

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. costaricensis*, 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 biseriata 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 micromorphological 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	<i>A. acidus</i>	Unknown substratum, Japan	AY585533	AY585533	AJ280009
CBS 121060 ^T	<i>A. aculeatinus</i>	Arabica green coffee bean, Thailand	EU159220	EU159241	EU159211
CBS 172.66 ^T	<i>A. aculeatus</i>	Tropical soil, unknown origin	AY585540	AJ964877	AJ279988
CBS 557.65 ^T	<i>A. awamori</i>	NRRL 4948 = WB 4948	AY820001	AJ964874	AM087614
CBS 101740 ^T	<i>A. brasiliensis</i>	Soil, Sao Paulo, Pedreira, Brazil	AY820006	AM295175	AJ280010
CBS 111.26 ^T	<i>A. carbonarius</i>	Paper, origin unknown	AY585532	AJ964873	DQ900605
CBS 119384 ^T	<i>A. coreanus</i>	Nuruk, Boun-up, Bounkun, Chungbuk Prov., Korea	FJ491693	FJ4916702	FJ491684
CBS 115574 ^T	<i>A. costaricaensis</i>	Soil, Taboga Island, Gauguin garden, Costa Rica	AY820014	EU163268	DQ900602
CBS 707.79 ^T	<i>A. ellipticus</i>	Soil, Costa Rica	AY585530	AM117809	AJ280014
CBS 122712 ^T	<i>A. eucalypticola</i>	<i>Eucalyptus</i> leaves, Australia	EU482435	EU482433	EU482439
CBS 119.49	<i>A. fijjensis</i>	<i>Lactuca sativa</i> , Palembang, Indonesia	FJ491689	FJ491701	FJ491679
CBS 313.89 ^T	<i>A. fijjensis</i>	Soil, Fiji	FJ491688	FJ491695	FJ491680
CBS 121.28 ^{NT}	<i>A. foetidus</i>	Awamori-koji alcoholic beverage, Ryuku island, Japan	FJ491690	FJ491694	FJ491683
CBS 114.49 ^T	<i>A. foetidus</i>	NRRL 341, Thom 5135.17; K. Yakoyama, Japan	EF661090	EF661155	EF661187
CBS 117.55 ^T	<i>A. heteromorphus</i>	Culture contaminant, Brazil	AY585529	AM421461	AJ280013
CBS 101889 ^T	<i>A. homomorphus</i>	Soil, near Dead Sea, Israel	AY820015	AM887865	EF166063
CBS 121593 ^T	<i>A. ibericus</i>	Grapes, Portugal	AM419748	AJ971805	AY656625
CBS 114.80 ^T	<i>A. indologenus</i>	Soil, India	AY585539	AM419750	AJ280005
CBS 114.51 ^T	<i>A. japonicus</i>	Saito 5087, origin unknown	AY585542	AJ964875	AJ279985
CBS 101883 ^T	<i>A. lacticoffeatus</i>	Coffee bean, South Sumatra, Indonesia	AY819998	EU163270	DQ900604
CBS 115657	<i>A. neoniger</i>	Desert sand, Namibia	FJ491692	FJ491699	FJ491681
CBS 115656 ^T	<i>A. neoniger</i>	Mangrove water, Mochima Bay, Venezuela	FJ491691	FJ491700	FJ491682
CBS 554.65 ^T	<i>A. niger</i>	Connecticut, USA	AY585536	AJ964872	AJ223852
CBS 112811 ^T	<i>A. piperis</i>	Black pepper, Denmark	AY820013	EU163267	DQ900603
CBS 121057 ^T	<i>A. sclerotiiicarbonarius</i>	Robusta coffee bean, Thailand	EU159229	EU159235	EU159216
CBS 127449 ^T	<i>A. saccharolyticus</i>	under a toilet seat made of treated oak wood, Gentofte, Denmark	HM853553	HM853554	HM853552
CBS 115572 ^T	<i>A. sclerotioniger</i>	Coffee bean, Karnataka, India	AY819996	EU163271	DQ900606
CBS 134.48 ^T	<i>A. tubingensis</i>	Origin unknown	AY820007	AJ964876	AJ223853
CBS 121591 ^T	<i>A. uvarum</i>	Healthy Cisternino grape, Italy	AM745751	AM745755	AM745751
CBS 113365 ^T	<i>A. vadensis</i>	Origin unknown	AY585531	EU163269	AY585549
CBS 115571	<i>A. violaceofuscus</i>	Marine environment, Bahamas	EU482434	EU482432	EU482440
CBS 123.27 ^{NT}	<i>A. violaceofuscus</i>	Soil, Puerto Rico; Thom 3522.30	FJ491685	FJ491698	FJ491678
CBS 122.35	<i>A. violaceofuscus</i> mut. <i>grisea</i>	WB 4880	FJ491687	FJ491696	FJ491676
CBS 102.23 ^T	<i>A. violaceofuscus</i>	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.

Extrrolite analysis

The isolates were grown on CYA and YES at 25 °C for 7 d. Extrrolites 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, secalonin acid D, neoxaline and other extrrolites from the collection at Department

of Systems Biology-DTU were used to confirm the identity of the detected extrrolites.

Physiological analysis

Aspergillus niger and *A. awamori* isolates were analysed using various methods. Elastase activity was studied on Czapek Dox minimal medium without NaNO₃, 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 μ L 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 % $(\text{NH}_4)_2\text{SO}_4$, 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, erythrit, galactit, L-valine, L- β -phenylalanine, L-tryptophane, 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, glycyglycine, 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 *Aspergillus 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 pre-denaturation 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). Phylogenetic 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).

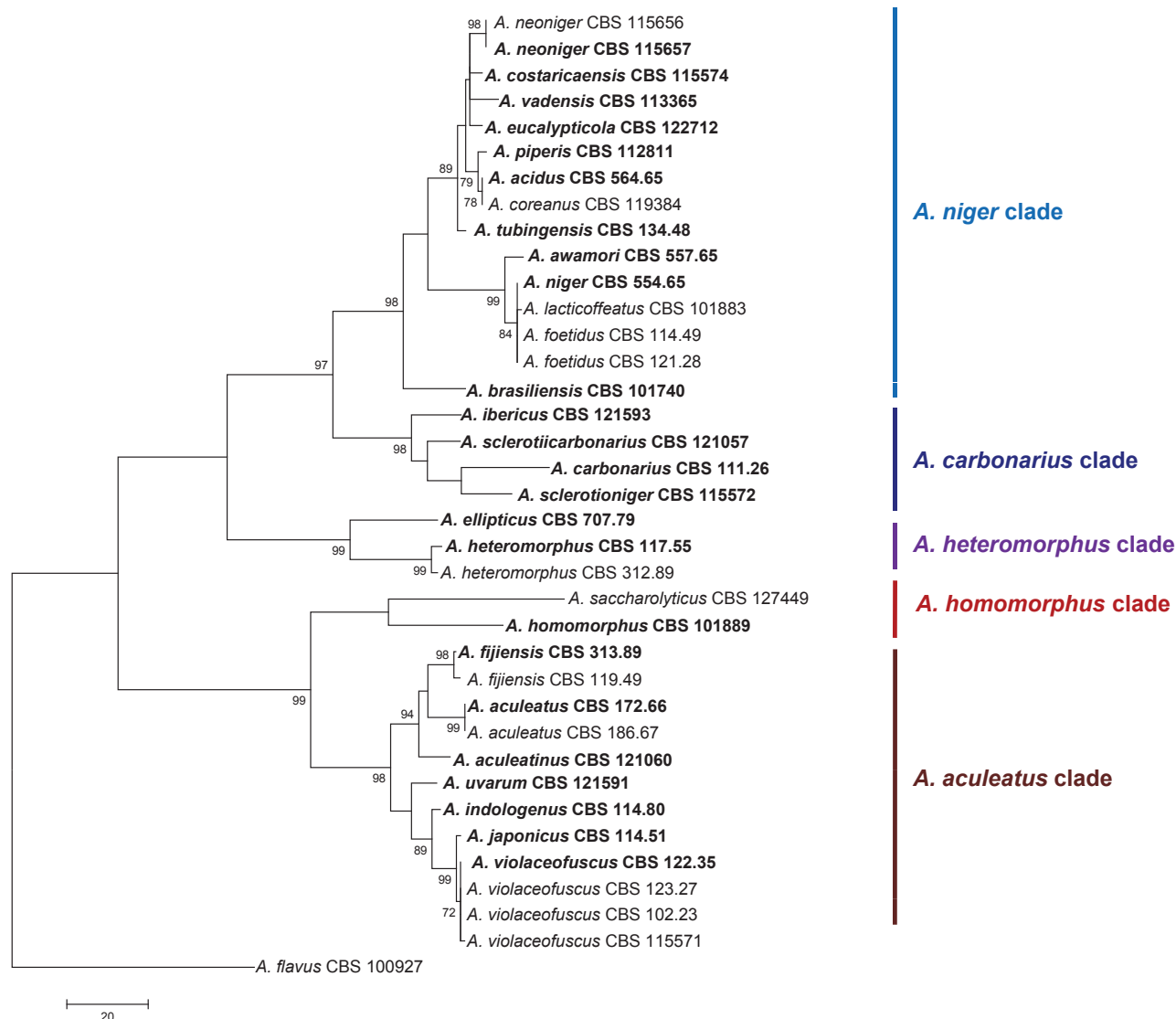


Fig. 1. One of the MP trees obtained based on phylogenetic analysis of calmodulin sequence data of *Aspergillus* section *Nigri*. Numbers above branches are bootstrap values. Only values above 70 % are indicated.

