles and excess conidia. Scanning electron microscopy (SEM) was performed with uncoated frozen samples of conidiophores

and conidia on agar plugs with a scanning electron microscope (JEOL 5600LV; JEOL) equipped with an Oxford CT1500 cryostation.

Extrolite analysis. The novel isolates were grown on CYA and YES at 25 °C for 7 days. Five plugs of each agar medium were taken and pooled into the same vial for extraction with 0.75 ml of a mixture of ethyl acetate/dichloromethane/methanol (3:2:1; by vol.) with 1 % (v/v) formic acid. The extracts were filtered and analysed by HPLC using alkylphenone retention indices and diode array UV-VIS detection as described by Frisvad & Thrane (1987), with minor modifications as described by Smedsgaard (1997). The column used was a 50×2 mm Luna C-18 (II) reversed phase column (Phenomenex) fitted with a 2×2 mm guard column. Standards from the collection at Biocentrum-DTU for ochratoxin A and B,

sclerotii carbonarius sp. nov. were unable to grow. In contrast, all isolates of the novel species grew very fast on CYA at 25 °C (CYA25) and YES. As with other species in *Aspergillus* section *Nigri*, no growth or germination was detected at 5 °C. There are two phenotypes of *Aspergillus sclerotii carbonarius* sp. nov., one with abundant sclerotium production and another producing less sclerotia. Considering the growth pattern of these two phenotypic isolates in MEA at different temperatures, heavily sclerotium-producing isolates stopped growing at a temperature of 33 °C while the isolates producing less sclerotia stopped growing at a temperature of 36 °C. Isolates of *A. sclerotii carbonarius* sp. nov. produced similar sclerotia when compared with *A. sclerotiumiger*. The distinguishing characteristic between these two species is the colour of the mycelium; *A. sclerotiumiger* has yellow mycelium, while the mycelium of *A. sclerotii carbonarius* is white. *A. carbonarius* can sometimes produce similar sclerotia, but these two species can be distinguished by the colour of their conidia; the conidia of *A. carbonarius* are carbon black, whereas conidia of *A. sclerotii carbonarius* are dark brown. From the SEM and light microscope images (Fig. 2), it is clear that this novel species has conspicuously spiny conidia when young and that verruculose conidia develop at maturity. The conidial ornamentation of *A. sclerotii carbonarius* is more similar to that of the recently described species, *A. ibericus* (Serra *et al.*, 2006) than to *A. carbonarius*.

The extrolite analysis showed that isolates of *A. sclerotii carbonarius* were able to produce pyranonigrin A, aurasperone B and some unique indol-alkaloids. These were prominent but unknown compounds in the HPLC analysis with retention indices 1475, 1676 and 1838 and were never observed with any other species in the section *Nigri*. The strains producing more sclerotia produced larger amounts of these indol-alkaloids.

Although two other closely related sclerotium-producing species, *A. carbonarius* and *A. sclerotiumiger*, are able to produce ochratoxin A (Samson *et al.*, 2004b), this toxin was absent in *A. sclerotii carbonarius*. It was suggested by Wicklow *et al.* (1996) that ochratoxin A was protecting the sclerotia of *A. carbonarius* from insects, but this hypothesis could not be confirmed for other black aspergilli, since ochratoxin A was not detected in several other sclerotium-producing species, including *A. sclerotii carbonarius*, *Aspergillus ellipticus*, *A. aculeatus*, *A. costaricensis*, *A. brasiliensis* and *A. piperis* (Samson *et al.*, 2004b; Varga *et al.*, 2007).

Another new taxon, which we propose here as *Aspergillus aculeatinus* sp. nov., was isolated from Arabica coffee beans from the North and Robusta coffee beans from the South of Thailand. It was difficult to differentiate this novel species from other uniseriate species using morphological or physiological characteristics since they could all grow in the temperature range of approximately 9–40 °C. They showed the same growth rate in different media as found for other uniseriate species. However, *A. aculeatinus* sp.

nov. could be distinguished based on the subglobose to ellipsoidal and smaller conidia (2–5 µm) compared with *A. aculeatus*/*A. japonicus* (4–5 µm; Fig. 3). Isolates of *A. aculeatinus* sp. nov. consistently produced neoxaline, secalononic acid D and secalononic acid F. Furthermore, strain ATCC 36411 (=CBS 115570) is a known producer of aculeacins (Mizuno *et al.*, 1977; Sato *et al.*, 1977) and so this antifungal extrolite (Mizoguchi *et al.*, 1977) is also among those that could potentially be produced by *A. aculeatinus* sp. nov. Strains of *A. aculeatinus* sp. nov. also produced a series of extrolites with characteristic retention indices and UV spectra. Even though some of these were unique for *A. aculeatinus*, their structures have not yet been elucidated.

