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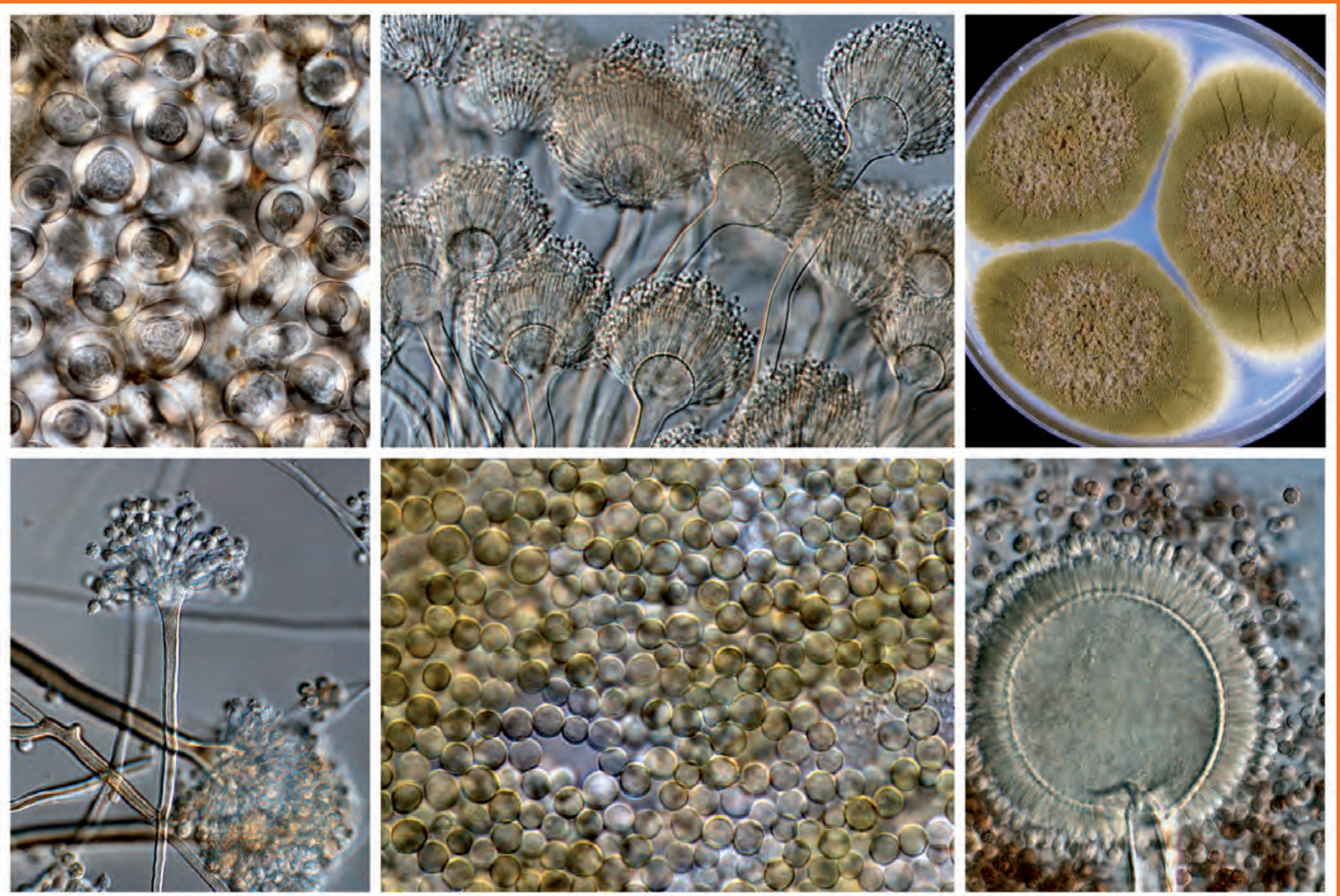
Taxonomic studies on the genus *Aspergillus*

Robert A. Samson, János Varga and Jens C. Frisvad



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Cover: Top from left to right: Young cleistothecium of *Talaromyces trachyspermus*, ascospores of *T. macrosporus*, conidiophore of *Penicillium sumatrense*. Bottom from left to right: Conidiophores of *P. calidicanium*, MEA Petri dish with 7 d old culture of *P. isariiforme*, scanning electron micrograph of ascospores of *P. shearii*.

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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,

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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).

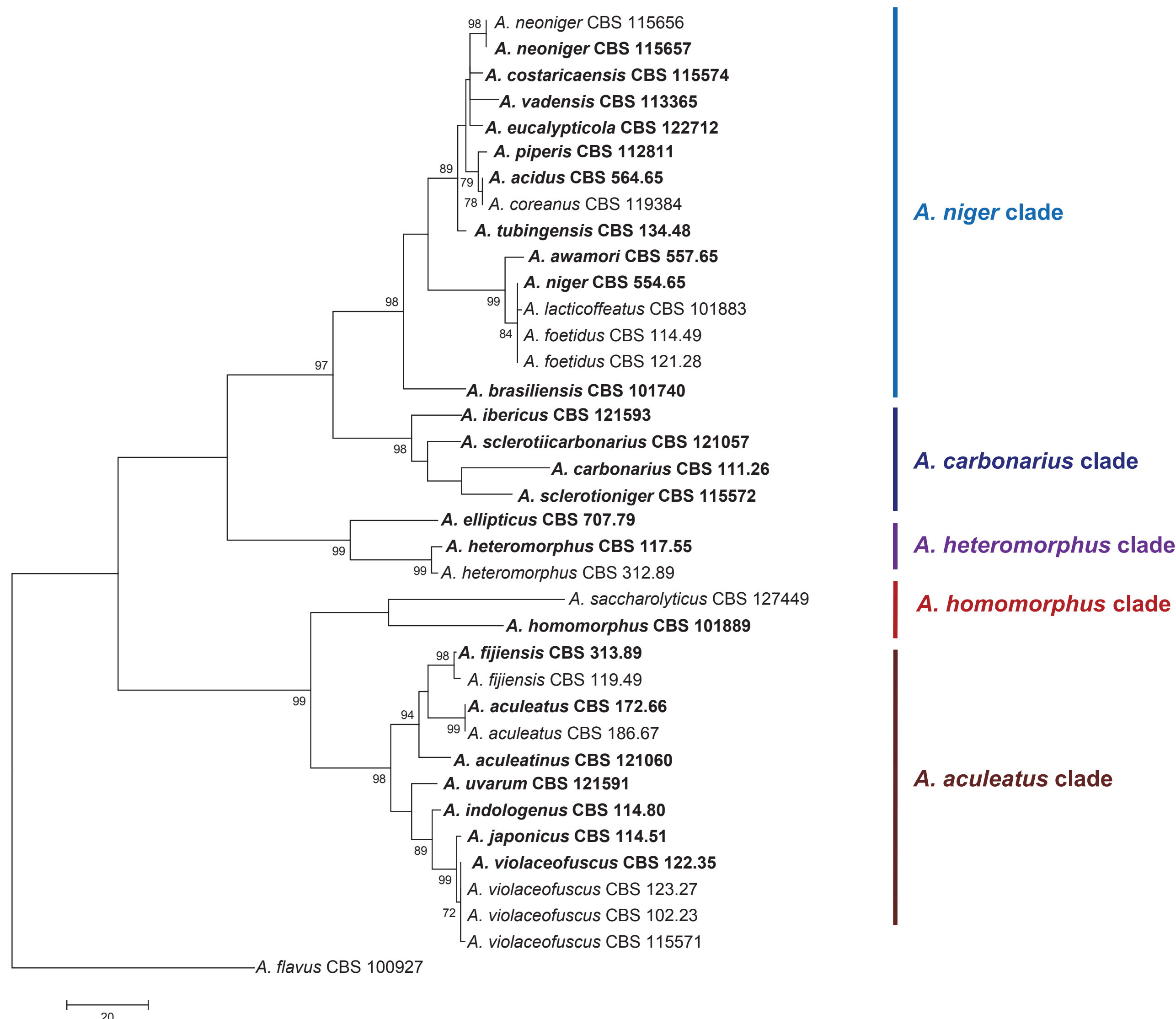


Fig. 1. One of the MP trees obtained based on phylogenetic analysis of calmodulin sequence data of *Aspergillus* section *Nigri.* Numbers above branches are bootstrap values. Only values above 70 % are indicated.

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

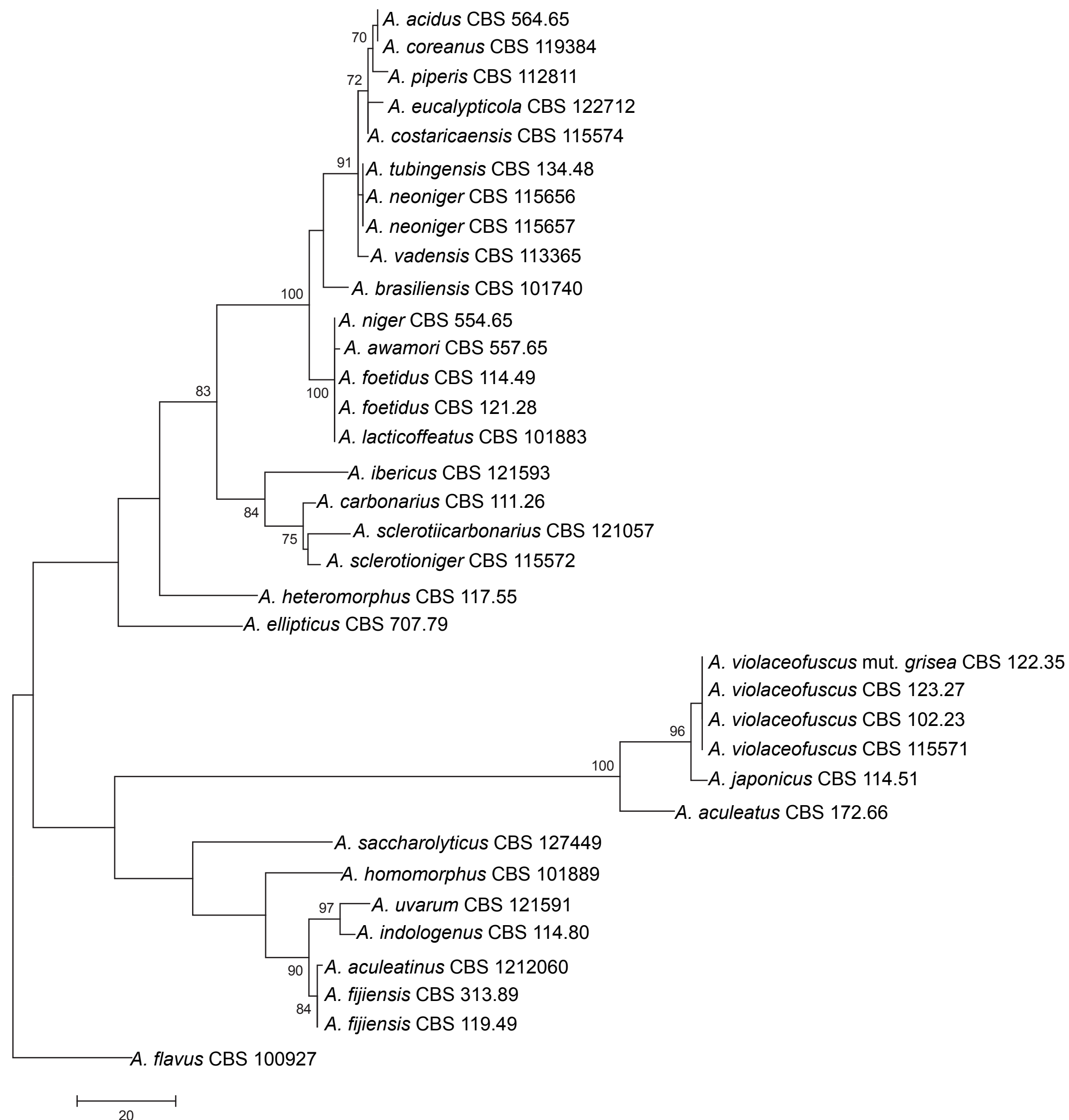


Fig. 2. The single MP tree obtained based on phylogenetic analysis of β-tubulin sequence data of *Aspergillus* section *Nigri.* Numbers above branches are bootstrap values. Only values above 70 % are indicated.

