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# New and revisited species in *Aspergillus* section *Nigri*

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**Abstract:** Four new species, *Aspergillus eucalypticola, A. neoniger, A. fijiensis* and *A. indologenus* are described and illustrated. *Aspergillus eucalypticola* was isolated from *Eucalyptus* leaf from Australia, and is related to *A. tubingensis* and *A. costaricaensis*, but could clearly be distinguished from them based on either β-tubulin or calmodulin sequence data. *Aspergillus eucalypticola* produced pyranonigrin A, funalenone, aurasperone B and other naphtho-γ-pyrones. *Aspergillus neoniger* is also a biseriate species isolated from desert sand in Namibia, and mangrove water in Venezuela, which produces aurasperone B and pyranonigrin A. *Aspergillus fijiensis* is a uniseriate species related to *A. aculeatinus*, and was isolated from soil in Fiji, and from *Lactuca sativa* in Indonesia. This species is able to grow at 37 °C, and produces asperparalines and okaramins. *Aspergillus indologenus* was isolated from soil, India. This species also belongs to the uniseriate group of black aspergilli, and was found to be related to, but clearly distinguishable from *A. uvarum* based on β-tubulin, calmodulin and ITS sequence data. *Aspergillus indologenus* produced the insecticidal compounds okaramins A, B, H, and two types of indol-alkaloids which have not been structure elucidated. Two other species, *A. violaceofuscus* and *A. acidus*, are revalidated based on molecular and extrolite data. *Aspergillus violaceofuscus* was found to be related to *A. japonicus*, and produced some of the same interesting indol-alkaloids as *A. indologenus*, and also produced several families of partially characterised extrolites that were also found in *A. heteromorphus*. *Aspergillus acidus* (previously known as *A. foetidus* var. *pallidus* and *A. foetidus* var. *acidus*) is also a valid species, while *A. foetidus* is a synonym of *A. niger* based on molecular and physiological data. Two other species described previously, *A. coreanus* and *A. lacticoffeatus*, were found to be colour mutants of *A. acidus* and *A. niger*, respectively. Methods which could be used to distinguish the two closely related and economically important species *A. niger* and *A. awamori* are also detailed. Although these species differ in their occurrence and several physiological means (elastase activities, abilities to utilise 2-deoxy-D-glucose as sole carbon source), our data indicate that only molecular approaches including sequence analysis of calmodulin or β-tubulin genes, AFLP analysis, UP-PCR analysis or mtDNA RFLP analysis can be used reliably to distinguish these sibling species. *Aspergillus* section *Nigri* now includes 26 taxa.

**Key words:** *Aspergillus* section *Nigri*, phylogeny, polyphasic taxonomy, extrolites.
**Taxonomic novelties:** *Aspergillus eucalypticola* Varga, Frisvad & Samson sp. nov., *Aspergillus fijiensis* Varga, Frisvad & Samson sp. nov., *Aspergillus indologenus* Frisvad, Varga & Samson sp. nov., *Aspergillus neoniger* Varga, Frisvad & Samson sp. nov.

## INTRODUCTION

The black aspergilli (*Aspergillus* section *Nigri*; Gams *et al.* 1985) are an important group of species in food mycology, medical mycology and biotechnology. Many species cause food spoilage, but on the other hand are also 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 since *A. niger* used under certain industrial conditions has been granted the GRAS (Generally Regarded As Safe) status by the Food and Drug Administration of the US 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.* 2004b, Ferracin *et al.* 2009). Black aspergilli are one of the more difficult groups concerning classification and identification, and several taxonomic schemes have been proposed. New molecular approaches have shown that there is a high biodiversity, but that species are occasionally difficult to recognise based solely on their phenotypic characters (Samson *et al.* 2007).

During a study of the genetic relationships among black aspergilli collected worldwide, four isolates have been identified which did not fit to any of the currently accepted 19 species of *Aspergillus* section *Nigri* (Samson *et al.* 2007, Noonim *et al.* 2008, Perrone *et al.* 2008). We used a polyphasic approach including sequence analysis of parts of the β-tubulin and calmodulin genes and the ITS region, macro- and micromorpholocigal analyses and examination of extrolite profiles of the isolates to describe four new species in this section. Besides, the applicability of various approaches for distinguishing the two closely related species *A. niger* and *A. awamori* has also been examined. The methods tested include morphological, physiological, ecological and molecular approaches.

## MATERIALS AND METHODS

### Isolates

The strains used in this study are listed in Table 1.

### Morphological analysis

For macromorphological observations, Czapek Yeast Autolysate (CYA) agar, Malt Extract Autolysate (MEA) agar, Yeast Extract Sucrose Agar (YES), Creatine Agar (CREA), and Oatmeal Agar (OA) were used (Samson *et al.* 2004a). The isolates were inoculated at three points on each plate of each medium and incubated at 25 °C and 37 °C in the dark for 7 d. For micromorphological observations,

**Table 1**. Isolates examined in this study.

| Accession No. | Species | Origin and information (abbreviation) | β-tubulin | calmodulin | ITS |
|---|---|---|---|---|---|
| CBS 564.65$^{T}$ | *A. acidus* | Unknown substratum, Japan | AY585533 | AY585533 | AJ280009 |
| CBS 121060$^{T}$ | *A. aculeatinus* | Arabica green coffee bean, Thailand | EU159220 | EU159241 | EU159211 |
| CBS 172.66$^{T}$ | *A. aculeatus* | Tropical soil, unknown origin | AY585540 | AJ964877 | AJ279988 |
| CBS 557.65$^{T}$ | *A. awamori* | NRRL 4948 = WB 4948 | AY820001 | AJ964874 | AM087614 |
| CBS 101740$^{T}$ | *A. brasiliensis* | Soil, Sao Paulo, Pedreira, Brazil | AY820006 | AM295175 | AJ280010 |
| CBS 111.26$^{T}$ | *A. carbonarius* | Paper, origin unknown | AY585532 | AJ964873 | DQ900605 |
| CBS 119384$^{T}$ | *A. coreanus* | Nuruk, Boun-up, Bounkun, Chungbuk Prov., Korea | FJ491693 | FJ4916702 | FJ491684 |
| CBS 115574$^{T}$ | *A. costaricaensis* | Soil, Taboga Island, Gauguin garden, Costa Rica | AY820014 | EU163268 | DQ900602 |
| CBS 707.79$^{T}$ | *A. ellipticus* | Soil, Costa Rica | AY585530 | AM117809 | AJ280014 |
| CBS 122712$^{T}$ | *A. eucalypticola* | *Eucalyptus* leaves, Australia | EU482435 | EU482433 | EU482439 |
| CBS 119.49 | *A. fijiensis* | *Lactuca sativa*, Palembang, Indonesia | FJ491689 | FJ491701 | FJ491679 |
| CBS 313.89$^{T}$ | *A. fijiensis* | Soil, Fiji | FJ491688 | FJ491695 | FJ491680 |
| CBS 121.28$^{NT}$ | *A. foetidus* | Awamori-koji alcoholic beverage, Ryuku island, Japan | FJ491690 | FJ491694 | FJ491683 |
| CBS 114.49$^{T}$ | *A. foetidus* | NRRL 341, Thom 5135.17; K. Yakoyama, Japan | EF661090 | EF661155 | EF661187 |
| CBS 117.55$^{T}$ | *A. heteromorphus* | Culture contaminant, Brazil | AY585529 | AM421461 | AJ280013 |
| CBS 101889$^{T}$ | *A. homomorphus* | Soil, near Dead Sea, Israel | AY820015 | AM887865 | EF166063 |
| CBS 121593$^{T}$ | *A. ibericus* | Grapes, Portugal | AM419748 | AJ971805 | AY656625 |
| CBS 114.80$^{T}$ | *A. indologenus* | Soil, India | AY585539 | AM419750 | AJ280005 |
| CBS 114.51$^{T}$ | *A. japonicus* | Saito 5087, origin unknown | AY585542 | AJ964875 | AJ279985 |
| CBS 101883$^{T}$ | *A. lacticoffeatus* | Coffee bean, South Sumatra, Indonesia | AY819998 | EU163270 | DQ900604 |
| CBS 115657 | *A. neoniger* | Desert sand, Namibia | FJ491692 | FJ491699 | FJ491681 |
| CBS 115656$^{T}$ | *A. neoniger* | Mangrove water, Mochima Bay, Venezuela | FJ491691 | FJ491700 | FJ491682 |
| CBS 554.65$^{T}$ | *A. niger* | Connecticut, USA | AY585536 | AJ964872 | AJ223852 |
| CBS 112811$^{T}$ | *A. piperis* | Black pepper, Denmark | AY820013 | EU163267 | DQ900603 |
| CBS 121057$^{T}$ | *A. sclerotiicarbonarius* | Robusta coffee bean, Thailand | EU159229 | EU159235 | EU159216 |
| CBS 127449$^{T}$ | *A. saccharolyticus* | under a toilet seat made of treated oak wood, Gentofte, Denmark | HM853553 | HM853554 | HM853552 |
| CBS 115572$^{T}$ | *A. sclerotioniger* | Coffee bean, Karnataka, India | AY819996 | EU163271 | DQ900606 |
| CBS 134.48$^{T}$ | *A. tubingensis* | Origin unknown | AY820007 | AJ964876 | AJ223853 |
| CBS 121591$^{T}$ | *A. uvarum* | Healthy Cisternino grape, Italy | AM745751 | AM745755 | AM745751 |
| CBS 113365$^{T}$ | *A. vadensis* | Origin unknown | AY585531 | EU163269 | AY585549 |
| CBS 115571 | *A. violaceofuscus* | Marine environment, Bahamas | EU482434 | EU482432 | EU482440 |
| CBS 123.27$^{NT}$ | *A. violaceofuscus* | Soil, Puerto Rico; Thom 3522.30 | FJ491685 | FJ491698 | FJ491678 |
| CBS 122.35 | *A. violaceofuscus* mut. *grisea* | WB 4880 | FJ491687 | FJ491696 | FJ491676 |
| CBS 102.23$^{T}$ | *A. violaceofuscus* | Received from D. Borrel, Strassbourg, France | FJ491686 | FJ491697 | FJ491677 |

$^{T}$ = Type, $^{NT}$ = Neotype

microscopic mounts were made in lactic acid from MEA colonies and a drop of alcohol was added to remove air bubbles and excess conidia.