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 antaformins, 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 biseriata 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. sclerotii carbonarius*). 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 biseriata *A. homomorphus* forms a distinct clade. All uniseriate species belong to the *A. aculeatus* clade, involving seven species (Fig. 1).

Extrolites

The extrolites produced 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

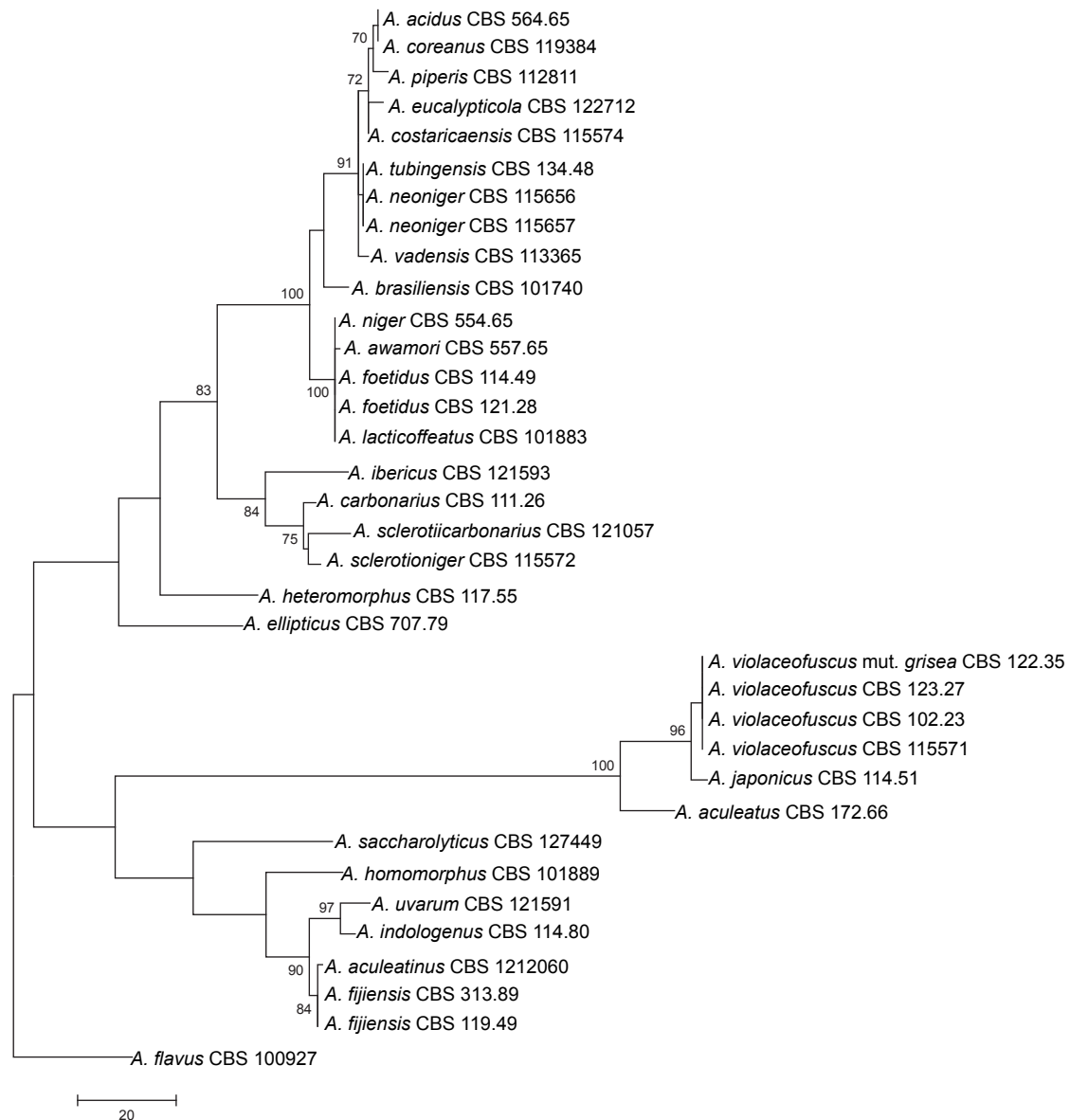


Fig. 2. The single MP tree obtained based on phylogenetic analysis of β -tubulin sequence data of *Aspergillus* section *Nigri*. Numbers above branches are bootstrap values. Only values above 70 % are indicated.

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. turingensis*, 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).

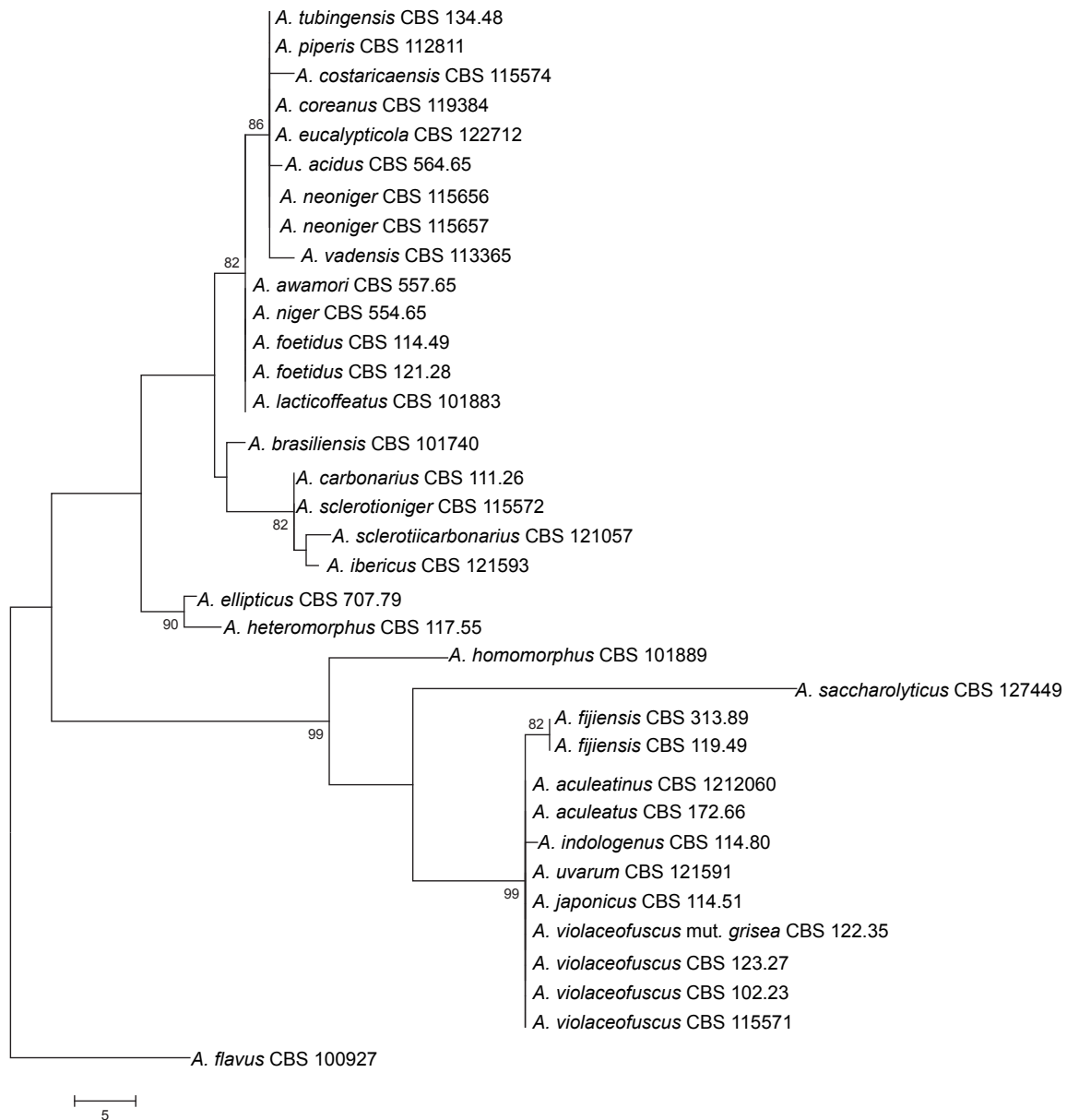
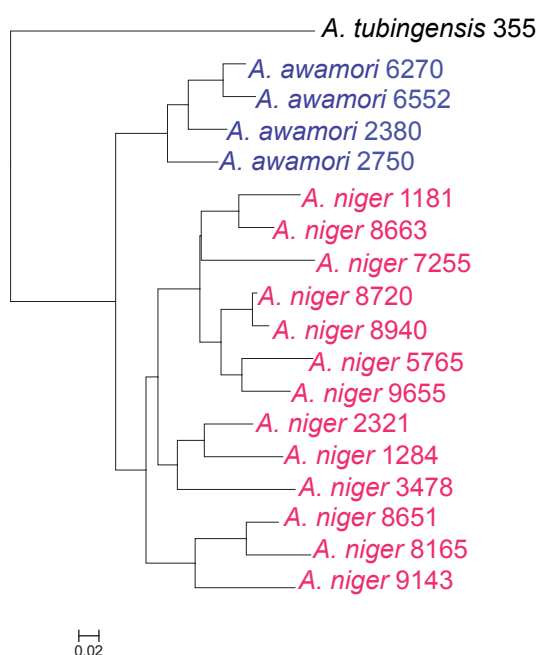