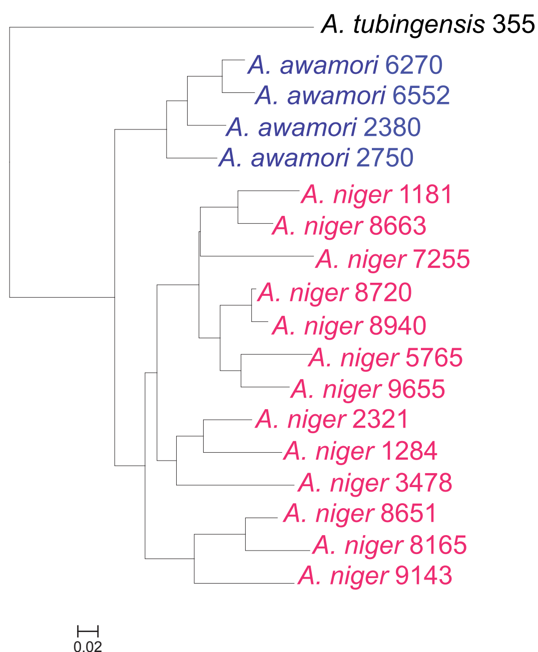
A and "MYC", and it is chemically very closely related to *A. eucalypticola*.

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).

A. tubingensis CBS 134.48
A. piperis CBS 112811
A. costaricaensis CBS 115574
A. coreanus CBS 119384
86
A. eucalypticola CBS 122712
A. acidus CBS 564.65
A. neoniger CBS 115656
A. neoniger CBS 115657
A. vadensis CBS 113365
82
A. awamori CBS 557.65
A. niger CBS 554.65
A. foetidus CBS 114.49
A. foetidus CBS 121.28
A. lacticoffeatus CBS 101883
A. brasiliensis CBS 101740
A. carbonarius CBS 111.26
A. sclerotioniger CBS 115572
82
A. sclerotiicarbonarius CBS 121057
A. ibericus CBS 121593
A. ellipticus CBS 707.79
90
A. heteromorphus CBS 117.55
A. homomorphus CBS 101889
A. saccharolyticus CBS 127449
82
A. fijiensis CBS 313.89
A. fijiensis CBS 119.49
99
A. aculeatinus CBS 1212060
A. aculeatus CBS 172.66
A. indologenus CBS 114.80
A. uvarum CBS 121591
99
A. japonicus CBS 114.51
A. violaceofuscus mut. grisea CBS 122.35
A. violaceofuscus CBS 123.27
A. violaceofuscus CBS 102.23
A. violaceofuscus CBS 115571
A. flavus CBS 100927
5

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.

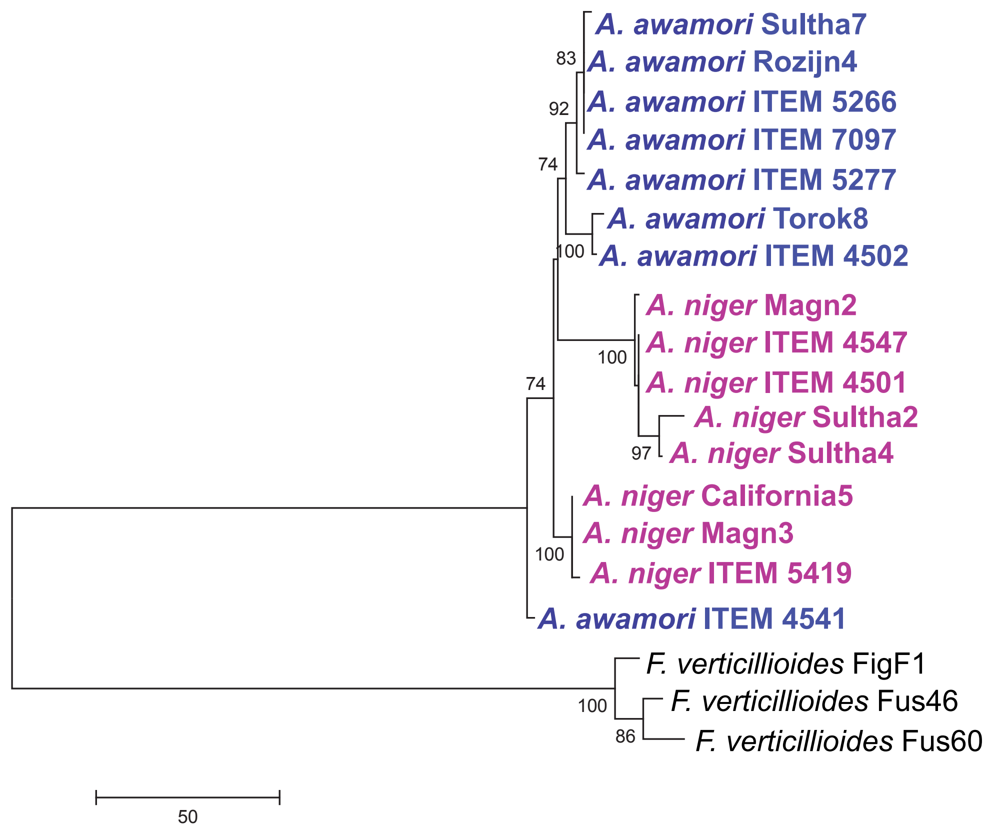


Fig. 5. One of the 73 MP trees obtained based on phylogenetic analysis of FUM8 sequence data of *A. niger* and *A. awamori* isolates (tree length: 411, consistency index: 0.961240, retention index: 0.980843). Numbers above branches are bootstrap values. Only values above 70 % are indicated.

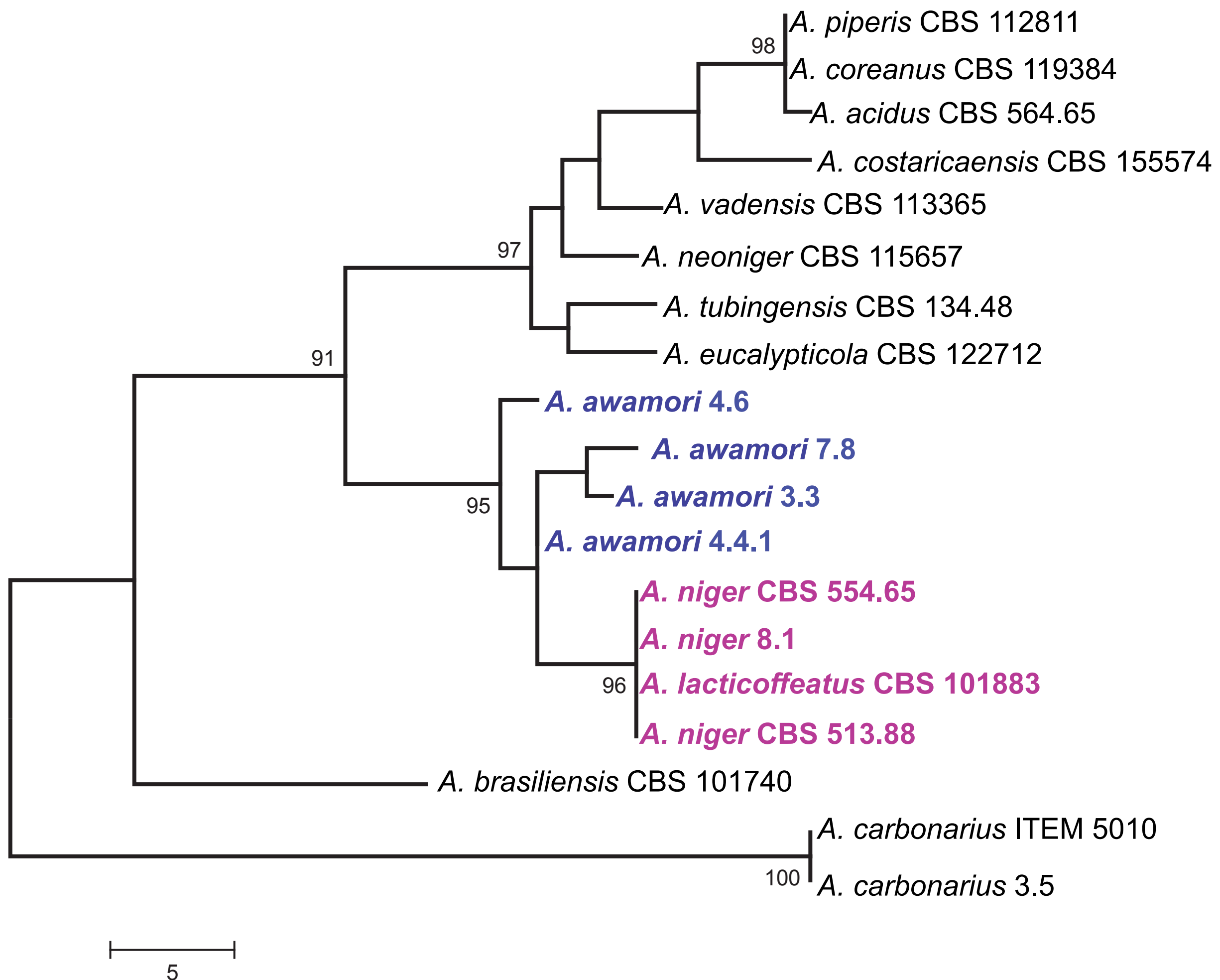


Fig. 6. One of the 153 MP trees obtained based on phylogenetic analysis of chloroperoxidase sequence data of *A. niger* and *A. awamori* isolates (tree length: 113, consistency index: 0.767677, retention index: 0.886700). Numbers above branches are bootstrap values. Only values above 70 % are indicated.

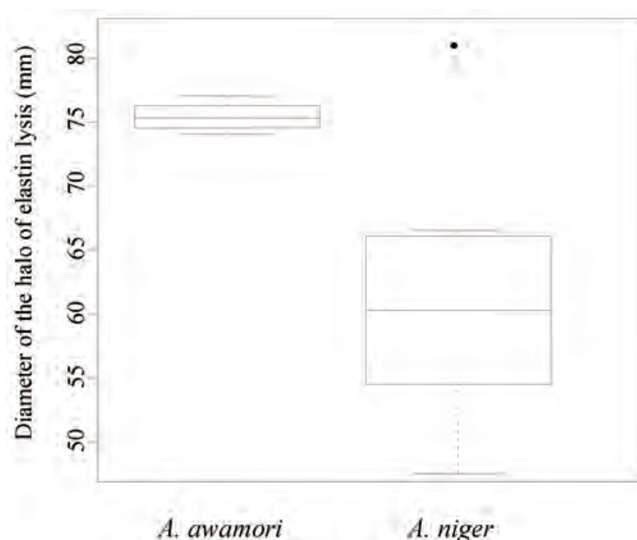


Fig. 7. The difference of elastin lysis between the isolates belonging to *A. niger* and *A. awamori*.

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 (χ^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).