## Extrolite analysis

The isolates were grown on CYA and YES at 25 °C for 7 d. Extrolites were extracted after incubation. Five plugs of each agar medium were taken and pooled together into same vial for extraction with 0.75 mL of a mixture of ethyl acetate/dichloromethane/methanol (3:2:1) (v/v/v) 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, 1993), with minor modifications as described by Smedsgaard (1997). The column used was a 50 x 2 mm Luna C-18 (II) reversed phase column (Phenomenex, Torrance, CA, USA) fitted with a 2 x 2 mm guard column. Standards of ochratoxin A and B, secalonic acid D, neoxaline and other extrolites from the collection at Department of Systems Biology-DTU were used to confirm the identity of the detected extrolites.

## Physiological analysis

*Aspergillus niger* and *A. awamori* isolates were analysed using various methods. Elastase activity was studied on Czapek Dox minimal medium without $NaNO_3$, with 0.05 % elastin (Sigma, St Louis, MO, USA) and 0.05 % Rose Bengal (Sigma, St Louis, MO, USA), buffered to pH 7.6 with NaOH (modified after Blanco *et al.* 2002). Conidial suspensions of *A. niger* and *A. awamori* strains were prepared from cultures grown on YPD agar slants. The suspensions were diluted in 1 mL bidistilled water. Plates were inoculated in a central spot with 20 µL of the conidium suspensions and were incubated for 7 d at 37 °C. The diameters of the halo of elastin lysis were measured. The experiment was repeated three times, and the average diameters were calculated for each strain. Statistical analysis was made using the R software package (http://

www.r-project.org/). The assumptions of ANOVA were tested using the diagnostic plots in R. According to Quantile-Quantile (QQ) Plot the data were not normally distributed, thus the Kruskal-Wallis test was applied to compare the average diameters between the two species.

Carbon source assimilation tests were performed on minimal medium (MM: 0.5 % $(NH_4)_2SO_4$, 0.1 % $KH_2PO_4$, 0.05 % $MgSO_4$, 2 % agar) with 0.2 % single carbon source. Conidial suspensions of eight *A. niger* and eight *A. awamori* strains were prepared from 5-d-old cultures grown on YPD agar slants. The suspensions were diluted in bidistilled water and conidia were filtered. An YPD plate was inoculated in 16 points with 15 µL of the conidium suspensions and was incubated for 3 d at 25 °C. Strains were replicated to the MM plates, which contained single carbon sources using a 16-pronged replicator. Plates were incubated for 7 d at 25 °C. The experiment was repeated twice and control series was made on MM plates without carbon source and YPD plates.

Thirty carbon sources were tested, which were selected based on previous carbon source utilisation experiments (Varga *et al.* 2000): glucose, D-xylose, galactose, D-lyxose, L-sorbose, L-rhamnose, lactose, eritrit, galactit, L-valine, L-β-phenilalanine, L-triptophane, L-treonine, L-serine, L-cysteine, L-asparagine acid, L-tyrosine, L-lysine, L-histidine, L-citrulline, cis-aconitic acid, vanillin, vanillin acid, L-ascorbic acid, D-glucoseamine, glycylglycine, salicin, pectin, melezitose, α-ketoglutaric acid.
Different growth patterns of the strains belonging to the two species were observed simply in the case of L-sorbose, so the test was extended to 2-deoxy-D-glucose because of the structural similarity of these two compounds.

## Genotypic analysis

The cultures used for the molecular studies were grown on malt peptone (MP) broth using 10 % (v/v) of malt extract (Oxoid) and 0.1 % (w/v) bacto peptone (Difco), 2 mL of medium in 15 mL tubes. The cultures were incubated at 25 °C for 7 d. DNA was extracted from the cells using the Masterpure™ yeast DNA purification kit (Epicentre Biotechnol.) according to the instructions of the manufacturer. The ITS region and parts of the β-tubulin and calmodulin genes were amplified and sequenced as described previously (Varga *et al.* 2007a–c).

Part of the FUM8 gene was amplified using primers vnF1 and vnR3 as described by Susca *et al.* (2010). Primer sets were also designed to target part of the chloroperoxidase gene of black aspergilli presumably taking part in ochratoxin biosynthesis. Construction of the primers was carried out by using the homologous sequences identified in the genomic sequences of *Aspegillus niger* CBS 513.88 and *Aspergillus carbonarius* ITEM 5010 isolates. The designed chloroperoxidase specific PCR primers were BCPOF (5'- CTGGGCGACTGCATCCAC – 3') and BCPOR (5'- TTCATCGTACGGCAGACGCT - 3') which generated specific amplicons of about 250 base pairs. Amplifications were performed on a PTC-0148 Mini48 thermocycler (BioRad, USA), using the following amplification steps: 4 min of initial denaturation at 94 °C followed by 35 amplification cycles of 20 s at 94 °C, 15 s at 62 °C and 30 s at 2 °C and a final extension step for 1 min at 72 °C.

DNA sequences were edited with the DNASTAR computer package and an alignment of the sequences and neighbour joining analyses were performed using the MEGA v. 4 software (Tamura *et al.* 2007). To determine the support for each clade, a bootstrap analysis was performed with 1 000 replications. *Aspergillus flavus* CBS 100927$^T$ was used as outgroup in these analyses.

Phylogenetic analysis of sequence data was also performed using PAUP* v. 4.0b10 (Swofford 2000). Alignment gaps were treated as fifth character state, parsimony uninformative characters were excluded and all characters were unordered and equal weight. Maximum parsimony analysis was performed for all data sets using the heuristic search option. To assess the robustness of the topology, 1 000 bootstrap replicates were run by maximum parsimony (Hillis & Bull 1993). Other measures including tree length, consistency index and retention index were also calculated. Sequences were deposited at GenBank under accession numbers listed in Table 1.

UP-PCR analyses were carried out according to Bulat *et al.* (2000). DNA was isolated as described in the literature (Leach *et al.* 1986). The primers used were L45, AS15inv, L15/AS19, AA2M2, L21, 3-2, AS4, AS15 (Lübeck *et al.* 1998, Bulat *et al.* 2000). The amplification process consisted of a predenaturation step for 1 min at 94 °C, followed by 35 cycles (30 s at 94 °C, 45 s at 55 °C, and 1 min at 72 °C), plus a final extension of 2 min at 72 °C. The amplification products were separated by electrophoresis in 1 % agarose gels, stained with ethidium bromide, and visualised under UV light. All amplifications were repeated at least two times. The faint bands which did not appear in all repeated experiments were not counted during cluster analysis.

Altogether 88 fragments were noted and a binomial matrix was created so that presence and absence of DNA fragments were scored as 1 or 0, respectively. Cluster analysis was carried out by using PHYLIP v. 3.67 software package (Felsenstein 2007). Phylogenetical tree was created by using neighbor-joining method (Saitou *et al.* 1987) with the program NEIGHBOR from the PHYLIP program package.

# RESULTS AND DISCUSSION

## Phylogenetic analysis of sequence data

The calmodulin data set consisted of 478 characters including 218 parsimony informative sites; MP analysis resulted in 33 most parsimonious trees (length = 621, consistency index = 0.620787, retention index = 0.873655), one of which is presented in Fig. 1. Of the aligned β-tubulin sequences, a portion of 468 positions including 221 parsimony informative characters was selected for the analysis; MP analysis of the sequence data resulted in 29 similar, equally most parsimonious trees (tree length = 464 steps, consistency index = 0.622172, retention index = 0.882394), one of which is shown in Fig. 2. The ITS data set consisted of 478 characters including 81 parsimony informative sites; MP analysis resulted in 211 equally most parsimonious trees (length = 143, consistency index = 0.837989, retention index = 0.957910), one of which is presented in Fig. 3.

All four species could be distinguished from the currently accepted species of *Aspergillus* section *Nigri* (Samson *et al.* 2007) based on either calmodulin or β-tubulin sequence data, and isolate CBS 114.80 also exhibited unique ITS sequences (Fig. 3). *Aspergillus eucalypticola* was found to be related to *A. costaricaensis*, however, their ITS sequences were different (Fig. 3). *Aspergillus violaceofuscus* and *A. indologenus* belonged to a clade including *A. uvarum* and *A. aculeatinus* on the tree based on β-tubulin sequence data (Fig. 1), and to a clade including *A. uvarum* and *A. japonicus* on the tree based on calmodulin sequence data (Fig. 2).

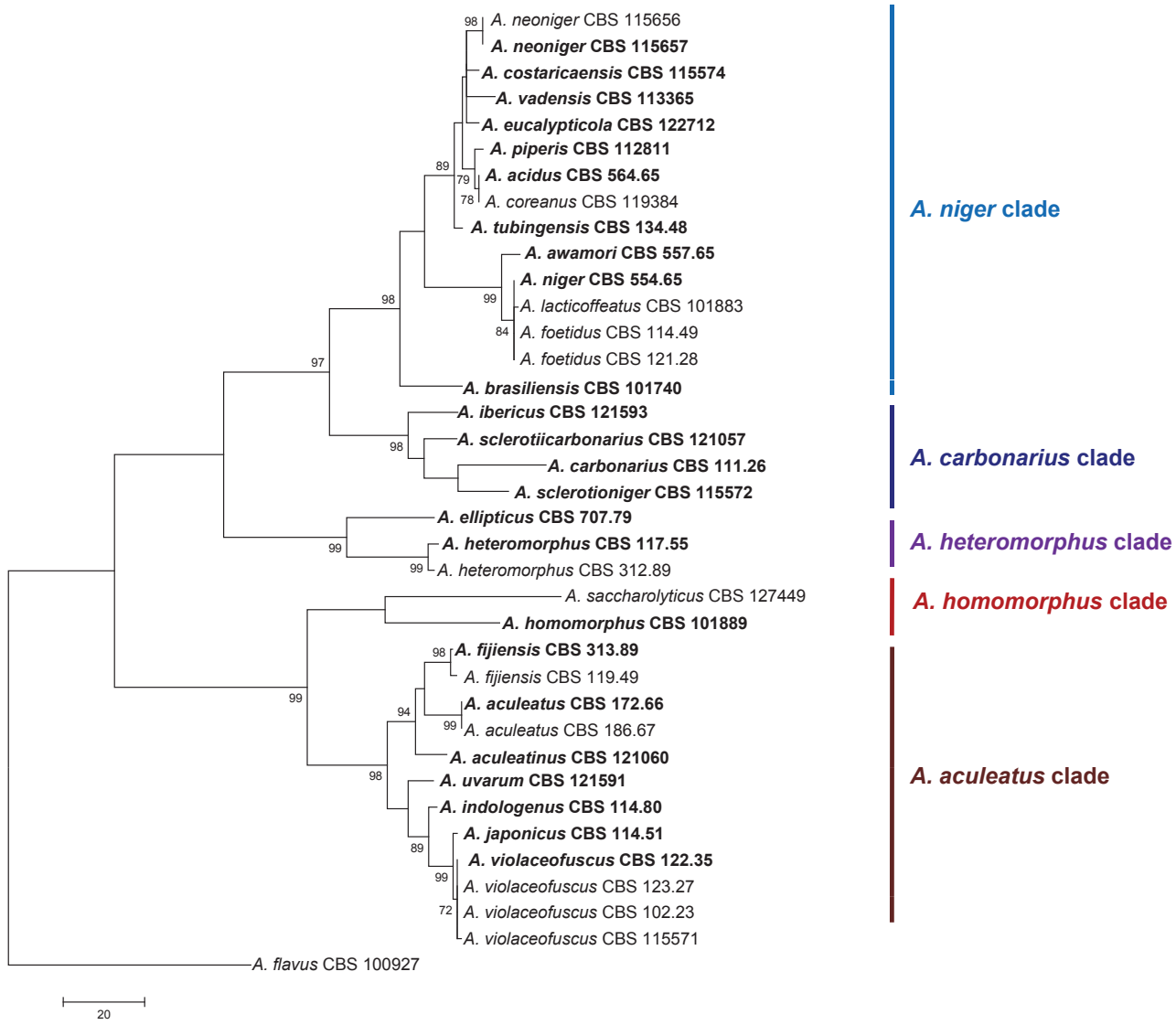



**Fig. 1.** One of the MP trees obtained based on phylogenetic analysis of calmodulin sequence data of *Aspergillus* section *Nigri*. Numbers above branches are bootstrap values. Only values above 70 % are indicated.