Fig. 3. One of the MP trees obtained based on phylogenetic analysis of ITS sequence data of *Aspergillus* section *Nigri*. Numbers above branches are bootstrap values. Only values above 70 % are indicated.



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.

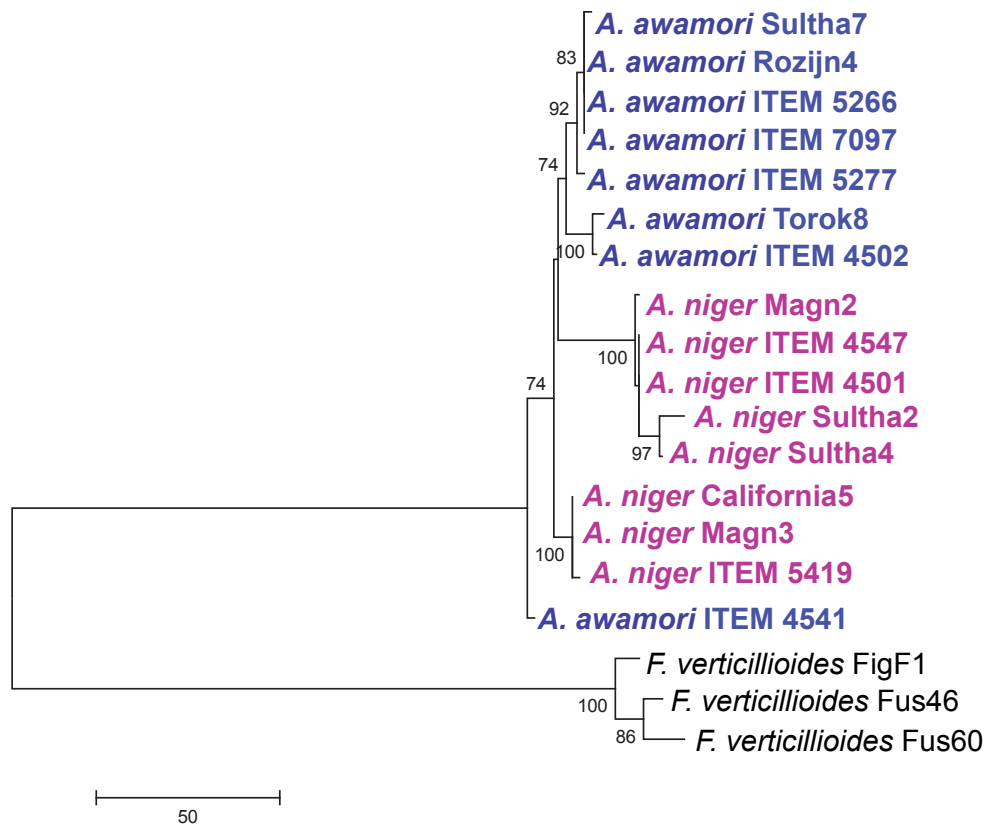


Fig. 5. One of the 73 MP trees obtained based on phylogenetic analysis of FUM8 sequence data of *A. niger* and *A. awamori* isolates (tree length: 411, consistency index: 0.961240, retention index: 0.980843). Numbers above branches are bootstrap values. Only values above 70 % are indicated.

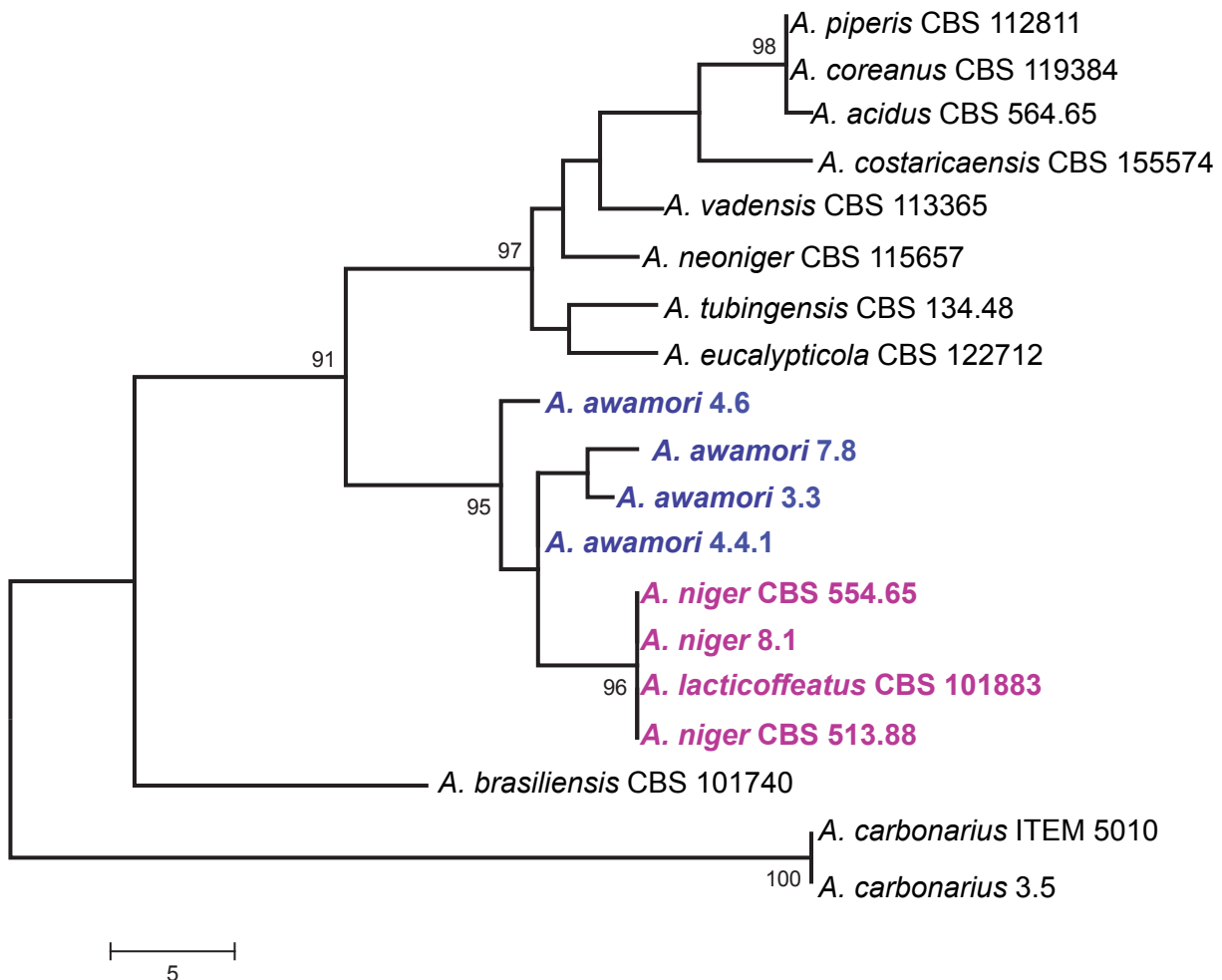


Fig. 6. One of the 153 MP trees obtained based on phylogenetic analysis of chloroperoxidase sequence data of *A. niger* and *A. awamori* isolates (tree length: 113, consistency index: 0.767677, retention index: 0.886700). Numbers above branches are bootstrap values. Only values above 70 % are indicated.