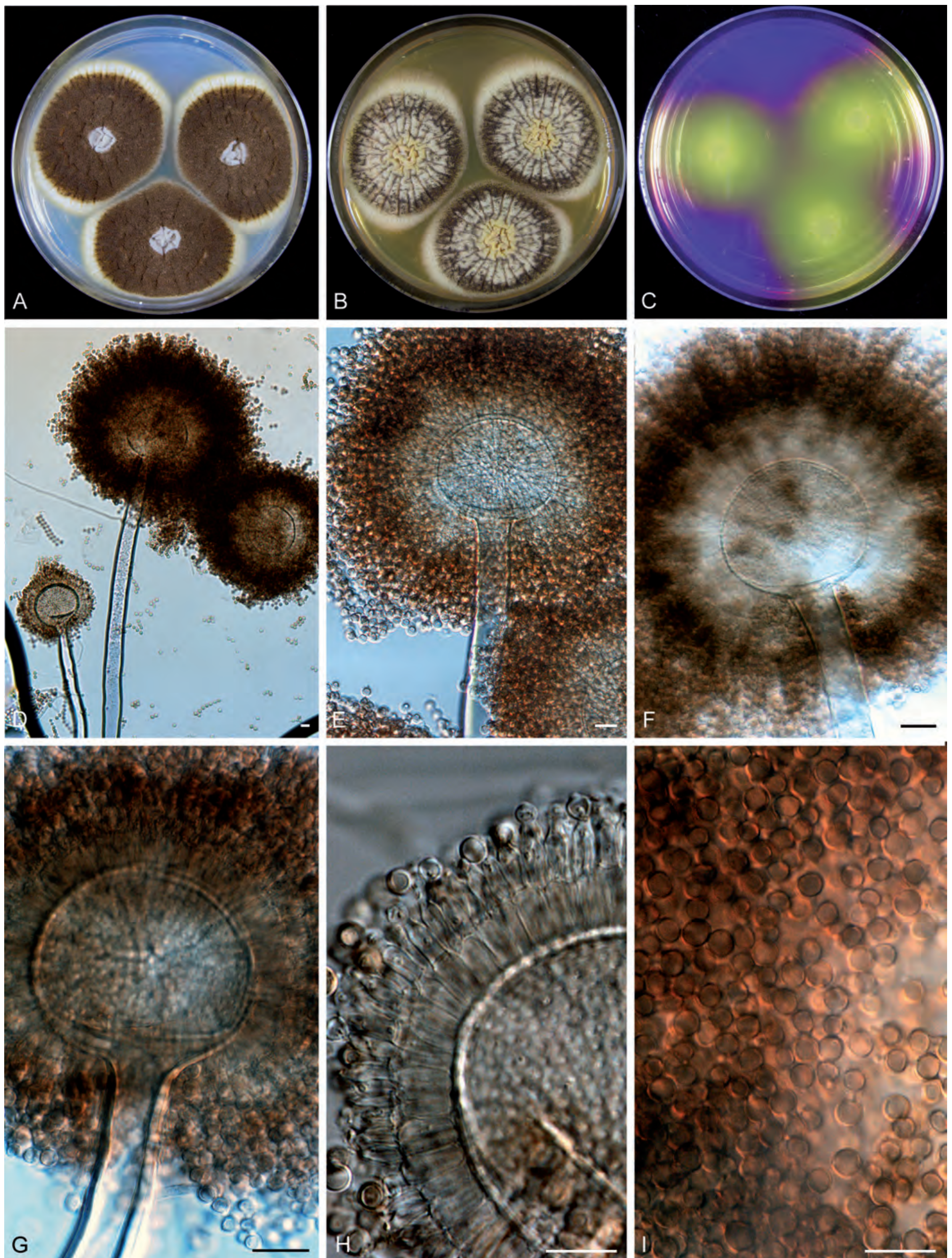


Fig. 8. *Aspergillus acidus* A–C. Colonies incubated at 25 °C for 7 d, A. CYA, B. MEA, C. CREA, D–I. Conidiophores and conidia. Scale bars = 10 µm.

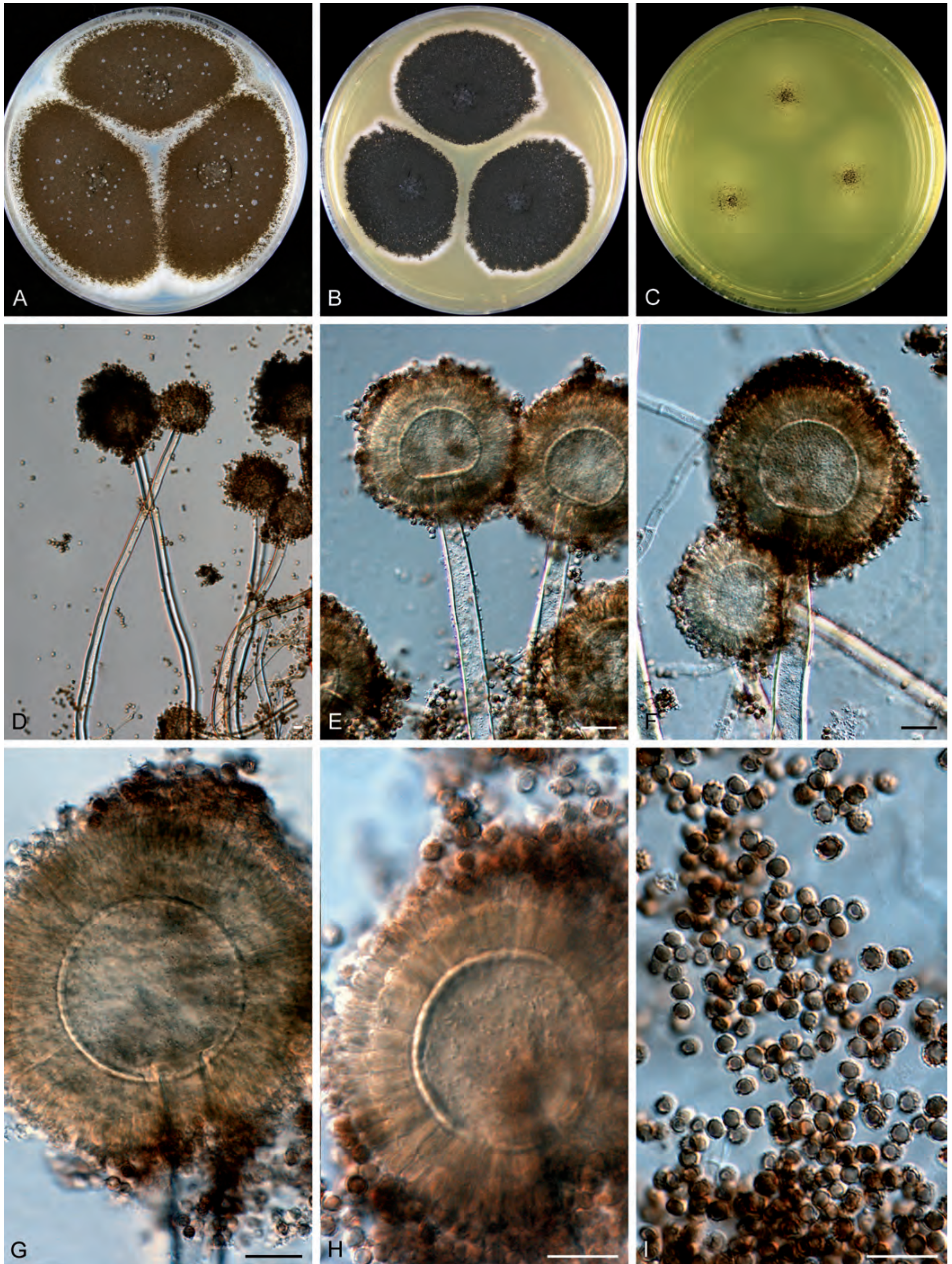


Fig. 9. *Aspergillus eucalypticola* sp. nov. A–C. Colonies incubated at 25 °C for 7 d, A. CYA, B. MEA, C. CREA, D–I. Conidiophores and conidia. Scale bars = 10 µm.

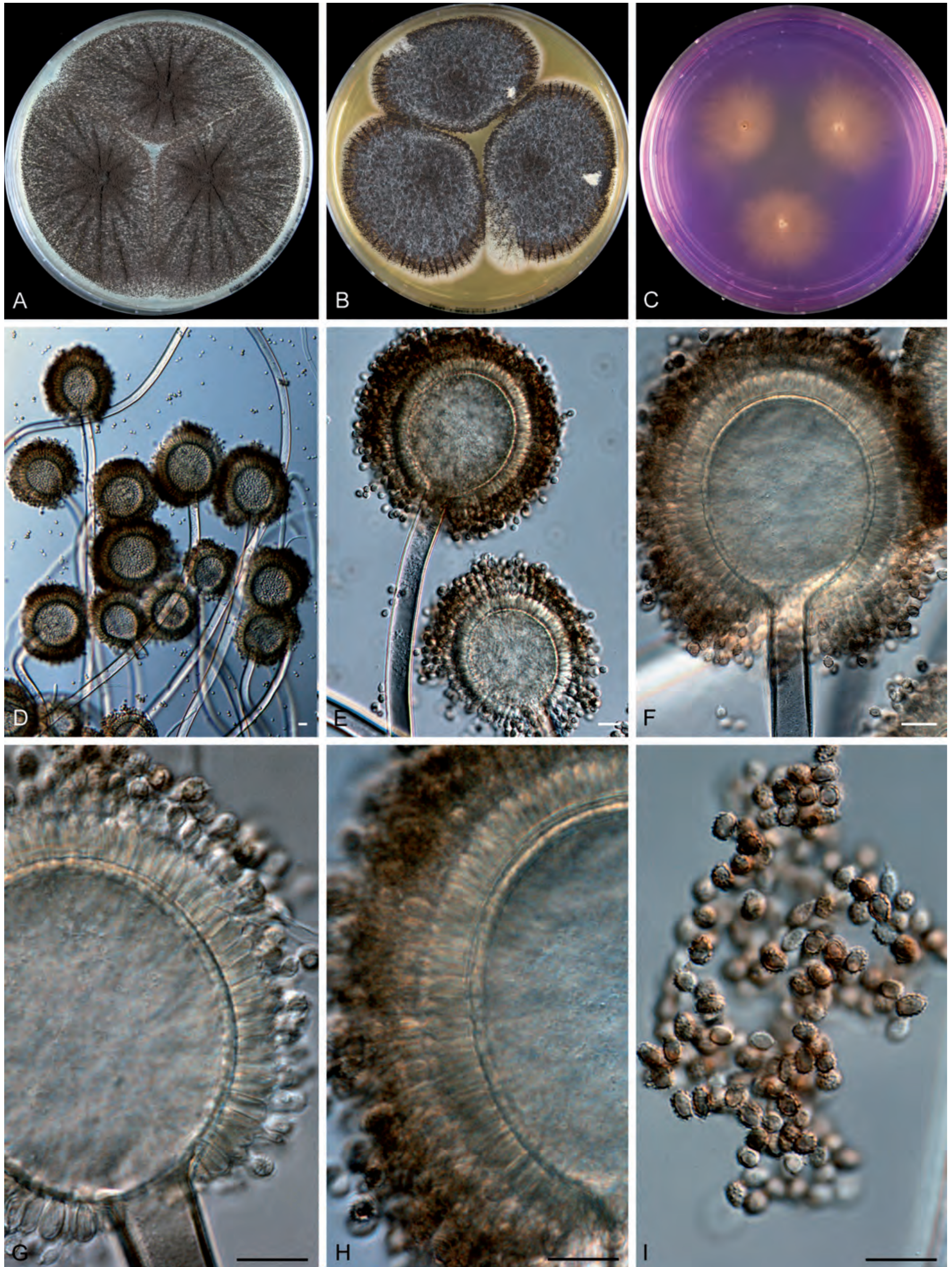


Fig. 10. *Aspergillus fijiensis* sp. nov. A–C. Colonies incubated at 25 °C for 7 d, A. CYA, B. MEA, C. CREA, D–I. Conidiophores and conidia. Scale bars = 10 μm.

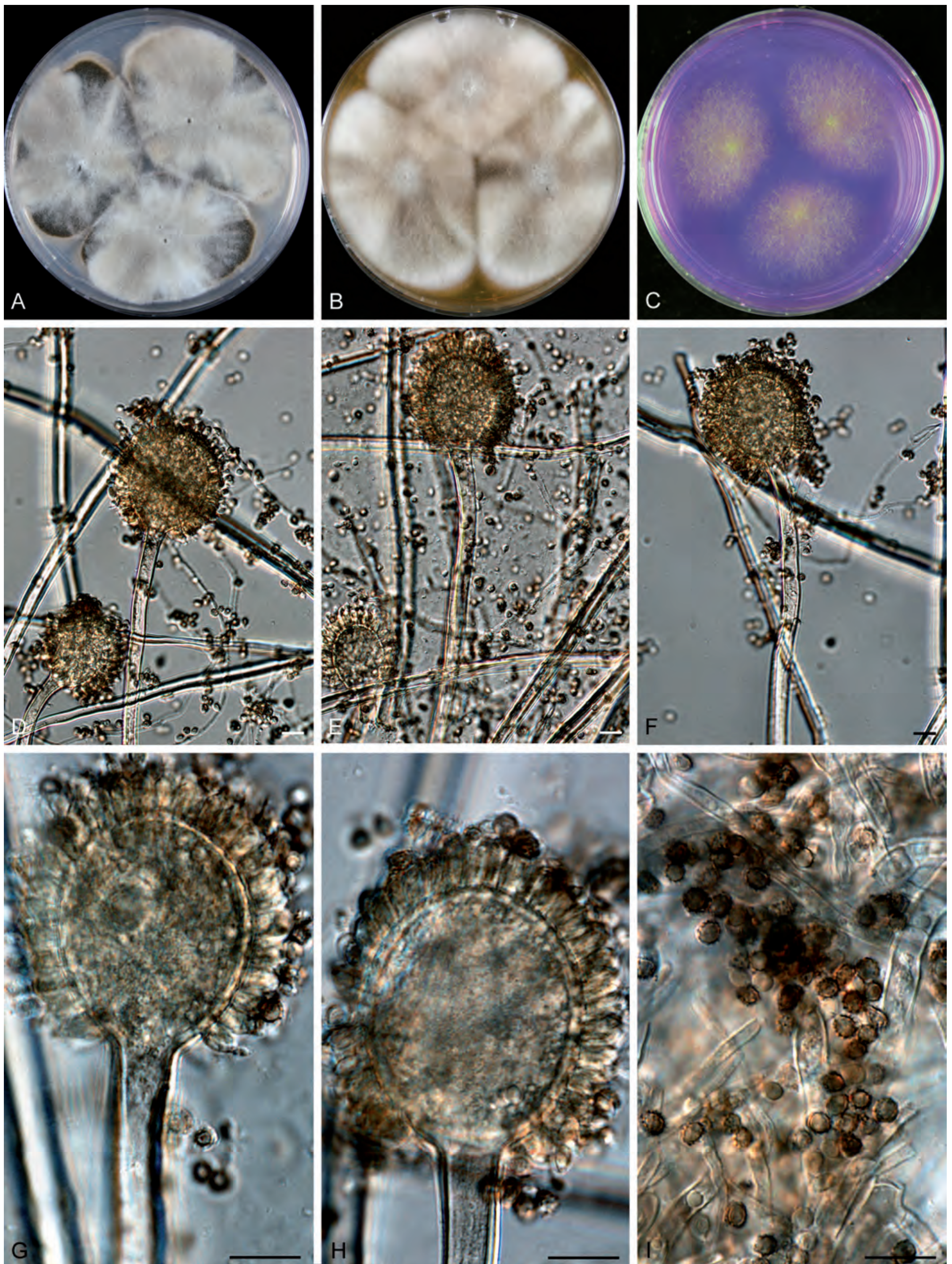


Fig. 11. *Aspergillus indologenus* sp. nov. A–C. Colonies incubated at 25 °C for 7 d, A. CYA, B. MEA, C. CREA, D–I. Conidiophores and conidia. Scale bars = 10 µm.