Sequence data also indicate that both the *A. foetidus* ex-type strain (CBS 114.49 = NRRL 341) and the neotype strain (CBS 121.28), and *A. lacticoffeatus* are synonyms of *A. niger*, while *A. coreanus* Yu *et al*. (2004) is a synonym of *A. foetidus* var. *acidus* (validly named *A. acidus* by Kozakiewicz 1989). *Aspergillus. lacticoffeatus* and *A. coreanus* differ from other black aspergilli in producing brownish conidia, and not producing naphtho-γ-pyrones. There appears to be a link between the black pigment and the naphtho-γ-pyrones via the gene PksA (Jørgensen *et al.* 2011, Chiang *et al.* 2011) Besides, *A. coreanus* also does not produce antafumicins, produced by some *A. acidus* isolates. *Aspergillus lacticoffeatus* also carries the fumonisin biosynthetic genes, similarly to *A. niger* (Meijer *et al.,* unpubl. data). Based on these observations, we consider *A. foetidus* and *A. lacticoffeatus* as synonyms of *A. niger*, and *A. coreanus* of *A. acidus*, respectively.

Our data indicate that *Aspergillus* section *Nigri* comprises 26 species including the new species *A. saccharolyticus* Sørensen *et al.* (2011). These taxa can be divided into five main clades (called series in Frisvad *et al.* 2007a; Fig. 1). The *A. niger* clade includes 10 biseriate species, and was divided into three subclades based on β-tubulin and calmodulin sequence data: the *A. tubingensis*, *A. niger* and *A. brasiliensis* subclades. Only *A. niger* is known to be able to produce ochratoxin A and fumonisins from this clade (Samson *et al.* 2004b, Frisvad *et al.* 2007b). Another main clade includes relatives of *A. carbonarius* (*A. ibericus, A. sclerotioniger* and *A. sclerotiicarbonarius*). These species series are characterised by relatively large conidia and two of them, *A. carbonarius* and *A. sclerotioniger* are able to produce ochratoxin A. *Aspergillus ellipticus* and *A. heteromorphus* form another clade, while the biseriate *A. homomorphus* forms a distinct clade. All uniseriate species belong to the *A. aculeatus* clade, involving seven species (Fig. 1).

## Extrolites

The extrolites produce by black *Aspergillus* species have been reviewed by Samson *et al.* (2007) and Nielsen *et al.* (2009). The new species described here produce a series of bioactive extrolites, especially the uniseriate species *A. indologenus* and *A. fijiensis*.

*Aspergillus eucalypticola* CBS 122712 produced pyranonigrin A, funalenone, aurasperone B and other naphtho-γ-pyrones, and an unknown extrolite ("MYC") in common with *A. neoniger* (CBS 115656 = IBT 20973 and CBS 115657 = IBT 23434). *Aspergillus neoniger* produced funalenone, naphtho-γ-pyrones, pyranonigrin

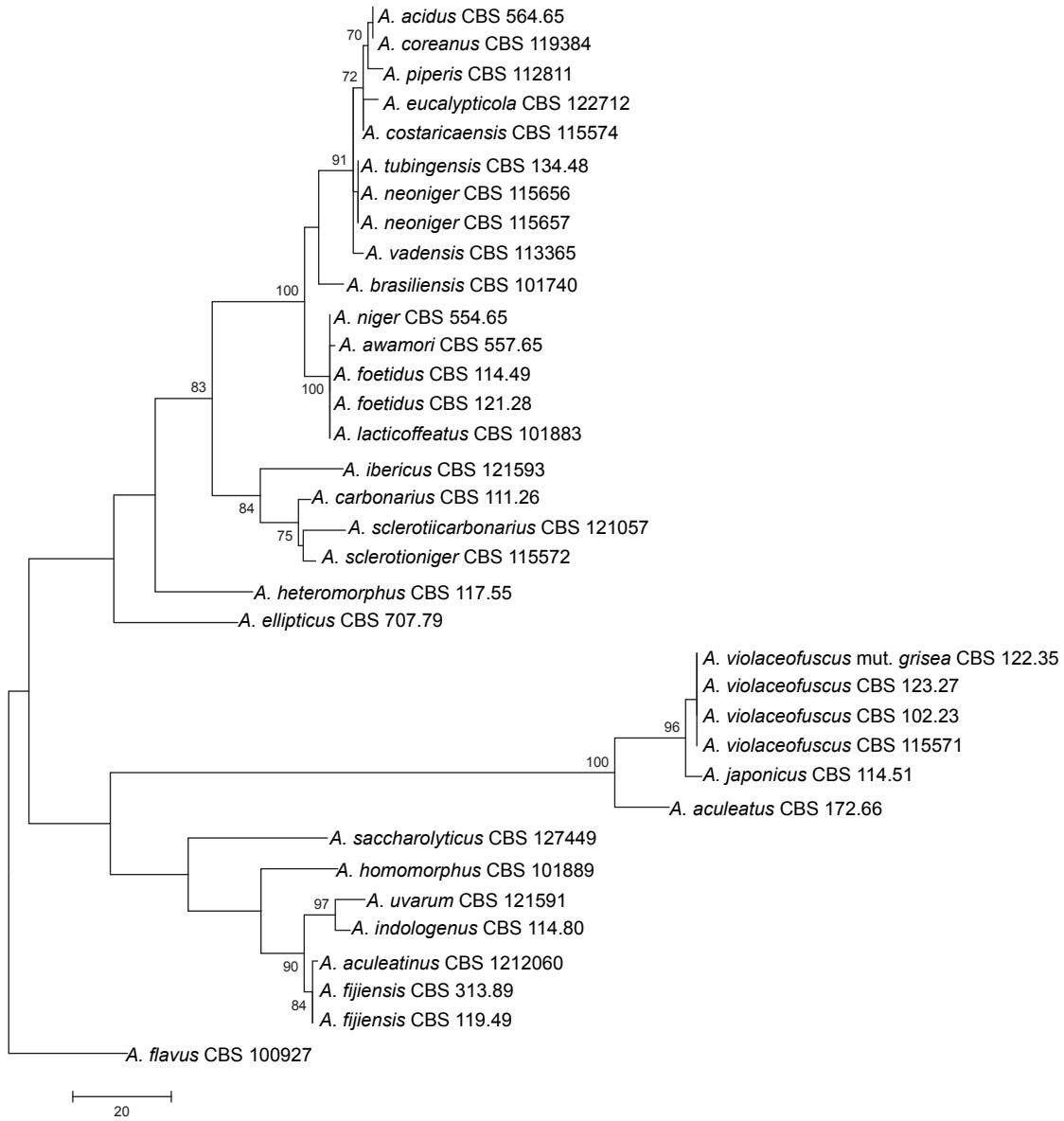



**Fig. 2.** The single MP tree obtained based on phylogenetic analysis of β-tubulin sequence data of *Aspergillus* section *Nigri.* Numbers above branches are bootstrap values. Only values above 70 % are indicated.

A and "MYC", and it is chemically very closely related to *A. eucalypticola*.

*Aspergillus indologenus* CBS 114.80 = IBT 3679 produced the insecticidal compounds okaramins A, B, H earlier also reported from an *A. aculeatus* isolate (Hayashi *et al.* 1999), partially characterised polar alkaloids, a series of very apolar sclerotial indol-alkaloids (related to aflavinins) and unique indol-alkaloids with similar UV spectra as the fumitremorgins. *Aspergillus violaceofuscus* produced some indol-alkaloids also found in *A. indologenus*, but it also produced several families of partially characterised extrolites that have also been found in *A. heteromorphus* ["SMIF", "PON", "SENGLAB" (a pyranonigrin-related compound), and yellow compounds with characteristic UV spectra]. *Aspergillus fijiensis* CBS 313.89 and CBS 119.49 produced asperparalins, secalonic acid D, F and the partially characterised "BAM", "PON" = "FIB1" & "FIB2", "GLABRINOL", "SENGLAB", and "YE1". CBS 313.89 in addition produced "DERH" and "YE2" and CBS 119.49 additionally produced neoxaline, and "TRU". Asperparaline A (= aspergillimide = VM55598), asperparaline B and C have earlier been reported from *Aspergillus japonicus* ATCC 204480 (Hayashi *et al.* 1997, 2000) and asperparaline A, 16-keto aspergillimide, VM54159, SB203105 and SB 200437 have been isolated from "a black *Aspergillus* with pink sclerotia" IMI 337664 (Banks *et al.* 1997) and neoxaline has been isolated from *A. japonicus* (Hirano *et al.* 1979). Based on the extrolite data, ATCC 204480 and IMI 337664 may indeed belong to *A. fijiensis*, but we have not examined these cultures yet.

Species related to *A. niger*, such as *A. eucalypticola* and *A. neoniger* and the well known species *A. carbonarius* and *A. tubingensis*, produce different combinations of pyranonigrins, tensidols, kotanins, fumonisins, funalenones, naphtho-γ-pyrones, ochratoxins, asperazines, and pyrophen (Samson *et al.* 2004b, 2007), while species related to *A. aculeatus*, *A. aculeatinus*, *A. japonicus*, *A. uvarum*, and the new species described here, *A. indologenus*, *A. fijiensis* and the revived *A. violaceofuscus* produce different combinations of asperparalins, okaramins, neoxaline, sclerotial indol-alkaloids, and secalonic acids (Parenicova *et al.* 2001, Samson *et al.* 2004b, Samson *et al.* 2007, Noonim *et al.* 2008).