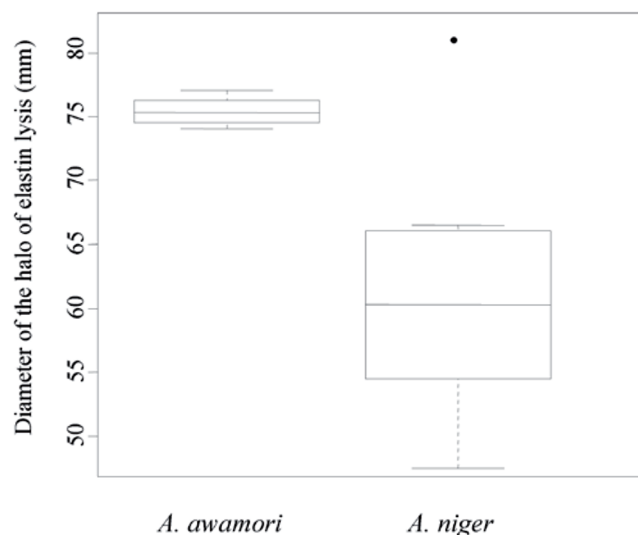


Fig. 7. The difference of elastin lysis between the isolates belonging to *A. niger* and *A. awamori*.

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 α -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 B₂, 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* in 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 Tunisia (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 Tunisia, 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 μ m, stipe smooth-walled to finely roughened, hyaline, 17–22 μ m. Conidia globose, 3–4 μ m, brown, smooth-walled to roughened. Sclerotia not observed.

Kozakiewicz (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 biseriatas, vesiculis globosis, 30–55 μ m diam, stipitibus levibus vel subtiliter exasperatis, hyalinis, 8–14 μ m latis. Conidiis globosis, 2.5–3.5 μ m, 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 μ m, stipe smooth-walled to finely roughened, hyaline, 8–14 μ m. Conidia globose, 2.5–3.5 μ m, 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. costaricensis*. 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 uniseriatas, vesiculis globosis vel ellipsoideis, 35–70 μ m diam, stipitibus levibus vel subtiliter exasperatis, hyalinis, 8–12 μ m latis. Conidiis ellipsoideis vel leniter fusiformibus, 3–3.5 \times 3.4–4 μ m, 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 μ m wide, stipe smooth-walled to finely roughened, hyaline, 8–12 μ m. Conidia ellipsoidal to slightly fusiform, 3–3.5 \times 3.4–4 μ m, 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 uniseriatas, vesiculis ellipsoideis, 20–45 μ m diam, stipitibus levibus vel subtiliter exasperatis, hyalinis, 5–11 μ m latis. Conidiis globosis, 3–4 μ m diam, brunneis, grosse exasperatis vel echinulatis. Sclerotia nulla.

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

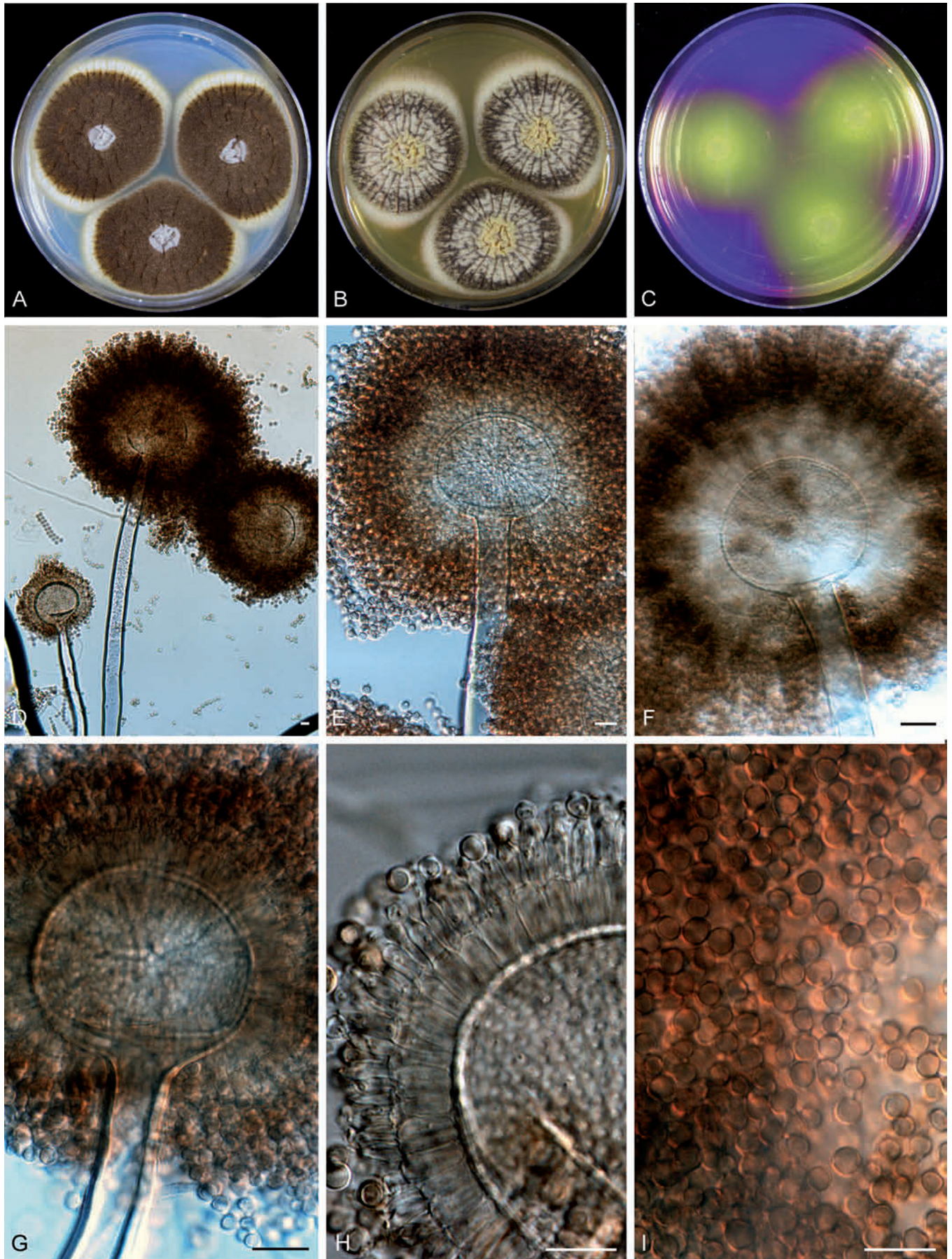


Fig. 8. *Aspergillus acidus* A–C. Colonies incubated at 25 °C for 7 d, A. CYA, B. MEA, C. CREA, D–I. Conidiophores and conidia. Scale bars = 10 μ m.