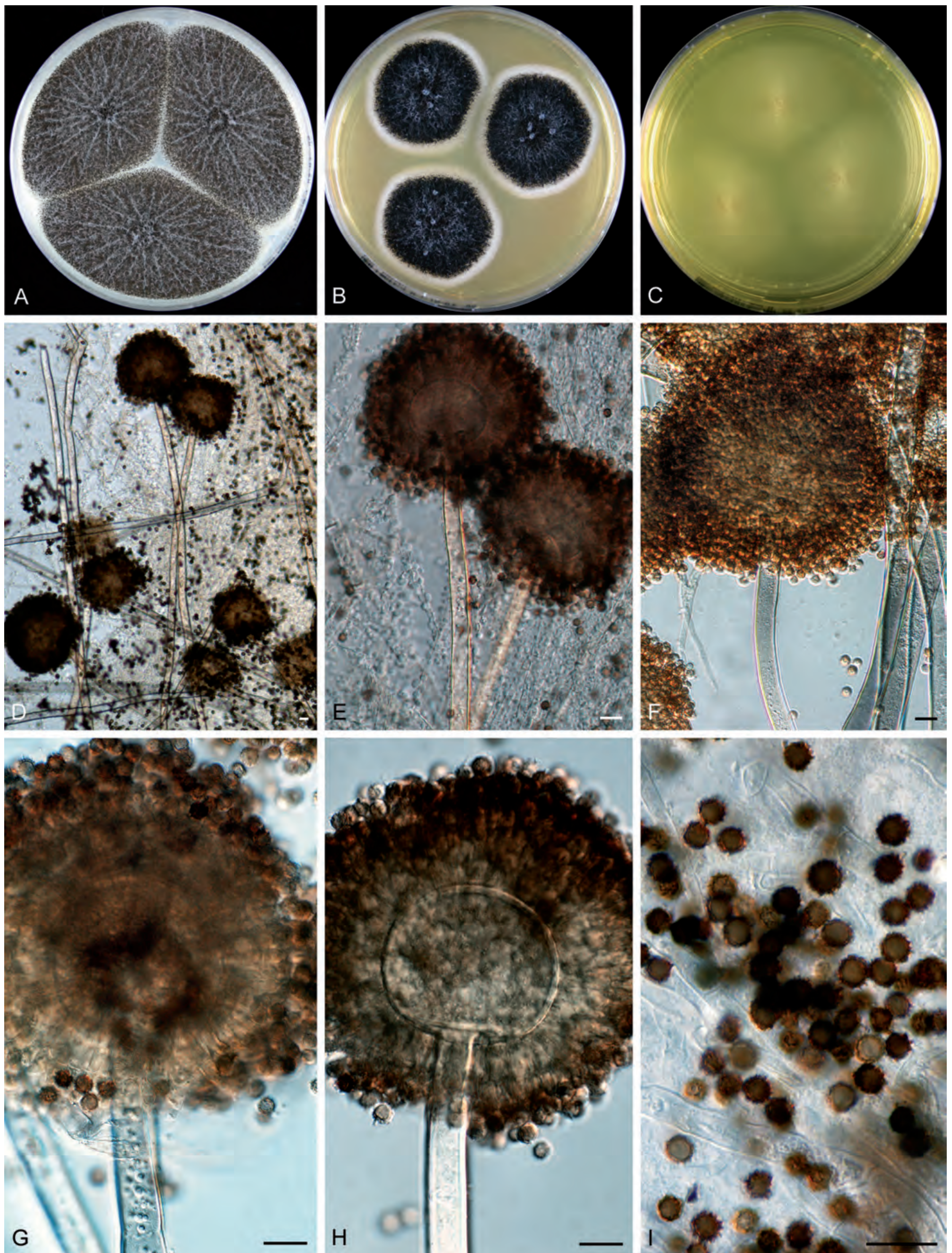


Fig. 12. *Aspergillus neoniger* sp. nov. A–C. Colonies incubated at 25 °C for 7 d, A. CYA, B. MEA, C. CREA, D–I. Conidiophores and conidia. Scale bars = 10 µm.

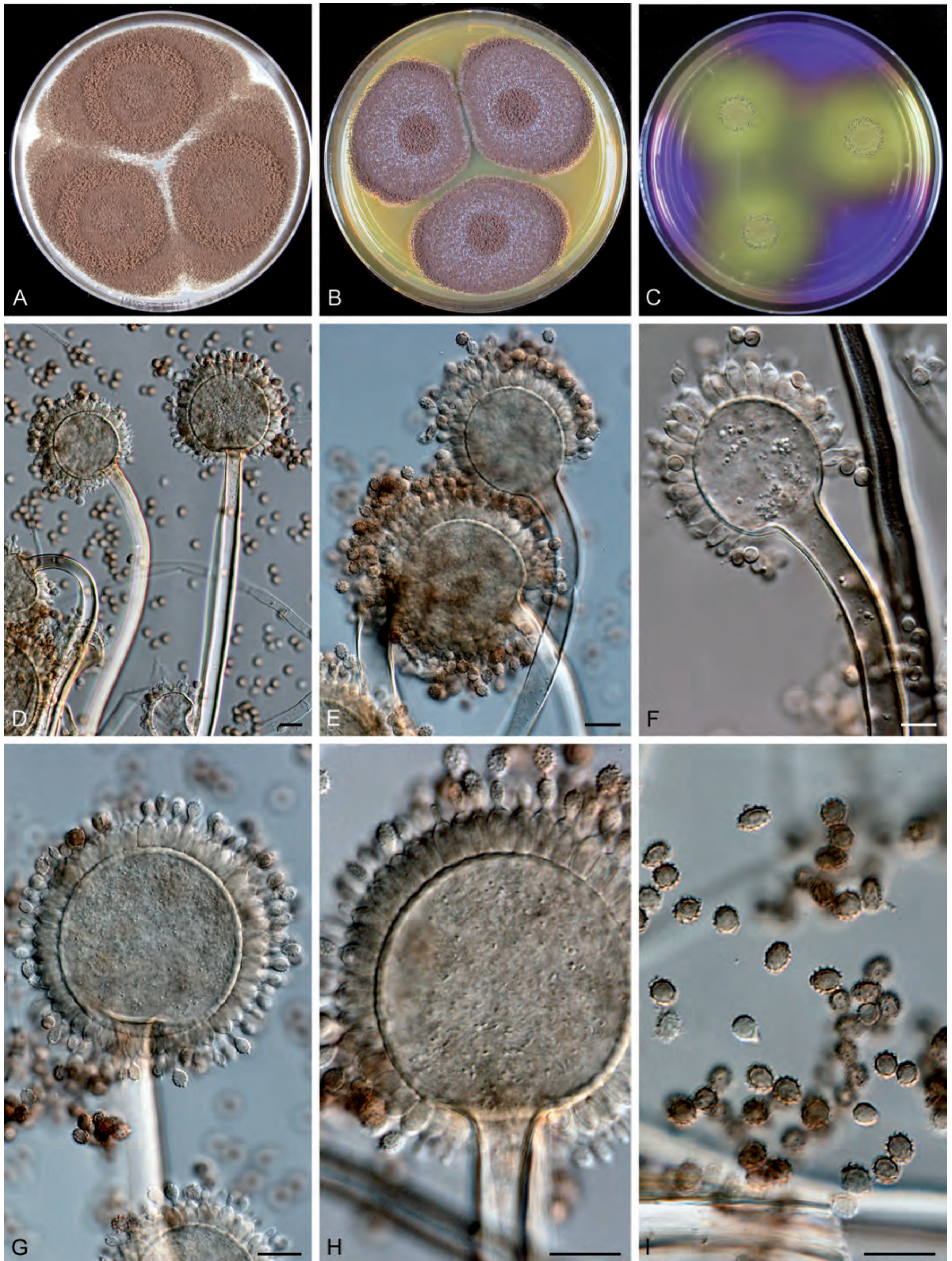


Fig. 13. *Aspergillus violaceofuscus* A–C. Colonies incubated at 25 °C for 7 d, A. CYA, B. MEA, C. CREA, D–I. Conidiophores and conidia. Scale bars = 10 µm.

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

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Growth and hydrolase profiles can be used as characteristics to distinguish *Aspergillus niger* and other black aspergilli

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Abstract: Wild type *Aspergillus niger* isolates from different biotopes from all over the world were compared to each other and to the type strains of other black *Aspergillus* species with respect to growth and extracellular enzyme profiles. The origin of the *A. niger* isolate did not result in differences in growth profile with respect to monomeric or polymeric carbon sources. Differences were observed in the growth rate of the *A. niger* isolates, but these were observed on all carbon sources and not specific for a particular carbon source. In contrast, carbon source specific differences were observed between the different species. *Aspergillus brasiliensis* is the only species able to grow on D-galactose, and *A. aculeatus* had significantly better growth on Locus Bean gum than the other species. Only small differences were found in the extracellular enzyme profile of the *A. niger* isolates during growth on wheat bran, while large differences were observed in the profiles of the different black aspergilli. In addition, differences were observed in temperature profiles between the black *Aspergillus* species, but not between the *A. niger* isolates, demonstrating no isolate-specific adaptations to the environment.

These data indicate that the local environment does not result in stable adaptations of *A. niger* with respect to growth profile or enzyme production, but that the potential is maintained irrespective of the environmental parameters. It also demonstrates that growth, extracellular protein and temperature profiles can be used for species identification within the group of black aspergilli.

INTRODUCTION

The genus *Aspergillus* consists of a large number of species, including several opportunistic pathogens (*e.g. A. fumigatus*, *A. terreus*), toxin producers (*e.g. A. flavus*, *A. parasiticus*) and industrial species (*A. niger*, *A. aculeatus*, *A. oryzae*). The genus is divided into several sections, such as the yellow and the black aspergilli. The black aspergilli (*Aspergillus* section *Nigri*) are cosmopolitan, and contain the most commonly used industrial species, *A. niger*.

Aspergillus niger has been collected from locations around the globe and is often among the most common species found in fungal communities, indicating that this species is able to propagate efficiently in a wide range of environments. *Aspergillus niger* and other black aspergilli grow predominantly on dead plant material, which consists mainly of cell walls. These cell walls contain polymeric components, such as cellulose, hemicellulose, pectin, lignin and proteins, of which the polysaccharides make up about 80 % of the biomass (de Vries & Visser 2001). *Aspergillus* cannot import polymeric compounds into the cell and therefore relies on enzymatic degradation to produce monomeric and small oligomeric carbon sources (de Vries & Visser 2001, de Vries 2003). Due to the large structural differences of the various plant polysaccharides, efficient degradation of these compounds relies on the production of a broad range of different enzymes. In addition, a tight regulatory system is required to ensure production of the right mixture of enzymes in the presence of a specific polysaccharide (de Vries & Visser 2001, de Vries 2003). Since different biotopes contain different plants (*e.g.* grasses *vs.* woods) and therefore different polysaccharides, different enzyme mixtures will be required for each biotope.