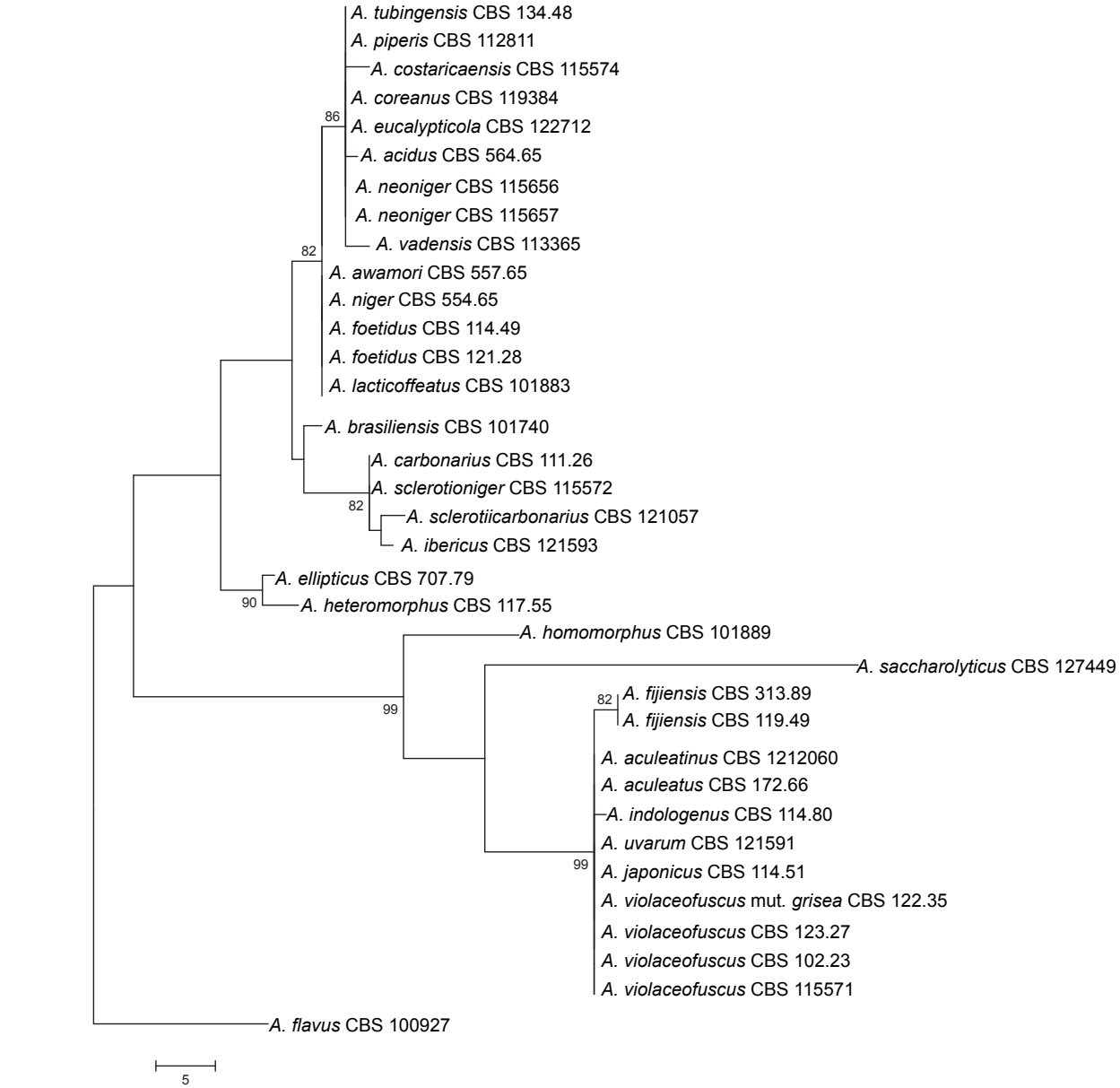
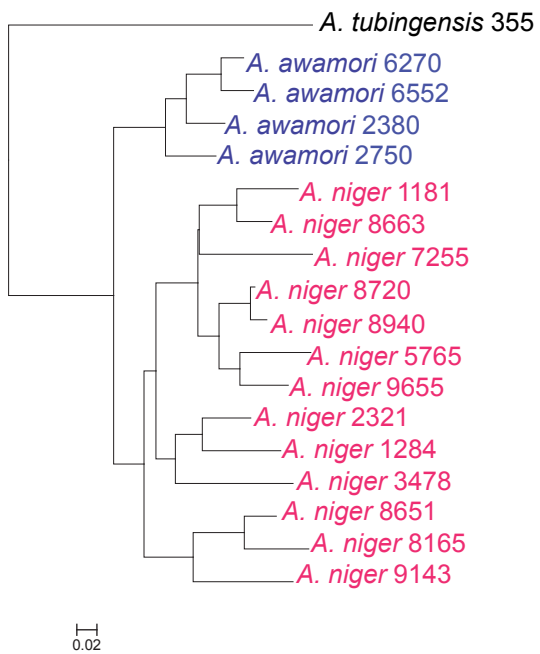



**Fig. 3.** One of the MP trees obtained based on phylogenetic analysis of ITS sequence data of *Aspergillus* section *Nigri*. Numbers above branches are bootstrap values. Only values above 70 % are indicated.




## Approaches to distinguish between isolates of the sibling species *A. niger* and *A. awamori*

*Aspergillus awamori* has recently been revalidated as a cryptic species within the *A. niger* species (Perrone *et al.* 2011). These species cannot be reliably separated from each other using either morphological or extrolite data. However, molecular data including sequence-based approaches using either β-tubulin, calmodulin or translation elongation factor a sequences and AFLP analysis were found to be useful for distinguishing these species (Perrone *et al.* 2011). *Aspergillus niger* and *A. awamori* are economically important as isolates of both species are able to produce fumonisins and/or ochratoxins (Varga *et al.* 2010, Perrone *et al.* 2011). In view of the importance of these species in mycotoxin contamination of various agricultural products (see below), we examined other possibilities which could be used for the easy identification of these species.

**Fig. 4.** Cluster analysis of *A. niger* and *A. awamori* isolates based on UP-PCR profiles.

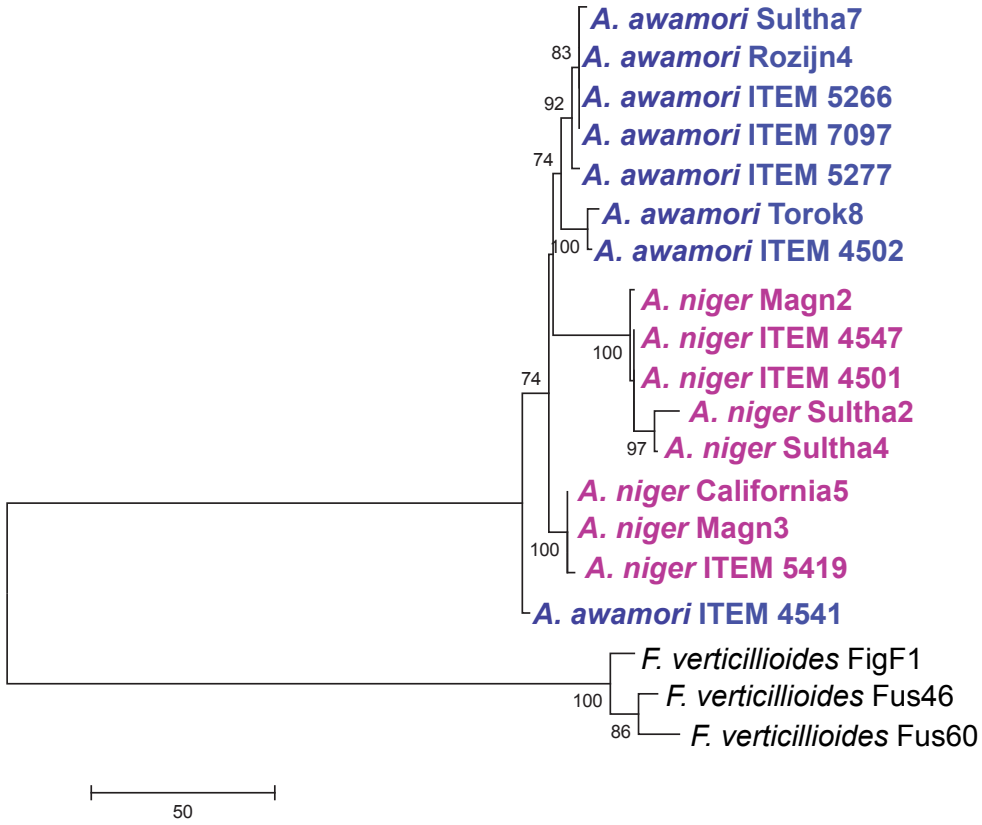



**Fig. 5.** One of the 73 MP trees obtained based on phylogenetic analysis of FUM8 sequence data of *A. niger* and *A. awamori* isolates (tree length: 411, consistency index: 0.961240, retention index: 0.980843). Numbers above branches are bootstrap values. Only values above 70 % are indicated.