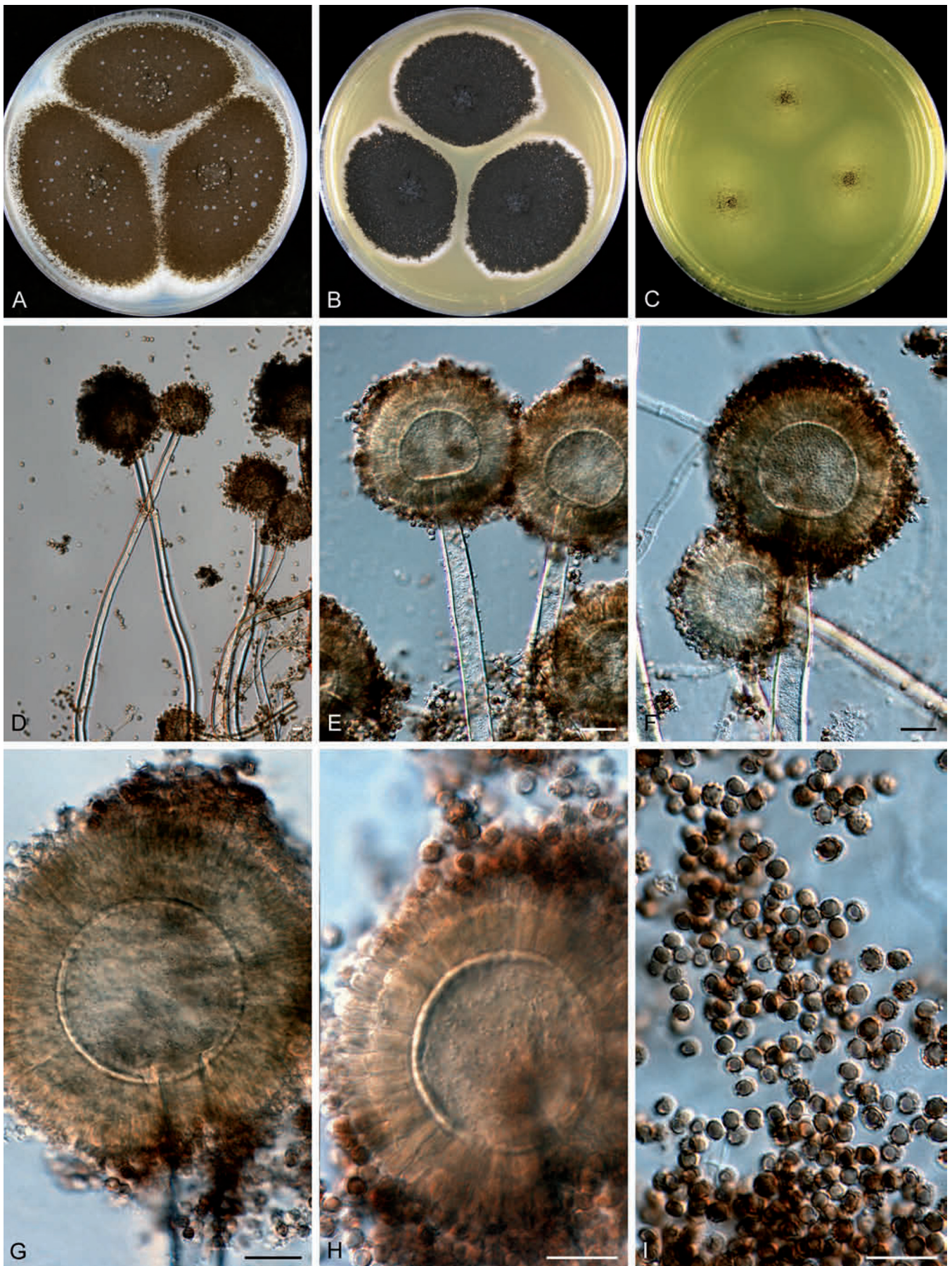


Fig. 9. *Aspergillus eucalypticola* sp. nov. A–C. Colonies incubated at 25 °C for 7 d, A. CYA, B. MEA, C. CREA, D–I. Conidiophores and conidia. Scale bars = 10 µm.

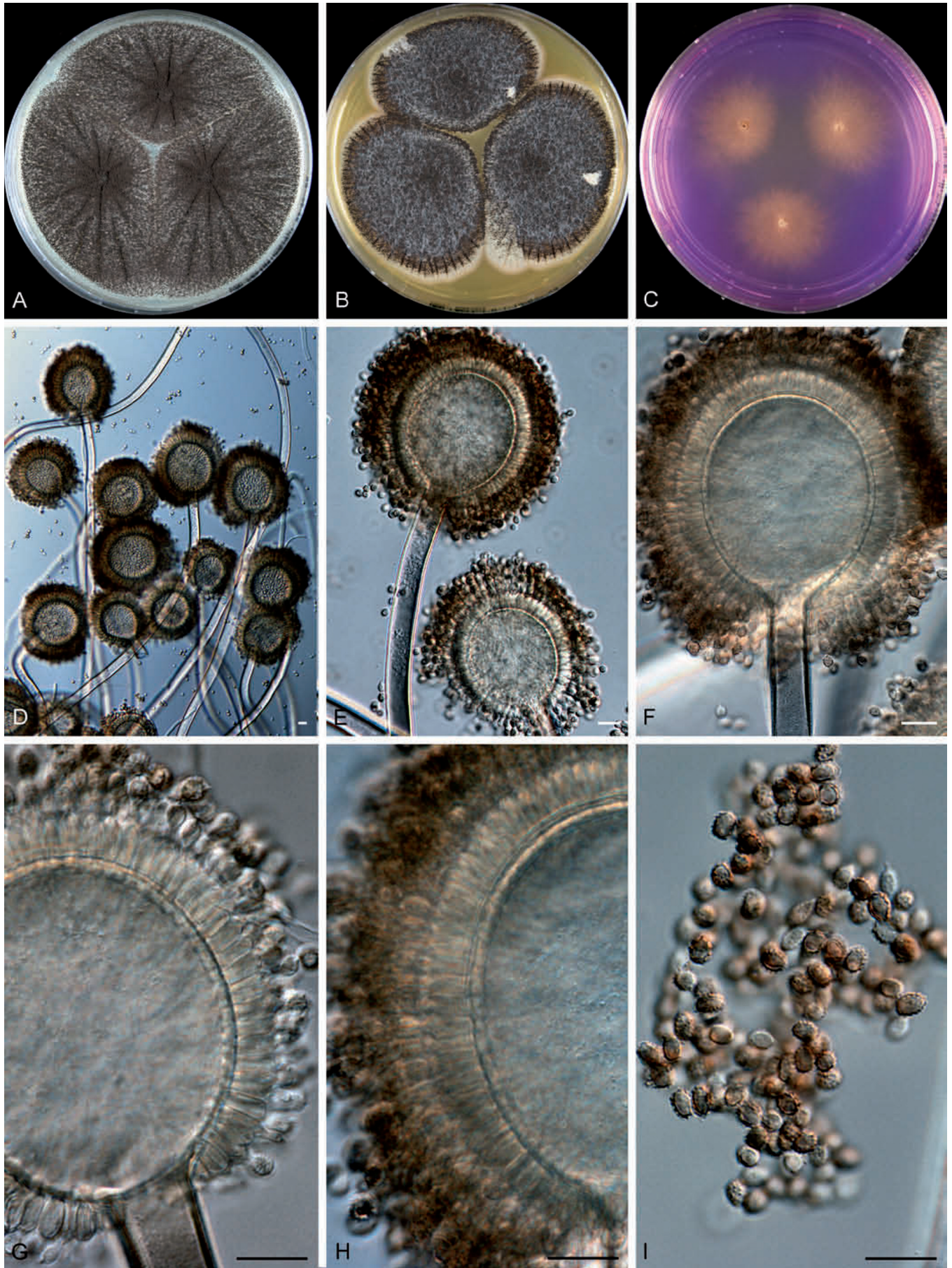


Fig. 10. *Aspergillus fijiensis* sp. nov. A–C. Colonies incubated at 25 °C for 7 d, A. CYA, B. MEA, C. CREA, D–I. Conidiophores and conidia. Scale bars = 10 µm.