In light of this, one might expect that *Aspergillus* isolates from different biotopes have adapted to the available carbon source and produce different mixtures of enzymes to optimally utilise the available nutrients. Individual strains that have adapted to their environment might therefore grow less efficient in a different biotope. To study whether adaptation to the environment occurs we have compared 14 *A. niger* isolates from different global locations with respect to physiology, growth on different carbon sources, enzyme production and temperature profiles. In addition we also compared the ex-type strains of 14 species of black aspergilli to determine whether the differences between these species are larger than the differences between *A. niger* isolates from different biotopes. It was shown previously that *A. niger* can be distinguished from the other black aspergilli by the ability to grow in the presence of 20 % tannic acid, while the other species would only tolerate up to 5 % (Rippel 1939, van Diepeningen 2004). In this study we test a variety of non-toxic naturally occurring carbon sources to identidy species-specific differences in carbon utilisation.

MATERIALS AND METHODS

Strains, media and growth conditions

All strains used in this study are listed in Table 1. Strains were grown on Malt Extract Agar (MEA) or Minimal Medium, pH 6.0 (MM) (de Vries *et al.* 2004) as indicated in the text. For growth on solid MM medium, 1.6 % (w/v) agar was added to the medium before autoclaving. For the generation of spore suspensions, strains were grown for 14 d on MEA plates at 25 °C except for *A. piperis* CBS 112811. This strain was cultivated at 37 °C, because it sporulated poorly at 25 °C. Temperature profiles were also obtained on MEA plates.

All strains and isolates were grown at 30 °C, for carbon source analysis. As a positive control, 1 % glucose was added to the MM media. Polysaccharides were added to a final concentration of 0.5 %, while monosaccharides were added to a final concentration of 25 mM.

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Table 1. Strains used in this study; T indicates type strain for that species.

Strain	Correct identification	Original identification	Origin (code)	β-tubulin Acc. No.	ITS Acc. No.
CBS 564.65 T	*A. acidus*		Japan (JAP)	FJ639280	FJ639329
CBS 106.47	*A. acidus*	*A. niger*	Switzerland (SWI)	FJ639281	FJ639330
CBS 124.49	*A. acidus*	*A. niger*	Central America (CA)	FJ639282	FJ639331
CBS 139.48	*A. acidus*	*A. niger*	Ukraine (UKR)	FJ639283	FJ639332
CBS 172.66 T	*A. aculeaus*		Tropics (TR)	FJ639271	FJ639320
CBS 101740 T	*A. brasiliensis*		Brasil (BRA)	FJ639272	FJ639321
CBS 246.65	*A. brasiliensis*	*A. niger*	Australia (AUS)	FJ639273	FJ639322
CBS 733.88	*A. brasiliensis*	*A. niger*	USA (USA)	FJ639274	FJ639323
CBS 116970	*A. brasiliensis*	*A. niger*	The Netherlands (NLD)	FJ639275	FJ639324
CBS 111.26 T	*A. carbonarius*		Unknown	FJ639276	FJ639325
CBS 115574 T	*A. costaricaensis*		Costa Rica (COS)	FJ639277	FJ639326
CBS 553.65	*A. costaricaensis*	*A. niger*	Costa Rica (COS)	FJ639278	FJ639327
CBS 707.79 T	*A. ellipicus*		Costa Rica (COS)	FJ639279	FJ639328
CBS 117.55 T	*A. heteromorphus*		Brasil (BRA)	FJ639284	FJ639333
CBS 101889 T	*A. homomorphus*		Israel (ISR)	FJ639285	FJ639334
CBS 114.51 T	*A. japonicus*		Unknown	FJ639286	FJ639335
CBS 101883 T	*A. niger*	*A. lacticoffeatus*	Indonesia (INA)	FJ639287	FJ639336
CBS 554.65 T	*A. niger*		USA (USA)	FJ639288	FJ639337
CBS 120.49	*A. niger*		USA (USA)	FJ639289	FJ639338
CBS 113.50	*A. niger*		Germany (GER)	FJ639290	FJ639339
CBS 139.54	*A. niger*		Namibia (NAM)	FJ639291	FJ639340
CBS 242.93	*A. niger*		The Netherlands (NLD)	FJ639292	FJ639341
CBS 101698	*A. niger*		Kenya (KEN)	FJ639293	FJ639342
CBS 101705	*A. niger*		Canada (CAN)	FJ639294	FJ639343
CBS 117785	*A. niger*		Morocco (MOR)	FJ639295	FJ639344
CBS 118725	*A. niger*		The Netherlands (NLD)	FJ639296	FJ639345
CBS 112.32	*A. niger*		Japan (JAP)	FJ639297	FJ639346
CBS 139.52	*A. niger*		Japan (JAP)	FJ639298	FJ639347
CBS 118.36	*A. niger*		USA (USA)	FJ639299	FJ639348
CBS 630.78	*A. niger*		South-Pacific Islands (SPI)	FJ639300	FJ639349
CBS 115989	*A. niger*		Nigeria (NIG) (DSM genome)	FJ639301	FJ639350
CBS 113.46	*A. niger*		USA (USA) (JGI genome)	FJ639302	FJ639351
CBS 112811 T	*A. piperis*		Denmark (DEN)	FJ639303	FJ639352
CBS 134.48 T	*A. tubingensis*		Unknown	FJ639305	FJ639354
CBS 126.52	*A. tubingensis*		Unknown	FJ639306	FJ639355
CBS 103.12	*A. tubingensis*	*A. niger*	Germany (GER)	FJ639307	FJ639356
CBS 116.36	*A. tubingensis*	*A. niger*	Russia (RUS)	FJ639308	FJ639357
CBS 122.49	*A. tubingensis*	*A. niger*	Japan (JAP)	FJ639309	FJ639358
CBS 130.52	*A. tubingensis*	*A. niger*	USA (USA)	FJ639310	FJ639359
CBS 121600	*A. tubingensis*	*A. niger*	Egypt (EGY)	FJ639311	FJ639360
CBS 626.66	*A. tubingensis*	*A. niger*	France (FRA)	FJ639312	FJ639361
CBS 522.85	*A. tubingensis*	*A. niger*	India (IND)	FJ639313	FJ639362
CBS 116417	*A. tubingensis*	*A. niger*	Iran (IRA)	FJ639314	FJ639363
CBS 425.65	*A. tubingensis*	*A. niger*	Japan (JAP)	FJ639315	FJ639364
CBS 161.79	*A. tubingensis*	*A. niger*	India (IND)	FJ639316	FJ639365
CBS 306.80	*A. tubingensis*	*A. niger*	Spain (SPA)	FJ639317	FJ639366
CBS 107.55	*A. tubingensis*	*A. niger*	Brasil (BRA)	FJ639318	FJ639367
CBS 113365 T	*A. vadensis*		Unknown	FJ639319	FJ639368

Plates were inoculated with 2 µL spore suspension of each strain. Cultivations for the crude polysaccharide assay were done with spore suspensions with a concentration of 5 × 10^4 spores/mL. For serial dilutions, spore suspensions of 5 × 10^6, 5 × 10^5, 5 × 10^4 and 5 × 10^3 spores/mL were used. For temperature profiles, a concentration of 5 × 10^5 spores/mL was used. Liquid cultures for enzyme analysis were performed in MM with 1 % wheat bran (WB) and were inoculated to a final concentration of 0.5 × 10^6 spores/mL and were incubated at 30 °C for 2 d. Liquid cultures for chromosomal DNA analysis were performed using malt peptone (MP) broth containing 10 % (v/v) malt extract and 0.1 % (w/v) bacto-peptone, and were incubated at 25 °C for 3–4 d. All standard chemicals and carbon sources were obtained from Sigma.

Molecular Biology methods

DNA was extracted from mycelial samples using the Masterpure yeast DNA purification kit according to the instructions of the manufacturer. Fragments containing the ITS region were amplified using the primers LS266 (GCATTCCCAAACAACTCGACTC) and V9G [TTACGTCCCTGCCCTTTGTA, (Gerrits van den Ende & de Hoog 1999)]. Amplification of part of the β-tubulin gene was performed using the primers Bt2a (GGTAACCAAATCGGTGCTGCTTTC) and Bt2b [ACCCTCAGTGTAGTGACCCTTGGC, (Glass & Donaldson 1995)]. Both strands of the PCR fragments were sequenced with the ABI Prism® Big Dye™ Terminator v. 3.0 Ready Reaction Cycle sequencing Kit. Samples were analysed on an ABI PRISM 3700 Genetic Analyzer and contigs were assembled using the forward and reverse sequences with the programme SeqMan from the LaserGene package. Sequences were aligned in Molecular Evolutionary Genetics Analysis (MEGA) v. 4 using CLUSTALW. The Phylogenetic trees were established with Maximum Parsimony method in MEGA v. 4. To determine the support for each clade, a bootstrap analysis was performed with 500 replications.

Enzyme assays and protein profiles

Extracellular hydrolytic activities were assayed using 0.01 % substrate, 20–40 µL sample and 25 mM sodium acetate pH 5.0 in a total volume of 100 µL. The mixtures were incubated for 1 h at 30 °C after which the reaction was stopped by adding 100 µL 0.25 M Na_2CO_3. Absorbance was measured at 405 nm in a microtiter plate reader. The activity was calculated using a standard curve of *p*-nitrophenol. The substrates used for enzyme assays were all obtained from Sigma and were *p*-nitrophenol-α-arabinofuranoside, *p*-nitrophenol-β-xylopyranoside, *p*-nitrophenol-β-galactopyranoside, *p*-nitrophenol-α-galactopyranoside, *p*-nitrophenol-β-glucopyranoside and *p*-nitrophenol-β-mannopyranoside to measure α-arabinofuranosidase, β-xylosidase, β-galactosidase, α-galactosidase, β-glucosidase and β-mannosidase, respectively. Culture filtrate samples were separated on 10 % SDS-PAGE gels and stained using silver-staining.

RESULTS

Identification of putative *A. niger* wild isolates

The CBS database was searched for *A. niger* isolates obtained from a wide variety of locations around the world, resulting in 34 isolates. In addition to these, the parent of the *A. niger* strain sequenced by DSM (Pel *et al.* 2007) and the strain sequenced by the Joint Genome Institute of the US Department of Energy (Baker 2006) were also included in the study. To confirm that these strains were true *A. niger* strains, the ITS and β-tubulin sequences of these strains were compared to those of the ex-type strains of the different black aspergilli (Fig. 1). This demonstrated that from the 34 isolates only 14 were *A. niger* strains. The other strains were members of *A. tubingensis* (13), *A. brasiliensis* (3), *A. acidus* (3) and *A. costaricaensis* (1). The 14 *A. niger* isolates as well as the sequenced strains were used for the rest of the study in comparison to the ex-type strains of the different black aspergilli, while the other isolates were eliminated from the study. The remaining *A. niger* isolates still represent a worldwide distribution.

Growth profiles of *A. niger* isolates and type strains from *Aspergillus* section *Nigri*

All *A. niger* isolates have similar growth profiles on monosaccharides (Table 2, Fig. 2). CBS 115989 grows significantly slower than the other isolates on all monomeric carbon sources. In contrast, carbon source specific differences were observed between the different black aspergilli (Table 2, Fig. 2). *Aspergillus brasiliensis* was the only species that was able to grow on D-galactose, and this species characteristic was confirmed for three other *A. brasiliensis* strains (data not shown). No or minimal growth was detected for *A. piperis*, *A. ellipticus* and *A. heteromorphus* on all carbon sources. Growth on L-rhamnose was only observed for *A. lacticoffeatus*, *A. niger*, *A. brasiliensis*, *A. tubingensis*, *A. costaricaensis* and *A. aculeatus* (Table 2, Fig. 2).

Growth on plant polysaccharides was also tested, as they are a major natural carbon source of aspergilli. The strain specific growth differences of the *A. niger* isolates observed on monomeric carbon sources were also observed on polysaccharides. All *A. niger* isolates grew best on starch and pectin, while slower growth was observed on xylan, arabinogalactan and Locust Bean gum (contains mainly galactomannan) (Table 3, Fig. 3). Very poor growth was observed on cellulose (Table 3, Fig. 3). In contrast, significant differences were observed when the *Aspergillus* ex-type strains were compared. Similar to the monomeric carbon sources, no growth was observed on any of the polysaccharides for *A. piperis* and *A. ellipticus*, but growth of *A. heteromorphus* on arabinogalactan and Locus Bean gum was better than on any of the monomeric carbon sources (Table 3, Fig. 3). Nearly all the other species preferred starch and pectin, as was observed for the *A. niger* isolates (Table 3, Fig. 3). An exception was *A. aculeatus*, which grew equally well on Locust Bean gum, pectin and starch. *Aspergillus niger*, *A. carbonarius*, *A. tubingensis*, *A. costaricaensis*, *A. homomorphus*, *A. aculeatus* and *A. japonicus* grew better on xylan than the other species, while significant growth on cellulose was only observed for *A. aculeatus*, *A. japonicus* and *A. homomorphus* (Table 3, Fig. 3).