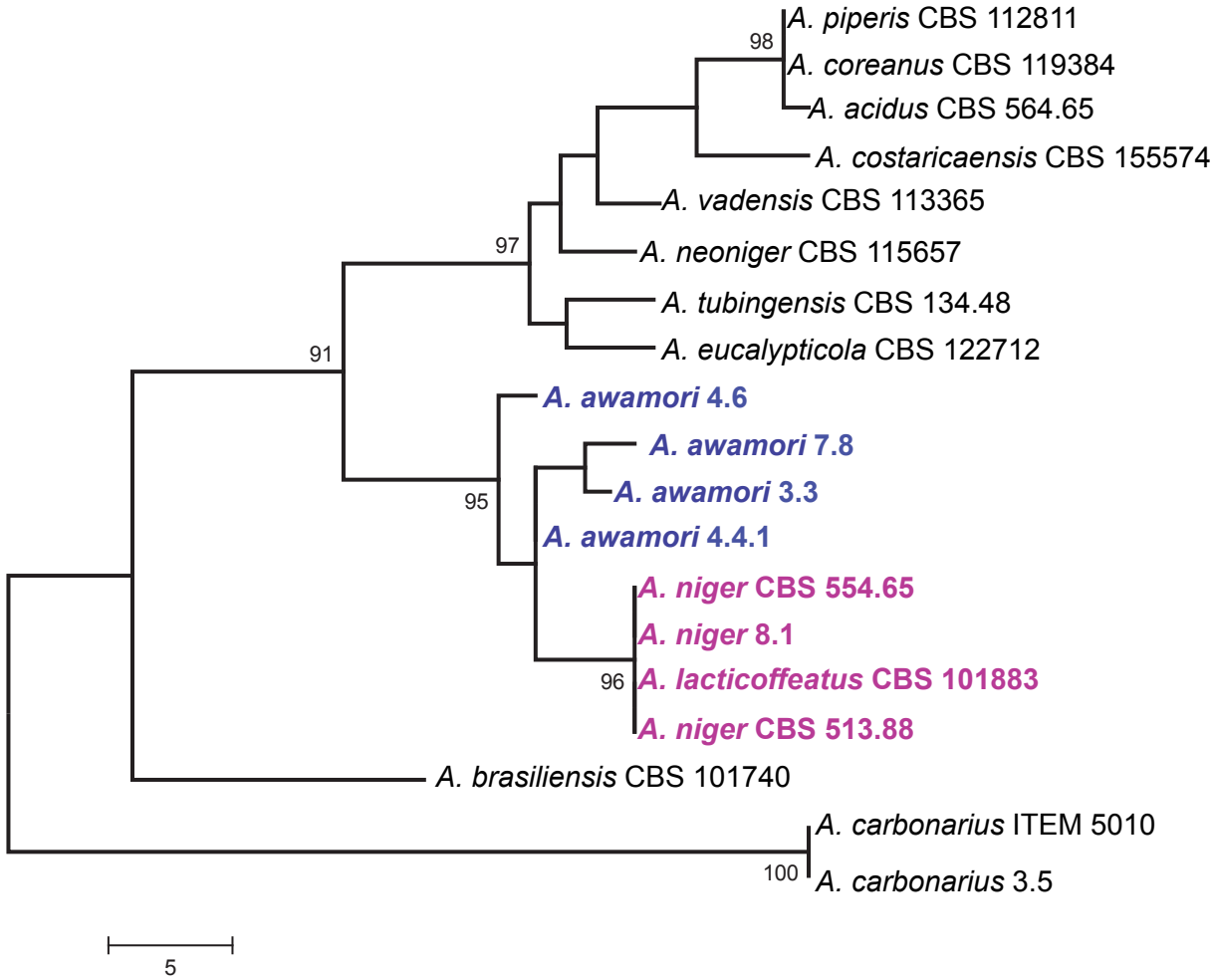



**Fig. 6.** One of the 153 MP trees obtained based on phylogenetic analysis of chloroperoxidase sequence data of *A. niger* and *A. awamori* isolates (tree length: 113, consistency index: 0.767677, retention index: 0.886700). Numbers above branches are bootstrap values. Only values above 70 % are indicated.

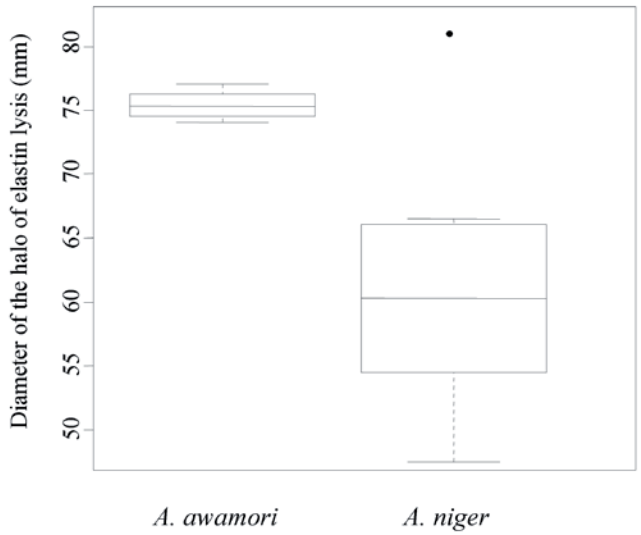



**Fig. 7.** The difference of elastin lysis between the isolates belonging to *A. niger* and *A. awamori*.

## Molecular approaches

UP-PCR analysis (Bulat *et al.* 2000) was found to be also useful for species delineation (Fig. 4). This technique is similar to RAPD, but is more reliable as it uses higher annealing temperatures and longer primers (Bulat *et al.* 2000). Besides, UP-PCR analysis is easier to perform than AFLP analysis. Similarly to AFLP analysis (Perrone *et al.* 2011), this technique could also be used successfully to separate the examined *A. niger* and *A. awamori* isolates into two clusters (Fig. 4).

Another possibility is the application of mitochondrial DNA RFLP analyses. This technique was previously used to assign isolates of the *A. niger* species aggregate to different haplotypes (Varga *et al.* 1993, 1994). Our study revealed that one of these types previously called mtDNA type 1c actually corresponds to *A. awamori* (data not shown).

Attempts have also been made to use sequences of mycotoxin biosynthetic genes for distinguishing *A. niger* from *A. awamori*. Susca *et al.* (2010) examined the presence of FUM8 encoding an a-oxoamine synthase in black aspergilli came from grapes. They found no strict correlation between the phylogenetic trees based on sequences of partial calmodulin gene and FUM8 (Fig. 5). Similar results were found in our laboratory using sequences of either FUM8, or another fumonisin biosynthetic gene, FUM1, encoding for a polyketide synthase taking part in fumonisin biosynthesis (Varga *et al.,* unpubl. data). It was suggested that, similarly to that observed in the trichothecene biosynthesis gene cluster of the *Fusarium graminearum* species complex, balancing selection could be responsible for maintaining sequence polymorphisms within the fumonisin gene cluster (Ward *et al.* 2002, Susca *et al.* 2010).

The applicability of another mycotoxin biosynthetic gene, a chloroperoxidase gene presumably taking part in ochratoxin biosynthesis was also examined for distinguishing *A. niger* and *A. awamori*. This gene has been found to take part in ochratoxin biosynthesis in *Penicillium verrucosum* and *P. nordicum* (Geisen 2007). Homologues of these genes were identified in the full genome sequences of *A. niger* and *A. carbonarius*, and primers were designed to amplify orthologues in species assigned to the *A. niger* species complex. Phylogenetic analysis of the sequence data indicate that sequences of a chloroperoxidase gene are useful for species delineation in the *A. niger* species aggregate (Fig. 6). *Aspergillus niger* and *A. awamori* could also be distinguished based on their chloroperoxidase sequences.

## Morphological and physiological approaches

Molecular methods are commonly used today for species identification among fungi. However, in accordance with the polyphasic species concept, other criteria have also been searched for. *Aspergillus niger* and *A. awamori* cannot be distinguished based on morphology alone. Regarding extrolite production, isolates of both species produce several metabolites in common including the mycotoxins ochratoxin A and fumonisin $B_2$, and they also share the production of pyranonigrin A, tensidol B, funalenone, malformins and naphtho-γ-pyrones. The growth rates of the isolates of these species are also similar at different temperatures (Varga *et al.*, unpubl. data).

Carbon source utilisation tests revealed that *A. niger* and *A. awamori* has very similar utilisation spectra (data not shown). Different growth of the strains belonging to the two species was observed only in the case of L-sorbose: *A. awamori* strains grew less intensively on this sugar than *A. niger* strains. Consequently, the test was extended to 2-deoxy-D-glucose because of the structural similarity of these two compounds. Of the 30 isolates examined, 13 of the examined *A. niger* isolates grew well, while 13 of the 15 examined *A. awamori* isolates failed to grow on 2-deoxy-D-glucose as sole carbon source (data not shown). Microscopical analysis of the colonies indicated that conidial germination was inhibited in the case of *A. awamori* isolates (data not shown). Furthermore 2-deoxy-D-glucose was earlier found to inhibit conidium germination in *Penicillium expansum* (Kazi *et al.* 1997).

Antifungal susceptibilities of the isolates has also been examined using five antifungal drugs including amphotericin B, fluconazole, itraconazole, ketoconazole and terbinafine (Szigeti *et al.* 2011). Species-specific differences were not observed between *A. niger* and *A. awamori* isolates. All isolates were highly susceptible to terbinafine, while exhibited moderate susceptibilities against amphotericin B, fluconazole and ketoconazole. However, in general, *A. niger* and *A. awamori* were found to have higher MICs for azoles than *A. tubingensis* (Szigeti *et al.* 2011).

Elastase production is treated as a virulence factor in *Aspergillus fumigatus*, contributing to the invasiveness of the fungus during infection (Denning *et al.* 1993, Kolattukudy *et al.* 1993, Blanco *et al.* 2002, Garcia *et al.* 2006). Elastase activities of the isolates were tested according to the method of Blanco *et al.* (2002). The data revealed that *A. awamori* isolates in general exhibit higher elastase activities in a much narrower range than *A. niger* (Fig. 7). We found significant difference between the elastase activities of the isolates belonging to the two species ($\chi^2$ = 8.017; p=0.0046; Kruskal-Wallis test). In general *A. awamori* isolates showed more intensive elastin lysis than *A. niger* isolates, although an outlier was found in the case of an *A. niger* strain, which exhibited very high elastase activities (Fig. 7). The high elastase activity of this isolate could be due to simple mutations as has been proposed for *A. fumigatus* recently (Alvarez-Perez *et al.* 2010).

## Ecology

Several differences have been found recently regarding the distribution of *A. niger* and *A. awamori* is various habitats. Both species have been found in various ratios in indoor air in various buildings in Southern Hungary and Serbia, although *A. awamori* was more frequently encountered in Serbia than in Hungary (Varga

J., unpubl. data). Similarly, both species were present on dried vine fruits collected in various countries (Varga *et al.* 2010). However, neither *A. niger* nor *A. awamori* could be isolated from pistachio nuts from Iran (Sedaghati *et al.* 2011), nor from dates from Iran and Tunesia (Varga J., unpubl. data). *Aspergillus awamori* was found to be the predominant black *Aspergillus* species on onions cultivated in Hungary, and is presumably the causative agent of black mold rot in this country (Varga *et al.,* unpubl. data), and was also found to be the causative agent of seed rot of *Welwitschia mirabilis* in Namibia (Varga *et al*., unpubl. data). However, *A. awamori* was not detected on figs from Tunesia, Turkey and Iran (Varga J., unpubl. data). Instead, *A. tubingensis* and *A. niger* were found to contaminate these fig samples.