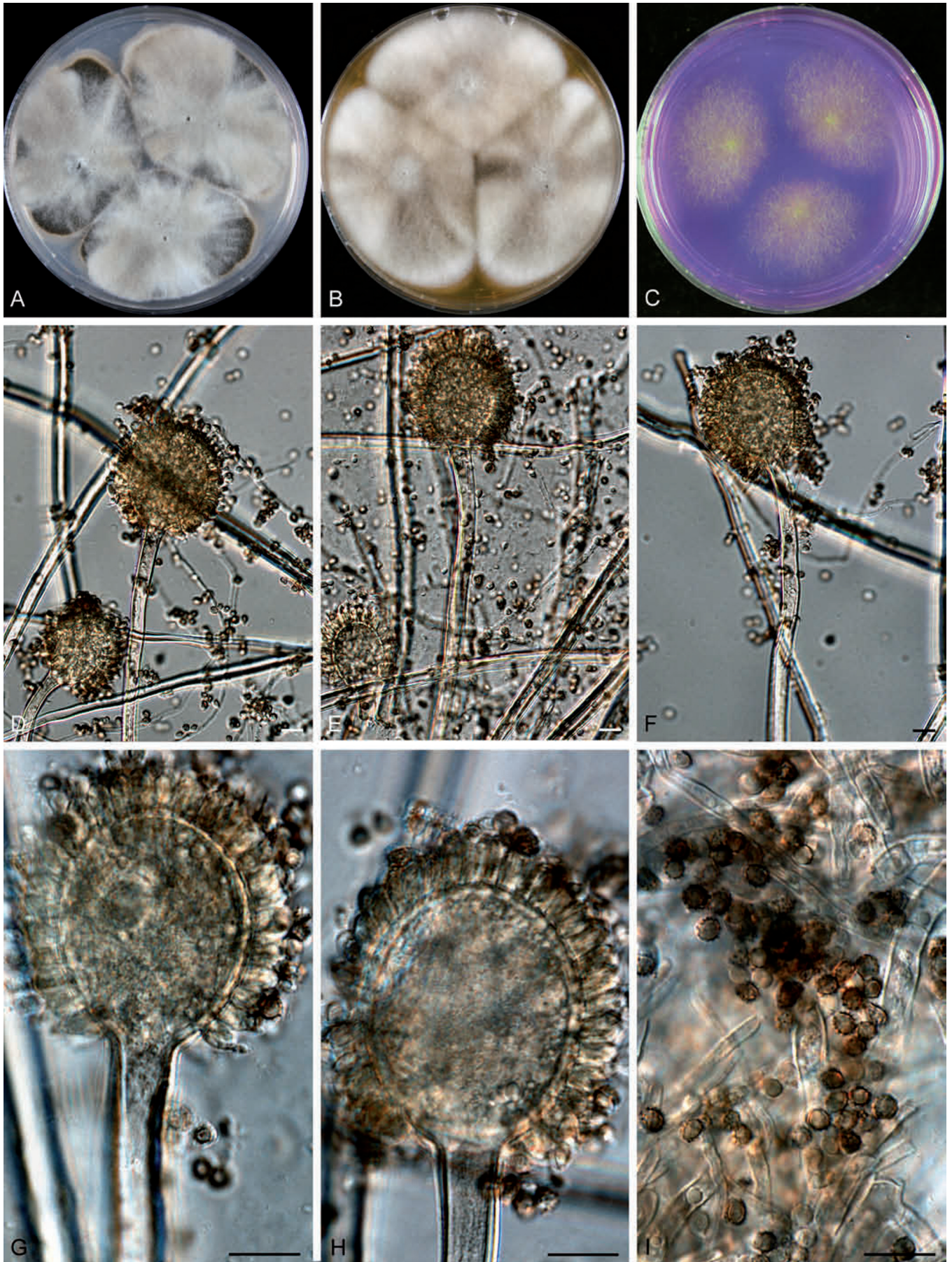


Fig. 11. *Aspergillus indologenus* sp. nov. A–C. Colonies incubated at 25 °C for 7 d, A. CYA, B. MEA, C. CREA, D–I. Conidiophores and conidia. Scale bars = 10 µm.

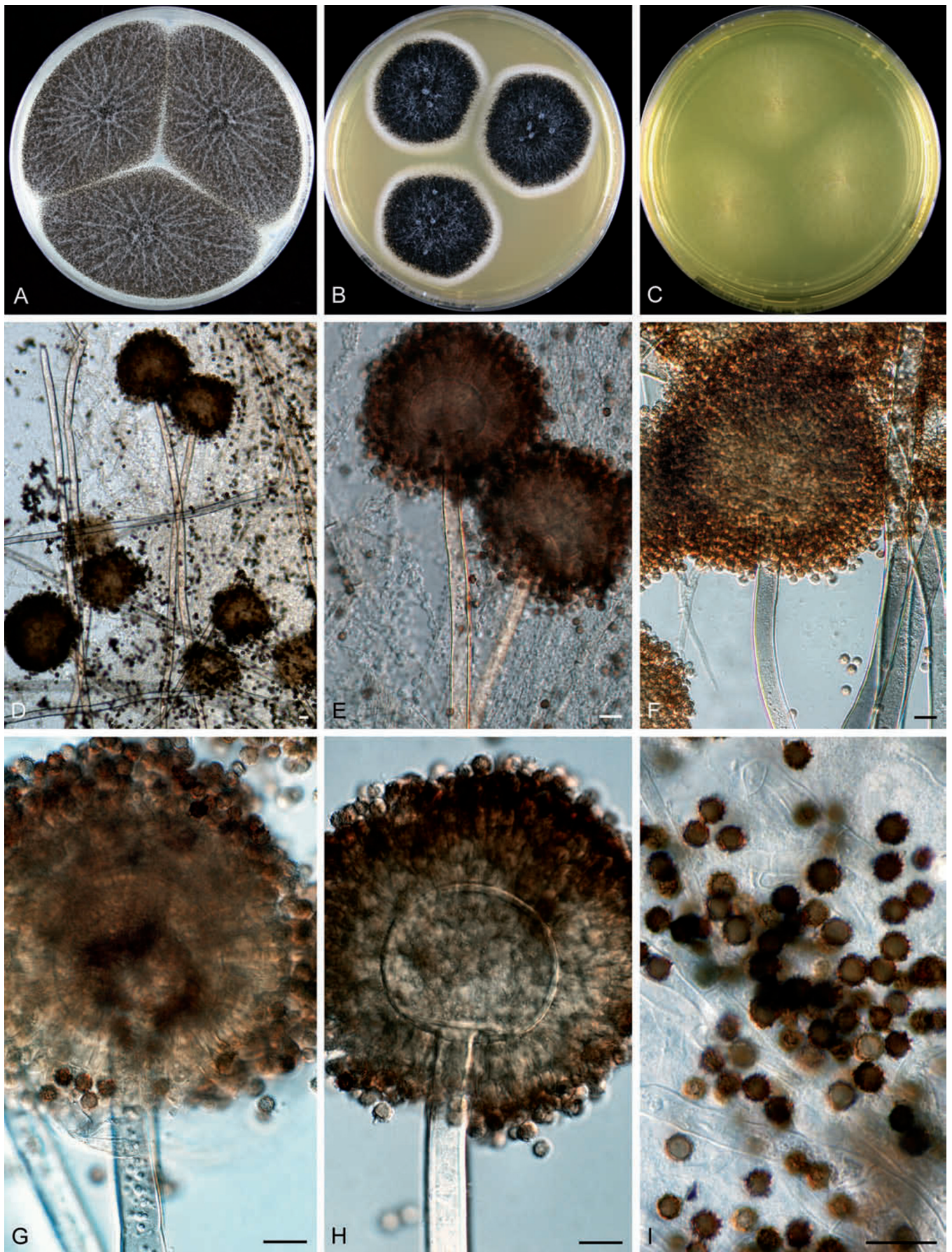


Fig. 12. *Aspergillus neoniger* sp. nov. A–C. Colonies incubated at 25 °C for 7 d, A. CYA, B. MEA, C. CREA, D–I. Conidiophores and conidia. Scale bars = 10 μm.