Protein and enzyme profiles of *A. niger* isolates and ex-type strains from *Aspergillus* section *Nigri*

Growth on polysaccharides is dependent on the production of extracellular enzymes that degrade these polymers to monomeric and small oligomeric compounds that can be taken up by the fungus. We therefore determined the extracellular protein profile and assayed the production of six polysaccharide hydrolases during growth on wheat bran: α-arabinofuranosidase (ABF, involved in xylan, xyloglucan and pectin degradation), β-xylosidase (BXL,

Table 2. Growth of the *A. niger* strains on monosaccharides in comparison to the ex-type strains of the black aspergilli. Glc = D-glucose, Gal = D-galactose, Rha = L-rhamnose, FRC = D-fructose, Xyl = D-xylose, Ara = L-arabinose.

Species	Strain	Glc	Gal	Rha	Frc	Xyl	Ara
A. acidus	CBS 564.65 T	+	-	-	+	+	±
A. aculeatus	CBS 172.66 T	+++	-	-	+++	+++	++
A. brasiliensis	CBS 101740 T	+++	+	+	+++	+++	+
A. carbonarius	CBS 111.26 T	+++	-	-	+++	+++	++
A. costaricaensis	CBS 115574 T	+++	-	+	+++	+++	++
A. ellipicus	CBS 707.79 T	-	-	-	-	-	-
A. heteromorphus	CBS 117.55 T	-	-	-	-	-	-
A. homomorphus	CBS 101889 T	++	-	+	++	++	+
A. japonicus	CBS 114.51 T	+++	-	+	+++	+++	++
A. lacicoffeaus	CBS 101883 T	+++	-	+	+++	+++	++
A. piperis	CBS 112811 T	-	-	-	-	-	-
A. tubingensis	CBS 134.48 T	+++	-	+	+++	+++	++
A. vadensis	CBS 113365 T	++	-	-	++	++	±
A. niger	CBS 554.65 T	++++	-	±	++++	++++	+
	CBS 120.49	+++	-	+	+++	+++	++
	CBS 113.50	++	-	±	++	++	±
	CBS 139.54	++	-	+	++	++	±
	CBS 262.65	+++	-	+	+++	+++	++
	CBS 242.93	+++	-	+	+++	+++	+
	CBS 101698	+++	-	±	+++	+++	+
	CBS 101705	+++	-	±	+++	+++	+
	CBS 117785	+++	-	±	+++	+++	+
	CBS 118725	+++	-	+	+++	+++	++
	CBS 112.32	+++	-	+	+++	+++	++
	CBS 139.52	++++	-	+	++++	++++	++
	CBS 118.36	++++	-	+	++++	++++	++
	CBS 630.78	++++	-	+	++++	++++	++
	CBS 115989	++	-	+	++	++	±
	CBS 113.46	+++	-	+	+++	+++	+

Table 3. Growth of the *A. niger* strains on polysaccharides in comparison to the ex-type strains of the black aspergilli. CEL = cellulose, ABG = arabinogalactan, LBG = locust bean gum (galactomannan), BWX = beechwood xylan, CP = citrus pectin.

Species	Strain	Starch	CEL	ABG	LBG	BWX	CP
A. acidus	CBS 564.65 T	+	-	+	+	-	+
A. aculeatus	CBS 172.66 T	+++	+	+	++	+	++
A. brasiliensis	CBS 101740 T	+++	-	+	+++	±	++
A. carbonarius	CBS 111.26 T	+++	-	+	+	+	++
A. costaricaensis	CBS 115574 T	+++	-	+	++	+	++
A. ellipicus	CBS 707.79 T	-	-	-	-	-	-
A. heteromorphus	CBS 117.55 T	-	-	±	±	-	±
A. homomorphus	CBS 101889 T	++	+	+	+	++	++
A. japonicus	CBS 114.51 T	+++	+	+	+	+	++
A. lacicoffeaus	CBS 101883 T	+++	-	+	+++	+	++
A. piperis	CBS 112811 T	-	-	-	-	-	-
A. tubingensis	CBS 134.48 T	+++	-	+	+	+	++
A. vadensis	CBS 113365 T	++	-	+	+	-	+
A. niger	CBS 554.65 T	++++	-	+	++	+	+++
	CBS 120.49	+++	-	++	++	++	+++
	CBS 113.50	+++	-	++	++	++	+++

Table 3. (Continued).

Species	Strain	Starch	CEL	ABG	LBG	BWX	CP
A. niger	CBS 139.54	++	-	+	+	+	++
	CBS 262.65	+	-	+	+	+	+
	CBS 242.93	++++	-	++	++	++	+++
	CBS 101698	+++	-	++	++	++	+++
	CBS 101705	+++	-	++	++	++	+++
	CBS 117785	+++	-	++	++	+	+++
	CBS 118725	+++	-	++	++	+	+++
	CBS 112.32	++	-	+	+	++	++
	CBS 139.52	+++	-	++	+	+	+++
	CBS 118.36	+++	-	++	+	+	+++
	CBS 630.78	+++	-	++	±	++	+++
	CBS 115989	+	-	+	+	+	±
	CBS 113.46	++	-	++	++	+	+++

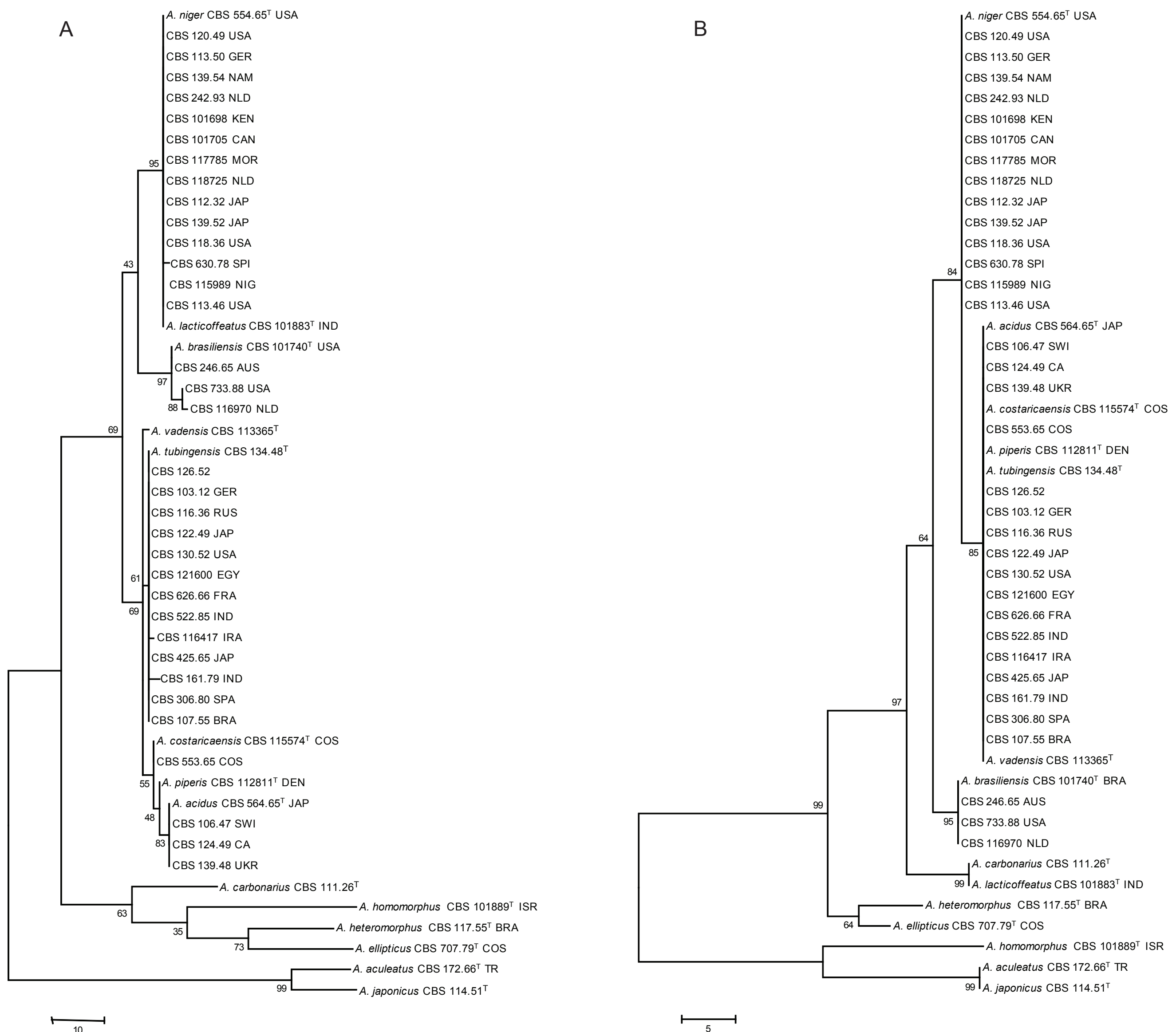

Fig. 1. Phylogeny of the strains used in this study. A. Maximum Parsimony tree based on the β-tubulin sequence. B. Maximum Parsimony tree based on the ITS sequence. The origin abbreviation refers to Table 1.

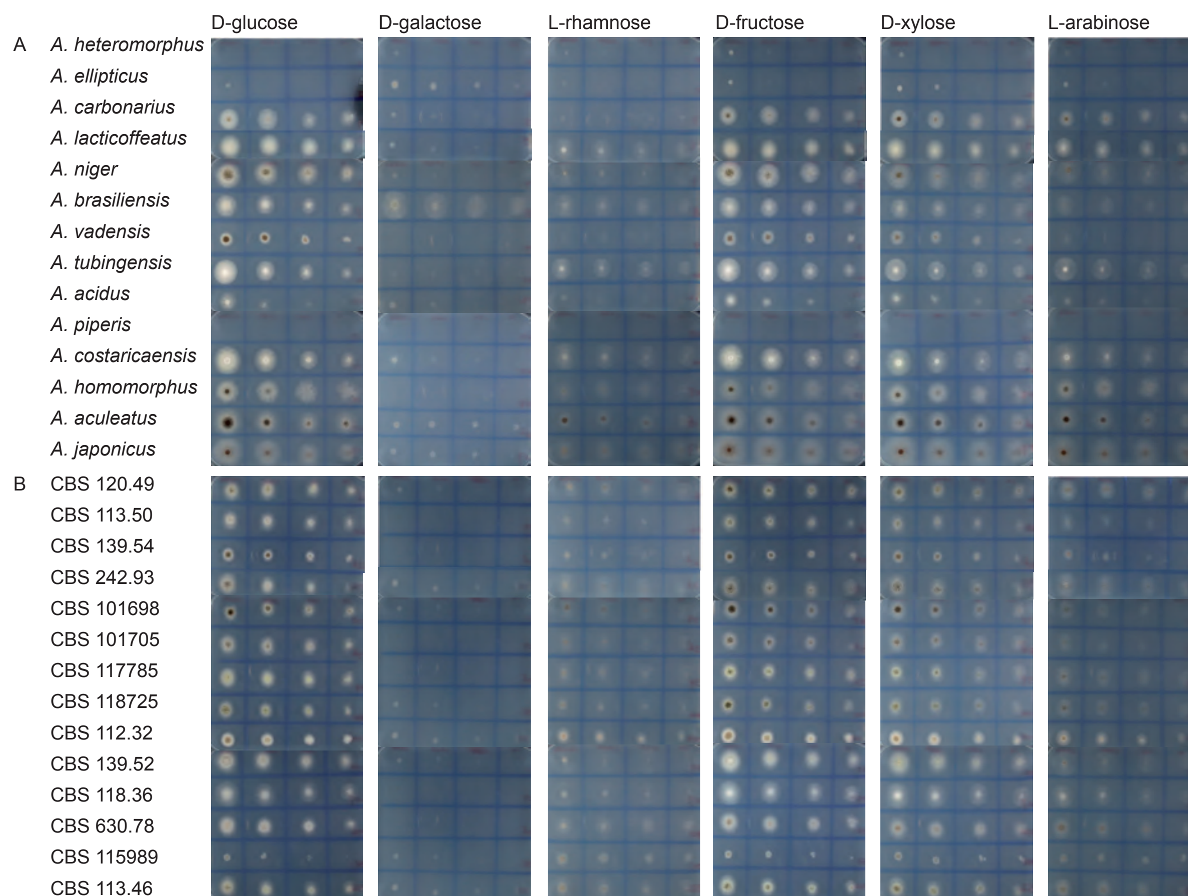

Fig. 2. Growth of ex-type strains of *Aspergillus* section *Nigri* (A) and *A. niger* isolates (B) on monomeric carbon sources. Strains were inoculated as serial dilutions (left to right) of 10000, 1000, 100 and 10 spores.