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. During analysis of part of the calmodulin gene, 485 characters were analysed. Among the 250 polymorphic sites, 196 were found to be parsimony-informative. The NJ tree based on partial calmodulin genes sequences is shown in Fig. 1. The topology of the tree is the same as one of the more than 416 most parsimonious trees constructed by the PAUP program (length: 627 steps, consistency index: 0.6475, retention index: 0.8637). The β -tubulin dataset included 405 characters, with 164 parsimony-informative characters. The topology of the NJ (see Supplementary Fig. S1 in IJSEM Online) was the same as that of one of the three most parsimonious trees (tree length: 442, consistency index: 0.6719, retention index: 0.8902). The ITS dataset included 470 characters with 74 parsimony-informative characters. The NJ tree shown in Supplementary Fig. S2 (see IJSEM Online) has the same topology as one of the 156 most parsimonious trees (tree length: 131, consistency index: 0.9160, retention index: 0.9865).

Based on the phylogenetic analysis of the sequence data for the three loci, isolates of *A. sclerotii carbonarius* sp. nov. formed a well-supported clade related to *A. carbonarius* and the related species *A. sclerotiumiger* and *A. ibericus* (Fig. 1, and Supplementary Figs S1 and S2). The isolates of *A. aculeatinus* sp. nov. were found to belong to a clade related to the uniseriate species *A. uvarum* in the tree based on β -tubulin gene sequence data and to *A. uvarum*, *A. aculeatus* and *A. japonicus* in the trees based on ITS and calmodulin gene sequence data. Apart from the isolates that were isolated from Thai coffee beans, one isolate that originated from garden soil from Japan was also found to belong to this novel species.

Our *Aspergillus* species concept is based on a polyphasic approach (Frisvad & Samson, 2004): A novel species should differ from any other species in diagnostic tests that encompass both phenotypic and phylogenetic features. Here, we have used morphological, physiological and chemotaxonomical features to determine the phenotype and have sequenced three genes in order to characterize the

Fig. 2. *Aspergillus sclerotiicarbonarius* sp. nov. CBS 121057^T. (a–c) Colonies on (a) CYA, 25 °C; (b) MEA, 25 °C; (c) YES, 25 °C. (d–f, h–i) Conidiophores; (g) sclerotia; (j–k) conidia. Bars, (d–h) 20 µm, (i) 1 mm, (j–k) 5 µm.

Fig. 3. *Aspergillus aculeatinus* sp. nov. CBS 121060^T. (a–c) Colonies on (a) CYA, 25 °C; (b) MEA, 25 °C; (c) YES, 25 °C. (d–h) Conidiophores; (i–j) conidia. Bars, (d–h) 10 µm, (i–j) 5 µm.

isolates. Because the isolates were unique in their morphology, extrolite profiles and phylogenetic features, we propose two novel species, *Aspergillus sclerotiicarbonarius* sp. nov. and *Aspergillus aculeatinus* sp. nov., to accommodate them.

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

Aspergillo carbonario similis sed massa conidiorum obscure brunnea et sclerotiis copiosis distinguendus; sclerotia (0.6–) 0.8–1.2 (–1.6) mm, (sub)globose vel ellipsoidea, flava vel aurantia vel rubrobrunnea, hyphis flavis oblecta. Conidia subglobosa, (5.1–) 6–8 (–9.5) × (4.8–) 5.8–7.8 (–8.5) µm.

Typus siccus in herb. CBS 121057^T (=IBT 28362^T), *et ex-typus vivus, isolatus Coffea canephora* var. *robusta*, Chumporn Provincia, Thailand.

Description of *Aspergillus sclerotiicarbonarius* Noonim, Frisvad, Varga & Samson sp. nov. (Fig. 2)

Aspergillus sclerotiicarbonarius (scler'o.ti.i.car.bon.ar.i.us. N.L. n. *sclerotium* sclerotium; L. adj. *carbonarius* of or relating to charcoal, and also a scientific epithet; N.L. masc. adj. *sclerotiicarbonarius* referring to similarity to *Aspergillus carbonarius* and the abundant production of sclerotia).