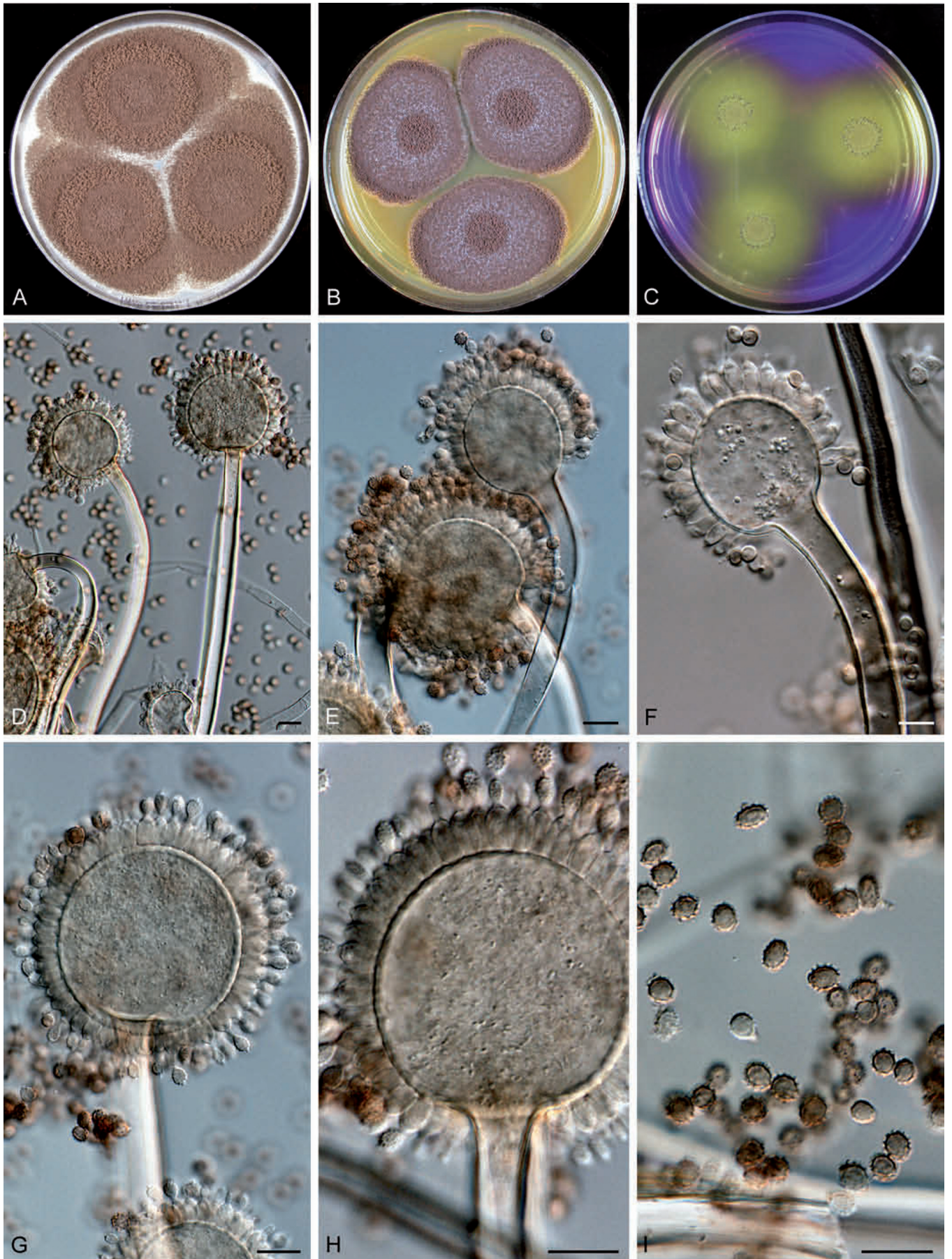


Fig. 13. *Aspergillus violaceofuscus* A–C. Colonies incubated at 25 °C for 7 d, A. CYA, B. MEA, C. CREA, D–I. Conidiophores and conidia. Scale bars = 10 μm.

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 conidia, while *A. indologenus* has conidia which are coarsely roughened 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. Morphologically 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*. *Aspergillus violaceofuscus* has typical ellipsoidal to fusiform conidia, which are coarsely roughened to echinulate.

ACKNOWLEDGEMENTS

This study was partly supported by the OTKA grant Nos. K 84077 and K 84122. We thank Dr Uwe Braun for the Latin diagnosis and his advice on nomenclatural issues.

REFERENCES

- Abarca ML, Accensi F, Cano J, Cabañes FJ (2004). Taxonomy and significance of black aspergilli. *Antonie Van Leeuwenhoek* **86**: 33–49.
- Alcazar-Fuoli L, Mellado E, Alastruey-Izquierdo A, Cuenca-Estrella M, Rodriguez-Tudela JL (2009). Species identification and antifungal susceptibility patterns of species belonging to *Aspergillus* section *Nigri*. *Antimicrobial Agents and Chemotherapy* **53**: 4514–4517.
- Alvarez-Perez S, Blanco JL, Alba P, Garcia ME (2010). Mating type and invasiveness are significantly associated in *Aspergillus fumigatus*. *Medical Mycology* **48**: 273–277.
- Alvarez-Perez S, Blanco JL, Lopez-Rodas V, Flores-Moya A, Costas E, Garcia ME (2010). Elastase activity in *Aspergillus fumigatus* can arise by random, spontaneous mutations. *International Journal of Evolutionary Biology* **2010**: Article ID 602457.
- Banks RM, Blanchflower SE, Everett JR, Manger BR, Reading C (1997) Novel antihelminthic metabolites from an *Aspergillus* species; the aspergillimides. *Journal of Antibiotics* **50**: 840–846.
- Blanco JL, Hontecillas R, Bouza E, Blanco I, Pelaez T, Muñoz P, Molina JP, Garcia ME (2002). Correlation between the Elastase Activity Index and Invasiveness of Clinical Isolates of *Aspergillus fumigatus*. *Journal of Clinical Microbiology* **40**: 1811–1813.
- Bulat SA, Lübeck M, Alekhina IA, Jensen DF, Knudsen IMB, Lübeck PS (2000). Identification of a universally primed-PCR-derived sequence characterized amplified region marker for an antagonistic strain of *Clonostachys rosea* and development of a strain-specific PCR detection assay. *Applied and Environmental Microbiology* **66**: 4758–4763.
- Chiang Y-M, Meyer KM, Praseuth M, Baker SE, Bruno KS, Wang CCC (2010). Characterization of a polyketide synthase in *Aspergillus niger* whose product is a precursor for both dihydroxynaphthalene (DHN) melanin and naphtho-γ-pyrone. *Fungal Genetics and Biology* **48**: 430–437.
- Denning DW, Elliott J, Keaney M (1993). Temperature-dependent expression of elastase in *Aspergillus* species. *Journal of Medical and Veterinary Mycology* **31**: 455–458.
- Felsenstein J (2007). PHYLIP (Phylogeny Inference Package). Version 3.67 Distributed by the author. Department of Genome Sciences and Department of Biology University of Washington, Seattle.
- Ferracin LM, Frisvad JC, Taniwaki MH, Iamanaka BT, Sartori D, Schaoavaloff ME, Fungaro MHP (2009). Genetic relationships among strains of the *Aspergillus niger* aggregate. *Brazilian Archives of Biology and Technology* **52**: 241–248.
- Frisvad JC, Larsen TO, Vries R de, Meijer M, Houbraken J, *et al.* (2007a). Secondary metabolite profiling, growth profiles and other tools for species recognition and important *Aspergillus* mycotoxins. *Studies in Mycology* **59**: 31–37.
- Frisvad JC, Smedsgaard J, Samson RA, Larsen TO, Thrane U (2007b). Fumonisin B₂ production by *Aspergillus niger*. *Journal of Agricultural and Food Chemistry* **55**: 9727–9732.
- Frisvad JC, Thrane U (1987). Standardized high performance liquid chromatography of 182 mycotoxins and other fungal metabolites based on alkylphenone retention indices and UV-VIS spectra (diode array detection). *Journal of Chromatography A* **404**: 195–214.
- Frisvad JC, Thrane U (1993). Liquid chromatography of mycotoxins. In: *Chromatography of mycotoxins: techniques and applications*. (Betina V, ed.). *Journal of Chromatography Library* **54**: 253–372.