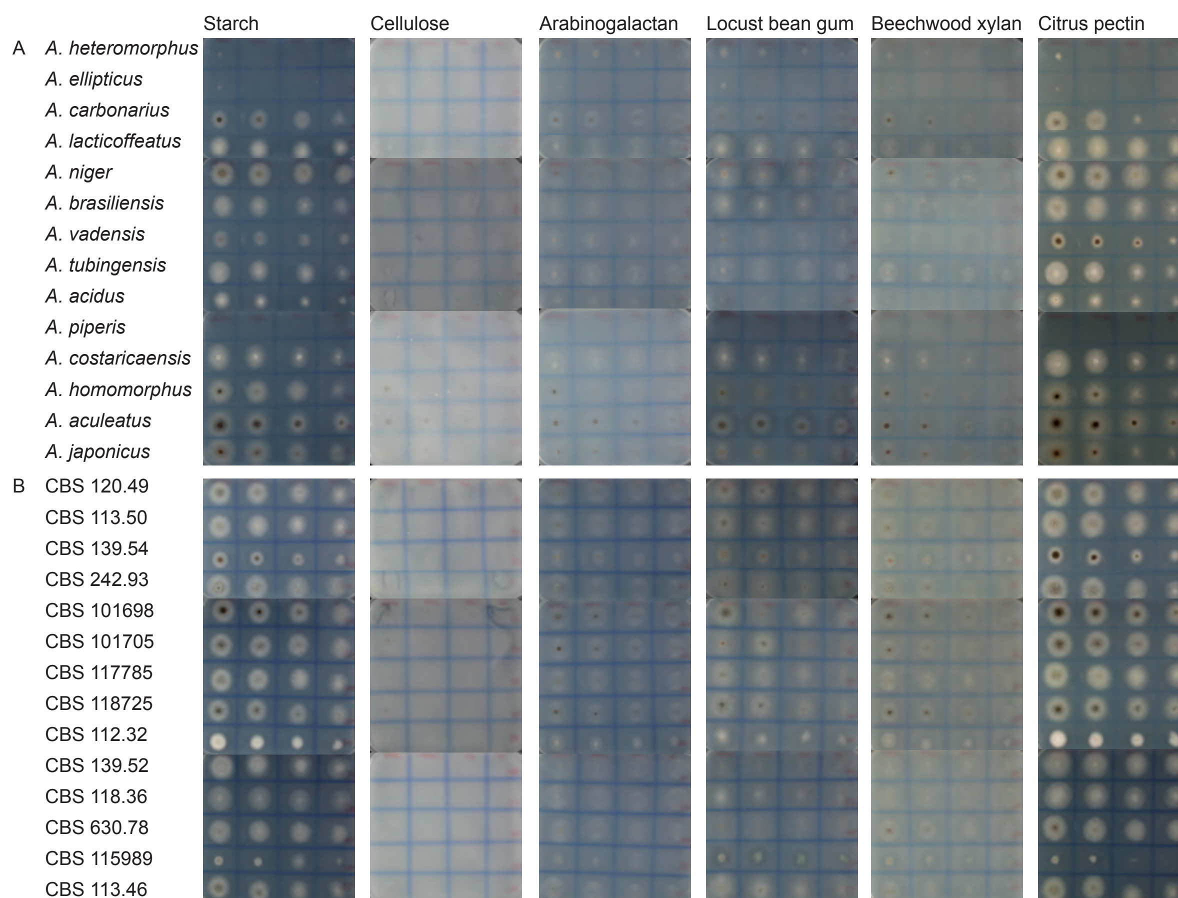

Fig. 3. Growth of ex-type strains of *Aspergillus* section *Nigri* (A) and *A. niger* isolates (B) on polymeric carbon sources. Strains were inoculated as serial dilutions (left to right) of 10000, 1000, 100 and 10 spores.

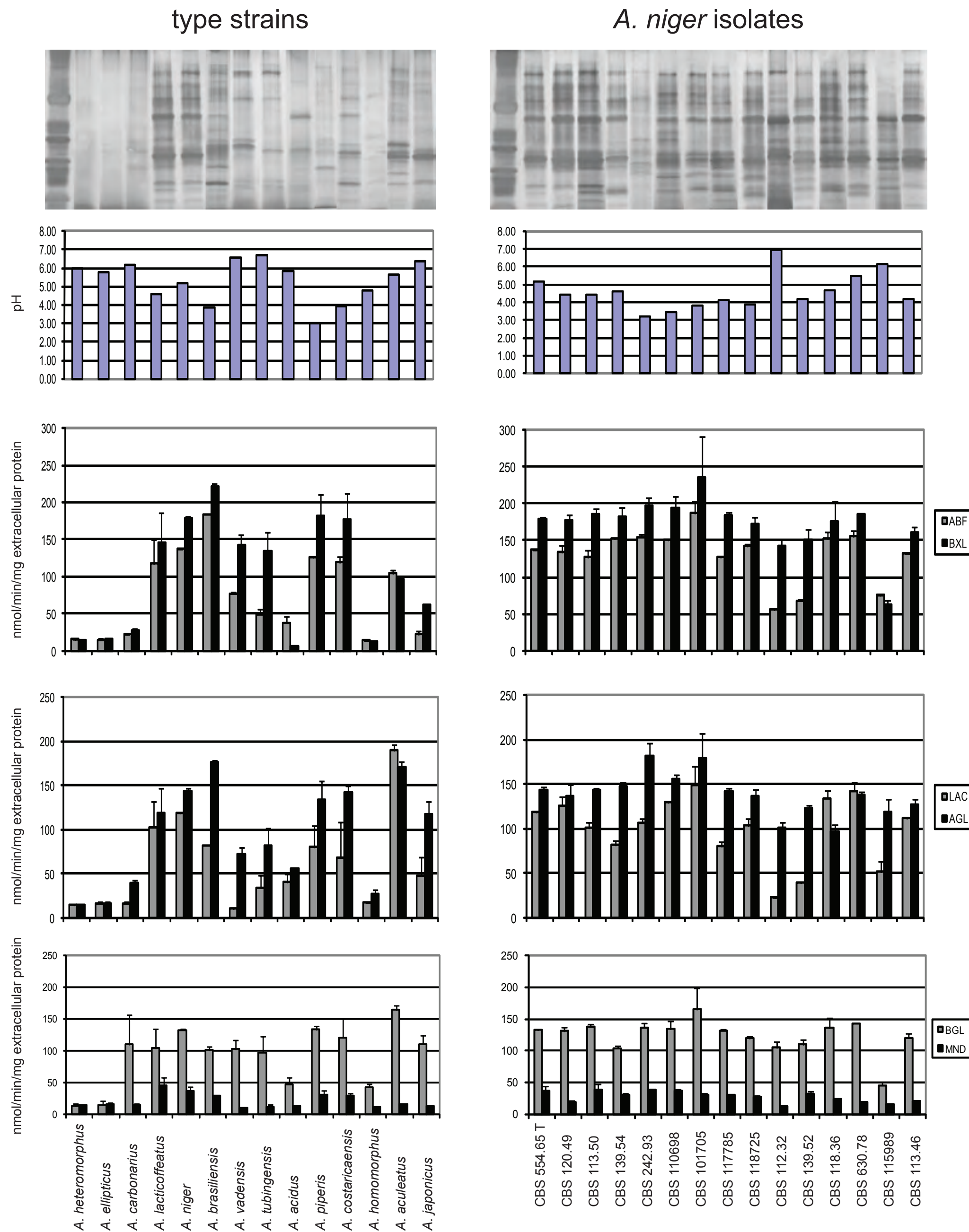

Fig. 4. SDS-PAGE profiles, pH, total secreted protein and hydrolytic activities of *A. niger* isolates and ex-type strains of *Aspergillus* section *Nigri* after growth on wheat bran. ABF = α-L-arabinofuranosidase, BXL = β-xylosidase, LAC = β-galactosidase, AGL = α-galactosidase, BGL = β-glucosidase, MND = β-mannosidase.

involved in xylan degradation), β-galactosidase (LAC, involved in xylan, xyloglucan, pectin and galactomannan degradation), α-galactosidase (AGL, involved in galactomannan degradation), β-glucosidase (involved in cellulose and galactoglucomannan degradation) and β-mannosidase (involved in galactomannan degradation). The protein profiles were highly similar for the *A. niger* isolates and *A. lacticoffeatus*, while significant differences were detected between the other species (Fig. 4). The pH at the moment of sampling varied both between the species and within the *A. niger* group, although most *A. niger* isolates acidified the medium (Fig. 4). The enzyme activity profiles of the *A. niger* isolates were also highly similar (Fig. 4). Some variation in activity levels were detected with CBS 112.32 and CBS 115989 often producing lower levels than the other *A. niger* isolates. Larger differences were observed between the different *Aspergillus* species (Fig. 4). *Aspergillus carbonarius*, *A. ellipticus* (poor growth), *A. acidus*, *A. heteromorphus* (poor growth) and *A. homomorphus* has significantly lower production of ABF, BXL, LAC, AGL, BGL and MND than the other species. The same applies for *A. japonicus* for ABF and BXL. The highest ABF and BXL activity was observed for *A. brasiliensis*, while the highest LAC and BGL activity was observed for *A. aculeatus* and the highest AGL activity for *A. brasiliensis* and *A. aculeatus* (Fig. 4). MND activity was low for all strains in comparison with the other enzyme activities.

Temperature profiles of the *A. niger* isolates and ex-type strains from *Aspergillus* section *Nigri*

The absence of growth of *A. piperis* and *A. ellipticus* on all carbon sources on solid media, but not in liquid media with wheat bran raised questions about the temperature tolerance of these species on solid media. To determine whether there were significant differences in the temperature profiles of the strains of this study, they were grown on MEA plates at temperatures ranging from

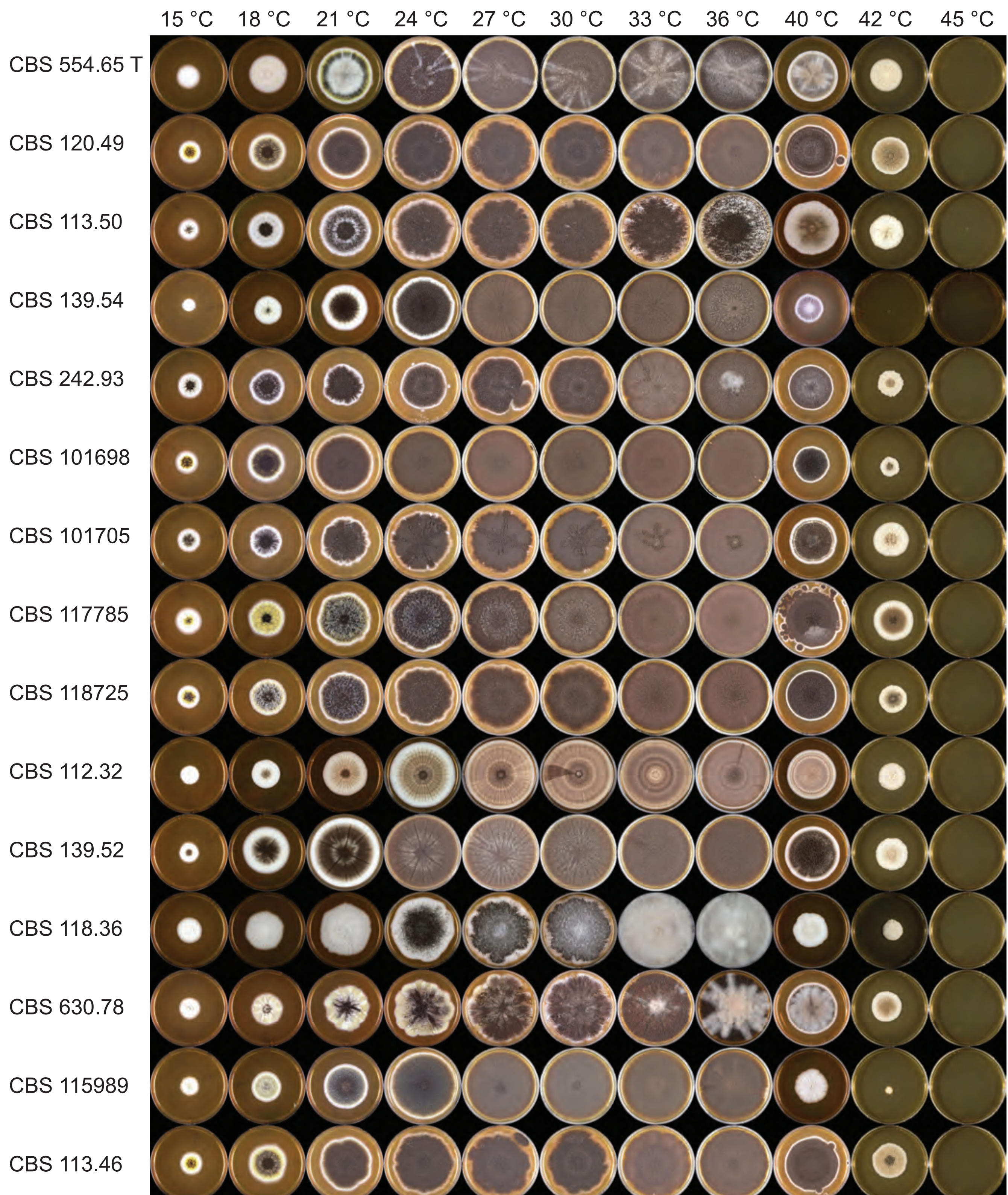

Fig. 5. Growth of the *A. niger* isolates at different temperatures. Pictures were taken after 10 d.