Regarding clinical significance, both species have been identified as causative agents of otomycosis in Iran and in Hungary, although at different frequencies, with *A. niger* being the dominant species in Iran, while *A. awamori* was most frequently identified in Hungary (Szigeti *et al.* 2011, unpubl. data).

In conclusion, *A. niger* and *A. awamori* are two very closely related species which seem to be in the course of speciation, similarly to the recently described species of *Fusarium graminearum sensu lato* (Ward *et al.* 2002, Starkey *et al.* 2007). Although these species differ in their occurrence on various substrates and several physiological characteristics (elastase activities, abilities to utilise 2-deoxy-D-glucose as sole carbon source), our data indicate that only molecular approaches including sequence analysis of calmodulin or β-tubulin genes, AFLP analysis, UP-PCR analysis or mtDNA RFLP analysis can be used reliably to distinguish these sibling species.

## Species descriptions

***Aspergillus acidus*** Kozak. Mycol. Pap. 161: 110 (1989) Fig. 8.

Culture ex-type: IMI 104688 = CBS 564.65, Japan, Nakazawa, 1936.

CYA, 7 d, 25 °C: 37–80 mm; MEA, 7 d, 25 °C: 43–68 mm; YES, 7 d, 25 °C: 38–80 mm; OA, 7 d, 25 °C: 38–55 mm; CYA, 7 d, 37 °C: 30–67 mm; CREA: poor growth but good acid production; CYAS: 16–69 mm (strong sporulation on all media, except CREA). Colony reverse colour on CYA: cream yellow, reverse colour on YES: yellow to cream yellow.

Conidiophores biseriate with globose vesicles 55–80 µm, stipe smooth–walled to finely roughened, hyaline, 17–22 µm. Conidia globose, 3–4 µm, brown, smooth-walled to roughened. Sclerotia not observed.

Kozakiewics (1989) proposed the name *A. acidus* for the variety *acidus* of *A. foetidus* on the basis on the verrucose conidium ornamentation as seen by scanning electron microscopy. This variety was recognised by Raper & Fennell (1965) as a variety of *A. citricus*. Al-Musallan (1980) however, could not distinguish this variety from the *A. niger* aggregate.

*Aspergillus acidus* seems to be the dominant black *Aspergillus* species on tea leaves (Mogensen *et al.* 2009), and has also been identified in human aspergillosis cases (Alcazar-Fuoli *et al.* 2009). *Aspergillus coreanus,* isolated from Korean fermented nuruk, was invalidly described because a Latin diagnosis was lacking. The ex-type strain of this species is morphologically different of *A. acidus*, because it was described with yellow green colonies. We observed that the colonies were light yellow brown.

***Aspergillus eucalypticola*** Varga, Frisvad & Samson, **sp. nov.** MycoBank MB560387. Fig. 9.

Conidiophoris biseriatis, vesiculis globosis, 30–55 µm diam, stipitibus levibus vel subtiliter exasperatis. hyalinis, 8–14 µm latis. Conidiis globosis, 2.5–3.5 µm, brunneis, levibus vel grosse exasperatis. Sclerotia nulla.

Typus: ex leaves of *Eucalyptus* sp., New South Wales, Australia, isolated by P.W. Crous, 2007. (CBS H-20627 -- holotypus, culture ex-type CBS 122712 = IBT 29274).

CYA, 7 d, 25 °C: 68–72 mm; MEA, 7 d, 25 °C: 46–51 mm; YES, 7 d, 25 °C: 70–80 mm; OA, 7 d, 25 °C: 45–50 mm; CYA, 7 d, 37 °C: 30–50 mm; CREA: poor growth but good acid production; CYAS: 50–54 mm (strong sporulation on all media, except CREA). Colony reverse colour on CYA: beige to cream yellow, reverse colour on YES: yellow.

Conidiophores biseriate with globose vesicles 30–55 µm, stipe smooth-walled to finely roughened, hyaline, 8–14 µm. Conidia globose, 2.5–3.5 µm, brown, smooth-walled to coarsely roughened. Sclerotia not observed.

*Aspergillus eucalypticola* was isolated from an *Eucalyptus* leaf from Australia, and resembles morphologically *A. tubingensis* and *A. costaricaensis*. It can be distinguished from these two taxa by the β-tubulin or calmodulin sequence data. *Aspergillus eucalypticola* produces pyranonigrin A, funalenone, aurasperone B and other naphtho-γ-pyrones.

***Aspergillus fijiensis*** Varga, Frisvad & Samson, **sp. nov.** MycoBank MB560388. Fig. 10.

Conidiophoris uniseriatis, vesiculis globosis vel ellipsoideis, 35–70 µm diam, stipitibus levibus vel subtiliter exasperatis, hyalinis, 8–12 µm latis. Conidiis ellipsoideis vel leniter fusiformibus, 3–3.5 × 3.4–4 µm, brunneis, grosse exasperatis vel echinulatis. Sclerotia nulla.

Typus: ex soil, Fiji Islands, K. Bundgaard. (CBS H-20628 -- holotypus, culture ex-type CBS 313.89 = IBT 13989).

Additional isolate: CBS 119.49 = IBT 4580, ex *Lactuca sativa*, Palembang, Indonesia).

CYA, 7 d, 25 °C: 71–78 mm; MEA, 7 d, 25 °C: 46–57 mm; YES, 7 d, 25 °C: 74–80 mm; OA, 7 d, 25 °C: 46–56 mm; CYA, 7 d, 37 °C: 12–25 mm; CREA: poor growth but moderate acid production; CYAS: 52–57 mm (strong sporulation on all media, except CREA). Colony reverse colour on CYA: beige to yellow, reverse colour on YES: yellow.

Conidiophores uniseriate with globose to ellipsoidal vesicles 35–70 µm wide, stipe smooth-walled to finely roughened, hyaline, 8–12 µm. Conidia ellipsoidal to slightly fusiform, 3–3.5 × 3.4–4 µm, brown, coarsely roughened to echinulate. Sclerotia not observed.

*Aspergillus fijiensis* is characterised by uniseriate conidial heads and is related to *A. aculeatinus*. It was isolated from soil in Fiji, and from *Lactuca sativa* in Indonesia. This species is able to grow at 37 °C, and produces asperparalines and okaramins.

***Aspergillus indologenus*** Frisvad, Varga & Samson, **sp. nov.** MycoBank MB560389. Fig. 11.

Conidiophoris uniseriatis, vesiculis ellipsoideis, 20–45 µm diam, stipitibus levibus vel subtiliter exasperatis, hyalinis, 5–11 µm latis. Conidiis globosis, 3–4 µm diam, brunneis, grosse exasperatis vel echinulatis. Sclerotia nulla.

Typus: ex soil India (CBS H-20629 -- holotypus, culture ex-type CBS 114.80 = IBT 3679).

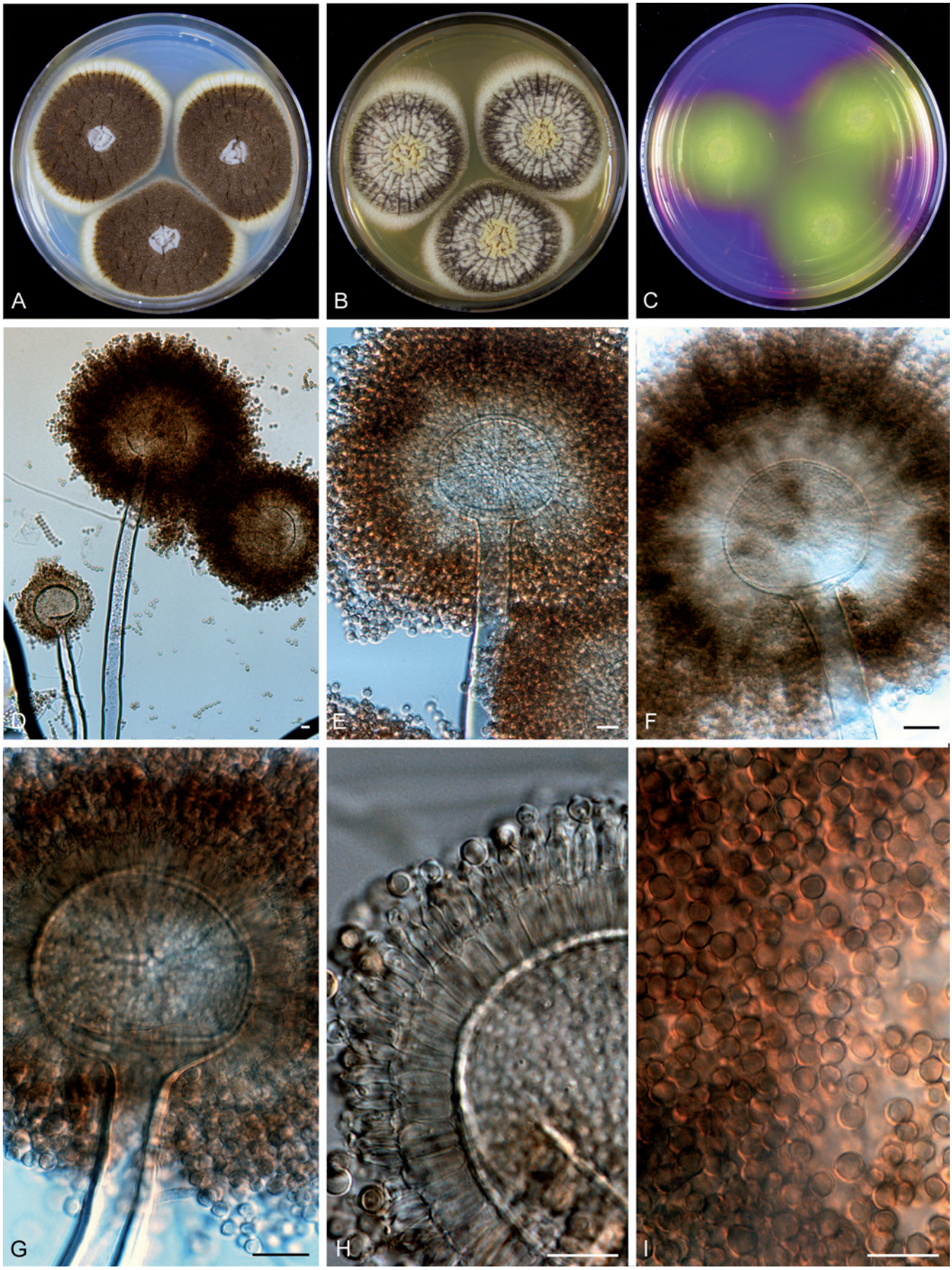



**Fig. 8.** *Aspergillus acidus* A–C. Colonies incubated at 25 °C for 7 d, A. CYA, B. MEA, C. CREA, D–I. Conidiophores and conidia. Scale bars = 10 µm.