Colony diameters at 7 days at 25 °C on various agars are: CYA, 85 mm; MEA, 48–64 mm; YES, >85 mm; CZA, 28–35, and OAT, 38–52 mm. No growth on CREA or CYA at 37 °C. On CYA25 and MEA, conidiophores are abundantly produced, conidial areas are dark brown; mycelium is white to light yellow; exudates abundantly present, sclerotia abundantly present in most isolates, medium size (minimum 0.6–) 0.8–1.2 (average dimensions) (–1.6 maximum) mm, (sub)globose to ellipsoidal, yellow to orange to red–brown covered by yellow mycelium. On YES agar, white to light yellow hyphae, moderate conidiophores and abundant sclerotia are produced. On OAT, similar to MEA and CYA25, but only few sclerotia are present (at the edge between colonies). On CZA, restricted growth, large irregular shaped yellow–orange sclerotia are produced. Reverse is light yellow on CYA, pinkish yellow and furrowed on MEA, and strikingly bright yellow on YES. Conidial heads radiate, splitting into poorly defined columns with age. Stipes are long (1200–) 1400–2400 (–2800) × (13–) 15–23 (–27) µm, walls thick, smooth, hyaline (light yellowish brown pigment on upper portion near conidial head); vesicles (45–) 55–75 (–90) µm wide, pyriform; biseriate; lightly packed metulae covering three quarters of the vesicle, measuring 2–4 µm (at base) to 4–8 µm (at top) × (28–) 32–39 (–45) µm; phialides 4–7 × 9–14 µm; conidia subglobose, (5.1–) 6–8 (–9.5) × (4.8–) 5.8–7.8 (–8.5) µm, conspicuously spiny when young, becoming verruculose when mature. Extrolites comprise pyranonigrin A, aurasperone B and three unique indol-alkaloids at retention indices 1475, 1676 and 1838. The

distinguishing features of this species are its inability to grow in CREA and on CYA at 37 °C, the rough (wrinkled) colony surface, heavily sclerotia production and the strikingly bright yellow reverse of the colony in YES. *A. sclerotiicarbonarius* isolates grow well at 25 °C, but not at 37 °C.

The type strain, CBS 121057^T (=IBT 28362^T), was isolated from surface-disinfected *Coffea canephora* var. *robusta* dried coffee cherries and green coffee beans from the Sawi District, Chumporn Province, Thailand. The Mycobank accession number is MB 504407.

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

Aspergillo aculeato similimus, sed conidiis paulo minoribus distinguendus; conidia subglobose vel ellipsoidea, 2–4 × 2.3–4.4 µm, echinulata.

Typus siccus in herb. CBS 121060^T (=IBT 29077^T), *et ex-typus vivus, isolatus Coffea arabica*, Chiangmai Provincia, Thailand.

Description of *Aspergillus aculeatinus* Noonim, Frisvad, Varga & Samson sp. nov. (Fig. 3)

Aspergillus aculeatinus (a.cul.e.a.ti.nus. L. adj. *aculeatus* furnished with stings or prickles, thorny, prickly, and also a scientific epithet; L. suff. *-inus -a -um* adjectival suffix used with the sense of belonging to; N.L. masc. adj. *aculeatinus* similar to *Aspergillus aculeatus*).

Colony diameters at 7 days and 25 °C on the following agars: CYA, >85 mm; MEA, 78–85 mm; YES, >85 mm; CZA, 33–43; OAT, 55–64 mm, and CREA, 13–52 mm. Colony diameter on CYA at 37 °C is 22–33 mm. On CYA and MEA, conidiophores are produced abundantly, conidial areas are light brown; sclerotia are present in some isolates but no sclerotia are observed in culture ex type, small (0.4–0.6), globose to subglobose, creamish to light yellow. Reverse of the colony is light yellow on CYA agar, light brown and furrowed on MEA and bright yellow on YES. Conidial heads radiate, splitting into poorly defined columns or 2–6 well-defined columns with age; stipes are short (minimum 380–) 500–620 (average dimensions) (–800 maximum) × (9–) 11–14 (–16) µm, walls thin, smooth, hyaline; vesicles (43–) 55–65 (–82) µm wide, globose; uniseriate; phialides flask-shaped and cover the entire surface of the vesicle, measuring 1.8–3.5 × 6.1–9.0 µm; conidia subglobose to ellipsoidal, 2–4 × 2.3–4.4 µm, echinulate. Strains of this species produce neoxaline, secalononic acid D, secalononic acid F and aculeacins. Distinguishing features of this species are small (2–5 µm) subglobose to ellipsoidal conidia and the production of unique extrolites (neoxaline, aculeacins, secalononic acid D and secalononic acid F)

The type strain, CBS 121060^T (=IBT 29077^T), was isolated from *Coffea arabica* (dried parchment and green coffee

beans), Muang District, Chiangmai Province, Thailand. The Mycobank accession number is MB 505075.

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