- Gams W, Christensen M, Onions AH, Pitt JI, Samson RA (1985). Infrageneric taxa of *Aspergillus*. In: *Advances in Penicillium and Aspergillus Systematics*. (Samson RA, Pitt JI, eds). New York, Plenum Press: 55–62.
- Garcia ME, Caballero J, Blanco I, Cruzado M, Costas E, Blanco JL (2006). Changes in the elastase activity and colonization ability of *A. fumigatus* after successive inoculations in mice. *Revista Iberoamericana de Micología* **23**: 221–223.
- Geisen R (2007). Molecular detection and monitoring of fungi in food. In: *Food Mycology. A Multifaceted Approach to Fungi and Food*. (Dijksterhuis J, Samson RA, eds). Boca Raton, CRC Press: 255–278.
- Hayashi H, Furutsuka K, Shiono Y (1999). Okaramins H and I, new okaramine congeners, from *Aspergillus aculeatus*. *Journal of Natural Products* **62**: 315–317.
- Hayashi H, Nishimoo Y, Nozaki H (1997). Asperparaline A, a new paralytic alkaloid from *Aspergillus japonicus* JV-23. *Tetrahedron Letters* **38**: 5655–5658.
- Hayashi H, Nishimoto Y, Akiyama K, Nozaki H (2000). New paralytic alkaloids, asperparalines A, B and C, from *Aspergillus japonicus* JV-23. *Bioscience Biotechnology Biochemistry* **64**: 111–115.
- Hillis DM, Bull JJ (1993). An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. *Systematic Biology* **42**: 182–192.
- Jørgensen TR, Park J, Arentshorst M, Welzen AM van, Lamers G, Kuyk PA van, Damveld RA, Nielsen KF, Frisvad JC, Ram AFJ (2010). The molecular and genetic basis of conidial pigmentation in *Aspergillus niger*. *Fungal Genetics and Biology* **48**: 544–553.
- Kazi S, Paterson RRM, Abo-Dhab NF (1997). Effect of 2-deoxy-D-glucose on mycotoxins from apples inoculated with *Penicillium expansum*. *Mycopathologia* **138**: 43–46.
- Kolattukudy PE, Lee JD, Rogers LM, Zimmerman P, Ceselski S, et al. (1993). Evidence for possible involvement of an elastolytic serine protease in aspergillosis. *Infection & Immunity* **61**: 2357–2368.
- Kozakiewicz Z (1989). *Aspergillus* species on stored products. *Mycological Papers* **161**: 1–188.
- Leach J, Finkelstein DB, Rambosck JA (1986). Rapid miniprep of DNA from filamentous fungi. *Fungal Genetics Newsletter* **33**: 32–33.
- Lübeck PS, Alekhina IA, Lübeck M, Bulat SA (1998). UP-PCR genotyping and rDNA analysis of *Ascochyta pisi* Lib. *Journal of Phytopathology* **146**: 51–55.
- Mogensen JM, Varga J, Thrane U, Frisvad JC (2009). *Aspergillus acidus* from Puerh tea and black tea does not produce ochratoxin A and fumonisin B₂. *International Journal of Food Microbiology* **132**: 141–144.
- Nielsen KF, Mogensen JM, Johansen M, Larsen TO, Frisvad JC (2009). Review of secondary metabolites and mycotoxins from the *Aspergillus niger* group. *Analytical and Bioanalytical Chemistry* **395**: 1225–1246.
- Noonim P, Mahakamchanakul W, Varga J, Frisvad JC, Samson RA (2008). Two new species of *Aspergillus* section *Nigri* from Thai coffee beans. *International Journal of Systematic and Evolutionary Microbiology* **58**: 1727–1734.
- Parenicova L, Skouboe P, Frisvad J, Samson RA, Rossen L, et al. (2001). Combined molecular and biochemical approach identifies *Aspergillus japonicus* and *Aspergillus aculeatus* as two different species. *Applied and Environmental Microbiology* **67**: 5621–5627.
- Perrone G, Stea G, Epifani F, Varga J, Frisvad JC, Samson RA (2011). *Aspergillus niger* contains the cryptic phylogenetic species *A. awamori*. *Fungal Biology* (in press).
- Perrone G, Varga J, Susca A, Frisvad JC, Stea G, et al. (2008). *Aspergillus uvarum* sp. nov., an uniseriate black *Aspergillus* species isolated from grapes in Europe. *International Journal of Systematic and Evolutionary Microbiology* **58**: 1032–1039.
- Raper KB, Fennell DI. 1965. The genus *Aspergillus*. Baltimore: Williams & Wilkins.
- Saitou N, Nei M (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4**: 406–425.
- Samson RA, Hoekstra ES, Frisvad JC (2004a). Introduction to food- and airborne fungi. 7th edition. Utrecht: Centraalbureau voor Schimmelcultures.
- Samson RA, Houbaken JAMP, Kuijpers AFA, Frank JM, Frisvad JC (2004b). New ochratoxin or sclerotium producing species in *Aspergillus* section *Nigri*. *Studies in Mycology* **50**: 45–61.
- Samson RA, Noonim P, Meijer M, Houbaken J, Frisvad JC, Varga J (2007). Diagnostic tools to identify black *Aspergillus*. *Studies in Mycology* **59**: 129–146.
- Sedaghati E, Nikkhab MJ, Zareb R, Fotuhifara KB, Kocsubé S, et al. (2011). Molecular identification of potentially mycotoxigenic black *Aspergillus* contaminating pistachio nuts in Iran. *Acta Alimentaria* **40**: 65–70.
- Smedsgaard J (1997). Micro-scale extraction procedure for standardized screening of fungal metabolite production in cultures. *Journal of Chromatography A* **760**: 264–270.
- Sørensen A, Lübeck PS, Lübeck M, Nielsen KF, Ahring BK, et al. (2011). *Aspergillus saccharolyticus* sp. nov., a new black *Aspergillus* species isolated in Denmark. *International Journal of Systematic and Evolutionary Microbiology* Published online Doi: 10.1099/ijs.0.029884-0.
- Starkey DE, Ward TJ, Aoki T, Gale LR, Kistler HC, et al. (2007). Global molecular surveillance reveals novel *Fusarium* head blight species and trichothecene toxin diversity. *Fungal Genetics and Biology* **44**: 1191–1204.
- Susca A, Proctor RH, Mule G, Stea G, Ritieni A, et al. (2010). Correlation of mycotoxin fumonisin B₁ production and presence of the fumonisin biosynthetic gene *fum8* in *Aspergillus niger* from grape. *Journal of Agricultural and Food Chemistry* **58**: 9266–9272.
- Swofford T (2000). *PAUP*: Phylogenetic analysis using parsimony*. v. 4.0. Sinauer Associates, Sunderland.
- Szigeti G, Sedaghati E, Zarei Mahmoudabadi A, Naseri A, Kocsubé S, et al. (2011). Species assignment and antifungal susceptibilities of black aspergilli recovered from otomycosis cases in Iran. *Mycoses* (in press).
- Tamura K, Dudley J, Nei M, Kumar S (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software v. 4.0. *Molecular Biology and Evolution* **24**: 1596–1599.
- Varga J, Due M, Frisvad JC, Samson RA (2007a). Taxonomic revision of *Aspergillus* section *Clavati* based on molecular, morphological and physiological data. *Studies in Mycology* **59**: 89–106.
- Varga J, Frisvad JC, Samson RA (2007b). Polyphasic taxonomy of *Aspergillus* section *Candidi* based on molecular, morphological and physiological data. *Studies in Mycology* **59**: 75–88.
- Varga J, Kevei F, Debets F, Kozakiewicz Z, Croft JH (1994). Mitochondrial DNA restriction fragment length polymorphisms in field isolates of the *Aspergillus niger* aggregate. *Canadian Journal of Microbiology* **40**: 612–621.
- Varga J, Kevei F, Fekete C, Coenen A, Kozakiewicz Z, Croft JH (1993). Restriction fragment length polymorphisms in the mitochondrial DNAs of the *Aspergillus niger* aggregate. *Mycological Research* **97**: 1207–1212.
- Varga J, Kevei F, Hamari Z, Tóth B, Téren J, et al. (2000). Genotypic and phenotypic variability among black *Aspergillus*. In: *Integration of modern taxonomic methods for Penicillium and Aspergillus classification*. (Samson RA, Pitt JI, eds). Amsterdam, Harwood Academic Publishers: 397–411.
- Varga J, Kocsubé S, Suri K, Szigeti G, Szekeres A, Varga M, Tóth B, Bartók T (2010). Fumonisin contamination and fumonisin producing black *Aspergillus* in dried vine fruits of different origin. *International Journal of Food Microbiology* **143**: 143–149.
- Varga J, Kocsubé S, Tóth B, Frisvad JC, Perrone G, et al. (2007c). *Aspergillus brasiliensis* sp. nov., a biseriata black *Aspergillus* species with world-wide distribution. *International Journal of Systematic and Evolutionary Microbiology* **57**: 1925–1932.
- Ward TJ, Bielawski JP, Kistler HC, Sullivan E, O'Donnell K (2002). Ancestral polymorphism and adaptive evolution in the trichothecene mycotoxin gene cluster of phytopathogenic *Fusarium*. *Proceedings of the National Academy of Sciences USA* **99**: 9278–9283.
- Yu TS, Yeo SH, Kim HS (2004). A new species of hypomyces, *Aspergillus coreanus* sp. nov., isolated from traditional Korean nuruk. *Journal of Microbiology and Biotechnology* **14**: 182–187.