**Fig. 9.** *Aspergillus eucalypticola* sp. nov. A–C. Colonies incubated at 25 °C for 7 d, A. CYA, B. MEA, C. CREA, D–I. Conidiophores and conidia. Scale bars = 10 µm.

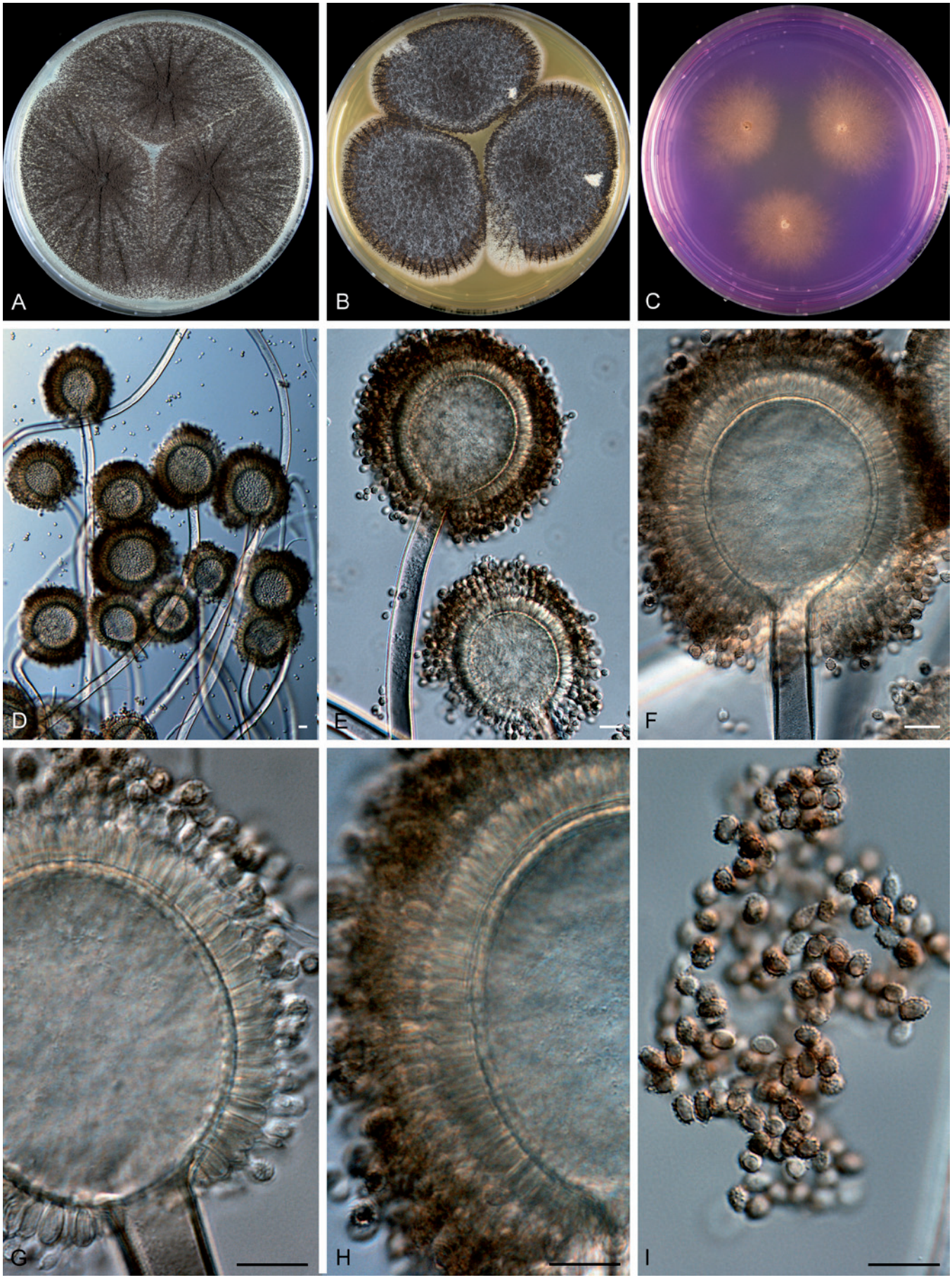



**Fig. 10.** *Aspergillus fijiensis* sp. nov. A–C. Colonies incubated at 25 °C for 7 d, A. CYA, B. MEA, C. CREA, D–I. Conidiophores and conidia. Scale bars = 10 µm.

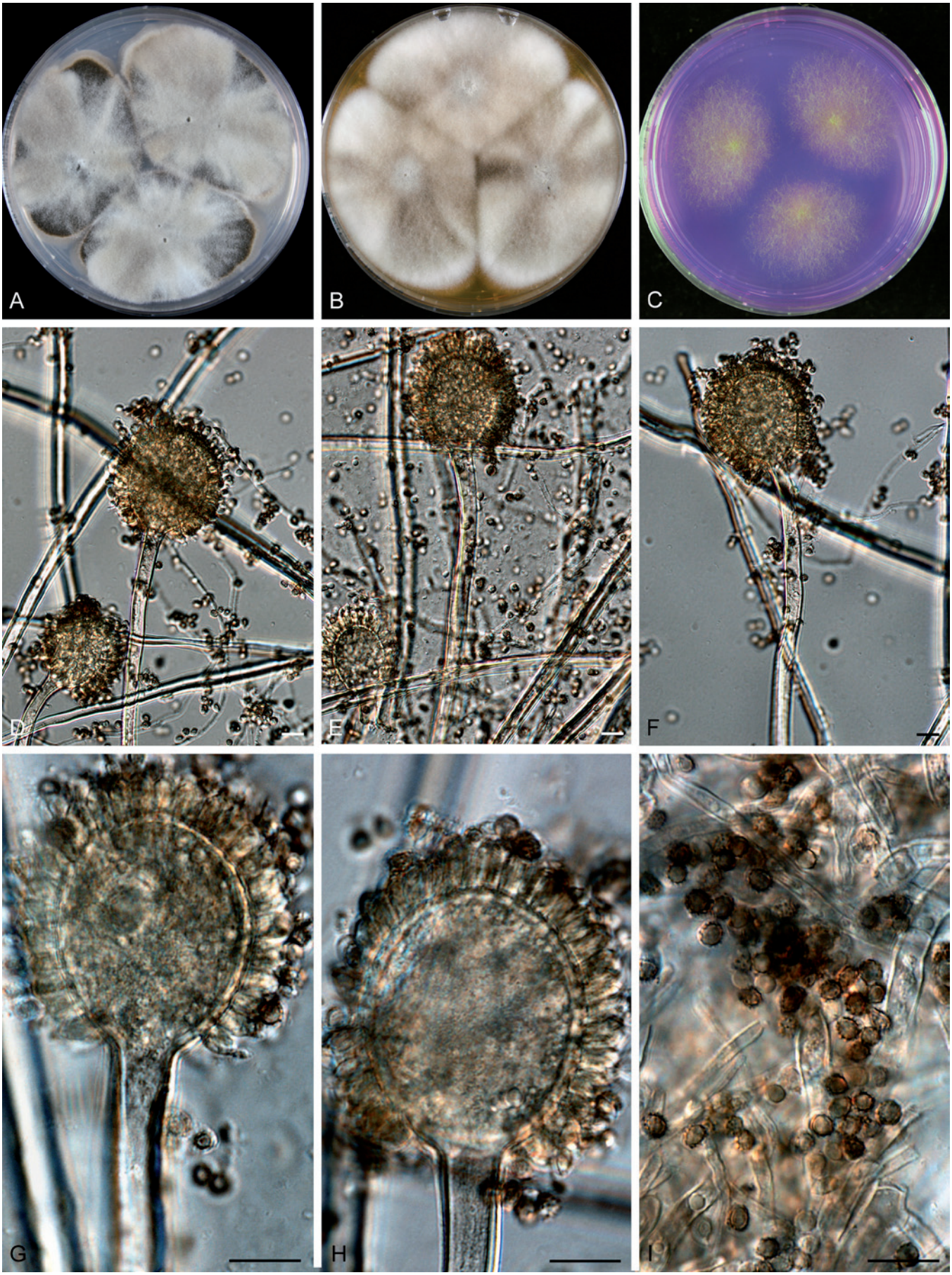



**Fig. 11.** *Aspergillus indologenus* sp. nov. A–C. Colonies incubated at 25 °C for 7 d, A. CYA, B. MEA, C. CREA, D–I. Conidiophores and conidia. Scale bars = 10 µm.

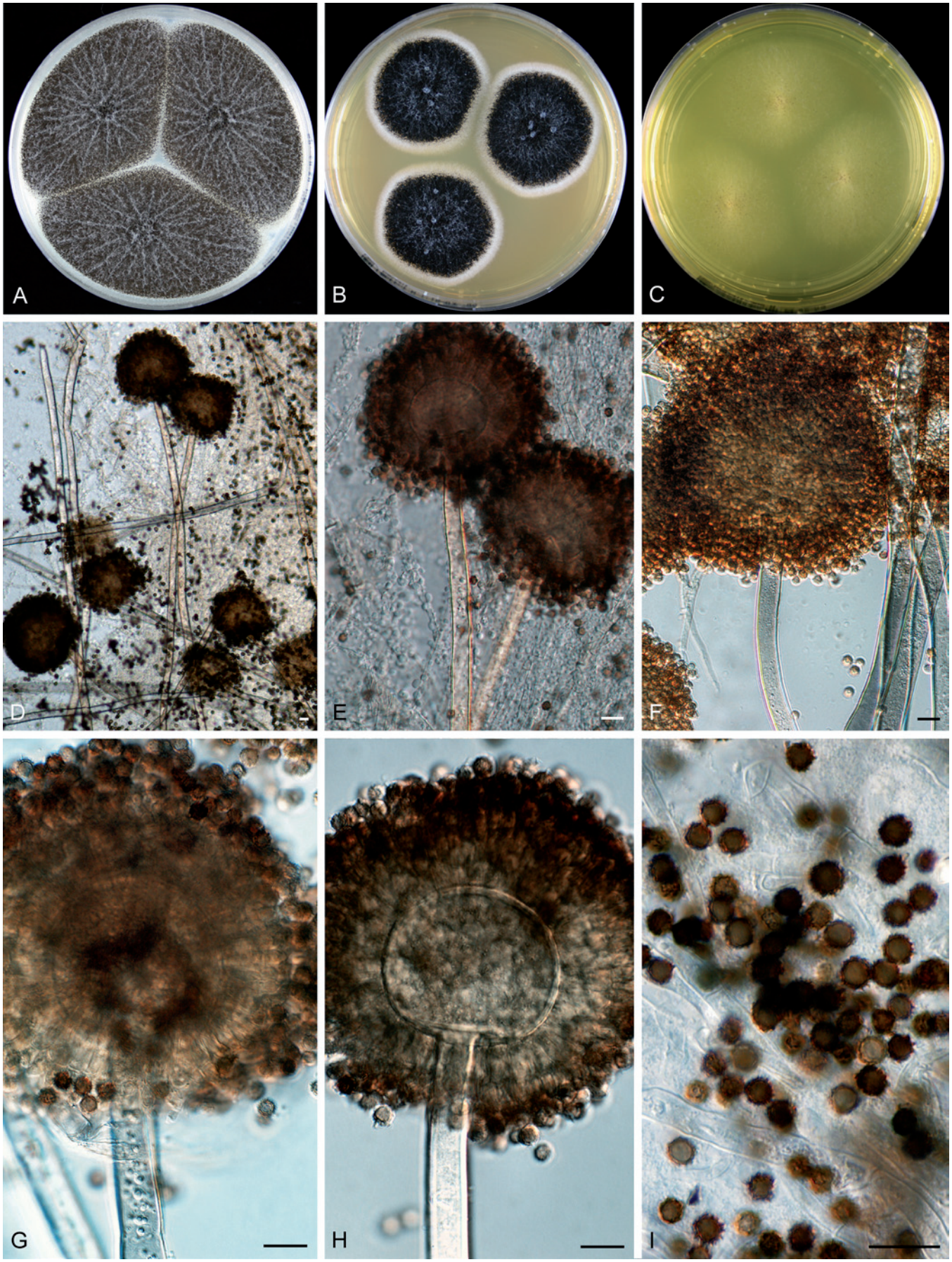



**Fig. 12.** *Aspergillus neoniger* sp. nov. A–C. Colonies incubated at 25 °C for 7 d, A. CYA, B. MEA, C. CREA, D–I. Conidiophores and conidia. Scale bars = 10 µm.

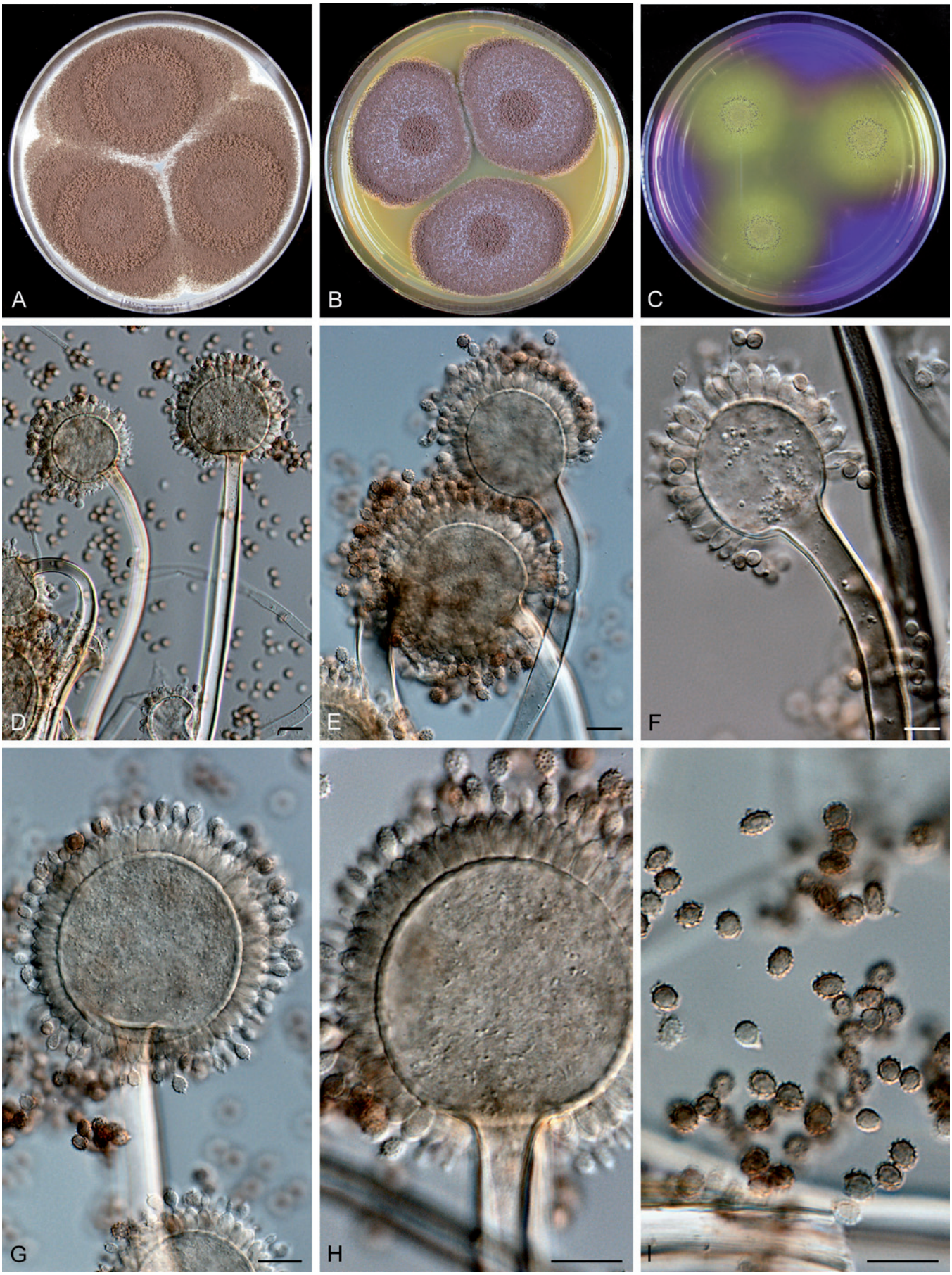



**Fig. 13.** *Aspergillus violaceofuscus* A–C. Colonies incubated at 25 °C for 7 d, A. CYA, B. MEA, C. CREA, D–I. Conidiophores and conidia. Scale bars = 10 µm.

CYA, 7 d, 25 °C: 63–70 mm; MEA, 7 d, 25 °C: 57–70 mm; YES, 7 d, 25 °C: 76–80 mm; OA, 7 d, 25 °C: 50–58 mm; CYA, 7 d, 37 °C: 20–33 mm; CREA: poor growth and no acid production; CYAS: 1–2 mm (strong sporulation on all media, except CYAS and CREA). Colony reverse colour on CYA: dark brown, reverse colour on YES: brown.

Conidiophores uniseriate with ellipsoidal vesicles 20–45 µm wide, stipe smooth-walled to finely roughened, hyaline, 5–11 µm. Conidia globose, 3–4 µm, brown, coarsely roughened to echinulate. Sclerotia not observed.

*Aspergillus indologenus* has uniseriate conidial heads and is related to, but clearly distinguishable from *A. uvarum* based on β-tubulin, calmodulin and ITS sequence data. *Aspergillus uvarum* has typical echinulate conida, while *A. indologenus* has conidia which are coarsely roughenmed to echinulate. *Aspergillus indologenus* produced the insecticidal compounds okaramins A, B, H, and two types of indol-alkaloids which have not been structure elucidated.

***Aspergillus neoniger*** Varga, Frisvad & Samson, **sp. nov.** MycoBank MB560390. Fig. 12.

Conidiophoris biseriatis, vesiculis globosis, 30–50 µm diam, stipitibus levibus vel subtiliter exasperatis, hyalinis, 8–12 µm. Conidiis globosis, 3.5–5 µm diam, brunneis, grosse exasperatis vel echinulatis. Sclerotia nulla.

Typus: ex *Verongia* species (sulphur sponge, Porifera), Morro of Garrapatá, Mochima Bay, Venezuela, isolated by E.K. Lyhne, 1997. (CBS H-20630 -- holotypus, culture ex-type CBS 115656 = IBT 20973 = Ven97 M64).

Other isolate: CBS 115657, ex desert soil, Namibia.

CYA, 7 d, 25 °C: 72–80 mm; MEA, 7 d, 25 °C: 54–61 mm; YES, 7 d, 25 °C: 74–80 mm; OA, 7 d, 25 °C: 50–72 mm; CYA, 7 d, 37 °C: 37–67 mm; CREA: poor growth but good acid production; CYAS: 50–54 mm (strong sporulation on all media, except CREA). Colony reverse colour on CYA: beige to cream yellow, reverse colour on YES: yellow.

Conidiophores biseriate with globose vesicles 30–50 µm, stipe smooth-walled to finely roughened, hyaline, 8–12 µm. Conidia globose, 3.5–5 µm, brown, coarsely roughened to echinulate. Sclerotia not observed.

*Aspergillus neoniger* is also a biseriate species isolated from desert sand in Namibia, and mangrove water in Venezuela. Morphologicaly it resembles *A. niger* and *A. tubingensis* and produces aurasperone B and pyranonigrin A. This species has also been identified in desert soil close to *Welwitschia mirabilis*, Namibia (Varga *et al.,* unpubl. data).

***Aspergillus violaceofuscus*** Gasperini, Atti della Societa Toscana di Scienze Naturali 2: 326 (1887). Fig. 13.

Culture ex-neotype: CBS 123.27 = Thom 3522.30, ex soil, Puerto Rico, deposited by M.B. Church.

CYA, 7 d, 25 °C: 50–77 mm; MEA, 7 d, 25 °C: 64–74 mm; YES, 7 d, 25 °C: 74–80 mm; OA, 7 d, 25 °C: 36–74 mm; CYA, 7 d, 37 °C: 0 mm (but 28–32 mm in CBS 122.35); CREA: poor growth and no acid production; CYAS: 0 mm (but 46–50 mm in CBS 115571) (strong sporulation on all media, except CYAS and CREA). Colony reverse colour on CYA: brown to cream yellow, reverse colour on YES: cream yellow.

Conidiophores uniseriate with globose vesicles 10–18 µm wide; stipe sometimes bent, smooth-walled to finely roughened, hyaline, 2.5–5 µm. Conidia ellipsoidal to slightly fusiform, 3.5–4 × 4–5.5 µm, brown, coarsely roughened to echinulate. Sclerotia not observed.

Raper & Fennell (1965) considered *A. violaceofuscus* as a possible synonym of *A. aculeatus*. In our study we have observed that the neotype strain is phylogenetically different from *A. aculeatus. Aspergilus violaceofuscus* has typical ellipsoidal to fusiform conidia, which are coarsely roughened to echinulate.

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