6 °C to 45 °C. All *A. niger* isolates had nearly identical temperature profiles, with 33–36 °C as optimal temperature (Fig. 6). More differences were observed between the different *Aspergillus* species (Fig. 5). *Aspergillus brasiliensis* grew very poorly at 15 °C. *Aspergillus ellipticus* only showed residual growth at 30 °C (Fig. 7), which was confirmed for a second *A. ellipticus* isolate (data not shown). *Aspergillus heteromorphus* showed only minimal growth at 33 °C, while the same was true at 36 °C for *A. japonicus*, *A. aculeatus*, *A. homomorphus* and *A. carbonarius*. The other species were still able to grow at 42 °C, but none of the species were able to grow at 45 °C.

DISCUSSION

Aspergillus niger is commonly found throughout the world and is therefore capable of growing in a large variety of biotopes with highly

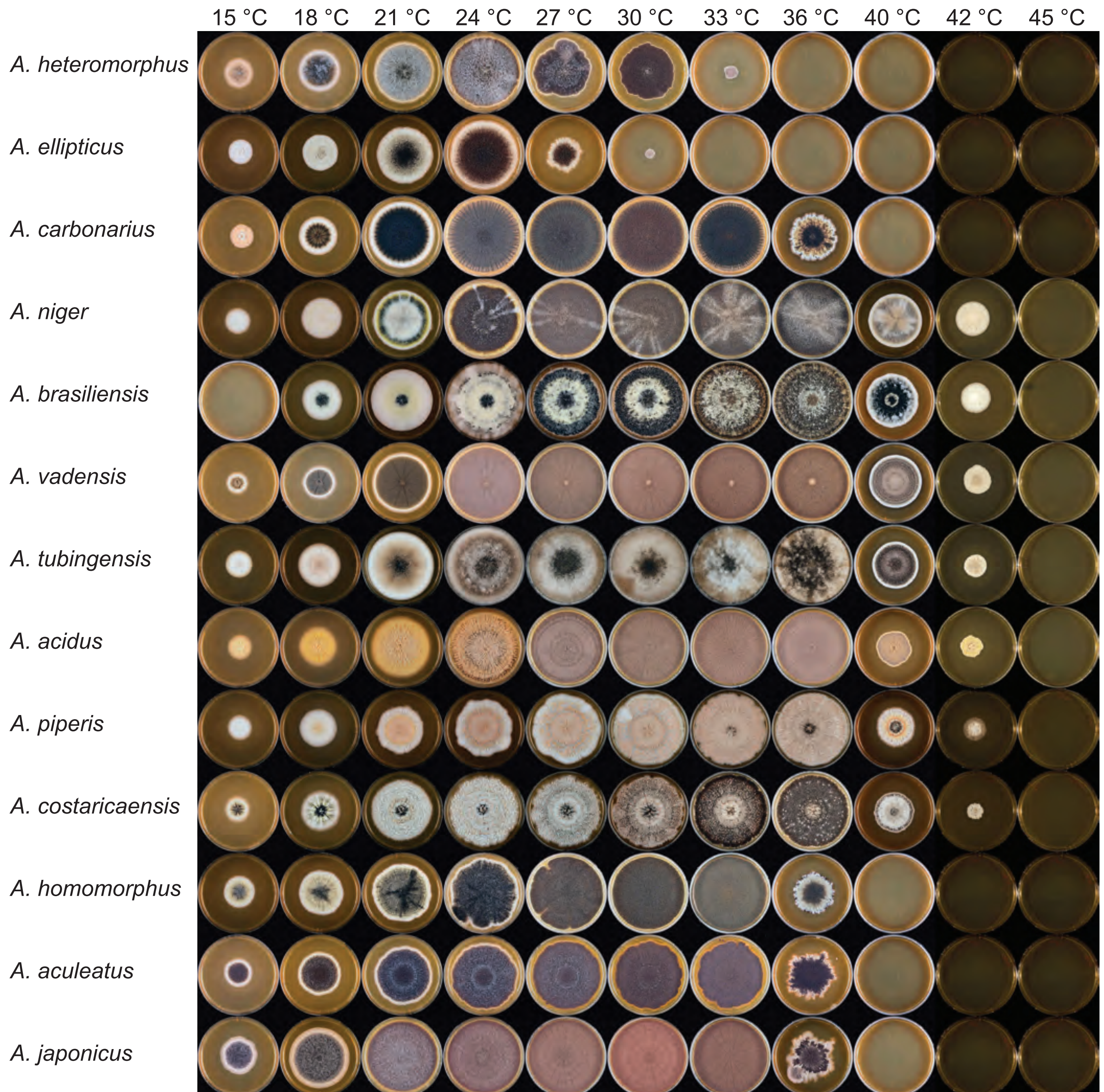

Fig. 6. Growth of ex-type strains of *Aspergillus* section *Nigri* at different temperatures. Pictures were taken after 10 d.

different environmental conditions, such as nature of available carbon sources and other nutrients, temperature and humidity. In this study we evaluated whether the global origin of an *A. niger* isolate affects its carbon source profile as this would indicate that the isolates adapt to their local environment. Sequence-based identification of the 34 *A. niger* isolates selected from the CBS database, demonstrated that only 14 were true *A. niger* strains. The others mainly belonged to species that were previously shown to be closely related to *A. niger* (Samson *et al.* 2007) and this result demonstrates that the classification based on morphology is not sufficient for species identification. A previous study (van Diepeningen 2004) demonstrated that 40 % of black aspergilli isolates from soil belong to *A. niger* and another 40 % to *A. tubingensis*, providing a similar species dispersion as obtained in our study.

The 14 remaining *A. niger* isolates still represent a global distribution as they include 3 isolates from North-America, 4 isolates from North-western Europe, 4 isolates from Africa, 2 isolates from Asia and 1 isolate from the South-Pacific islands. As the climates and biotopes are very different in these areas it can be concluded that the strains were isolated from significantly different environments. Unfortunately, for most isolates the material they were collected from was not indicated, so it is impossible to describe the strains based on their natural carbon source at the moment of isolation.

Although some *A. niger* isolates grow faster than others, no carbon source specific differences were found between the strains, either on monomeric or polymeric carbon sources. This indicates that the ability to grow on the range of carbon sources tested in this study is maintained among all the isolates, even though they were isolated from environments that differ strongly in their carbon source composition. It can therefore be concluded that adaptation to the natural environment does not occur at the genetic level for *A. niger* and its ability to utilise various carbon sources. It could

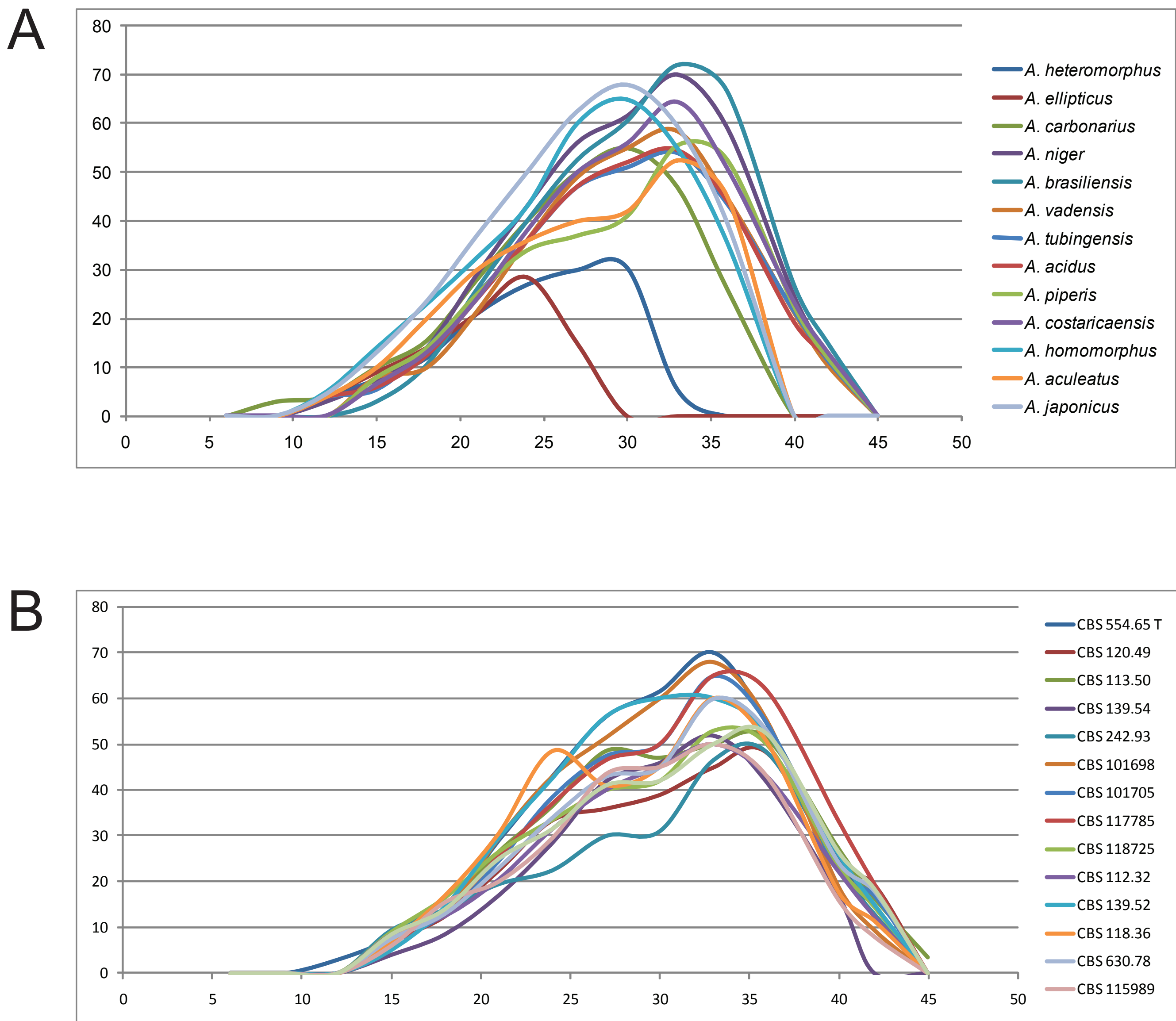

Fig. 7. Growth curves of the type strains (A) and the *A. niger* isolates (B). Growth curves were determined by the colony diameter (mm) after 4 d incubation.

be that metabolic adaptation occurs during growth in different environments, but this does not result in a permanent alteration of the ability of the strain to consume a wide range of carbon sources. A previous study (van Diepeningen 2004) suggested that the air-borne and UV-resistant characteristics of the spores result in world-wide well-mixed population of *A. niger* isolates. Wind-based distribution would result in highly varied biotopes for the spores of a particular isolate. Specialisation to specific carbon sources would then be a disadvantage to an isolate. A recent study by Rokas *et al*. (2007) compared the two *A. niger* strains that were used for genome sequencing, CBS 513.88 (a descendent from CBS 115989) and ATCC 1015 (CBS 113.48). They identified differences between the strains at the level of colony morphology. Another study (Pal *et al.* 2007) demonstrated that the two strains were heterokaryon incompatible, indicating that they do not have a (recent) clonal relation. Non clonal linkages often vary in gene expression and growth rates that in some cases can be attributed to the occurrence of dsRNA mycoviruses (van Diepeningen *et al.* 2006). In the current study the main difference between CBS 115989 and CBS 113.46 was the slower growth of CBS 115989, which confirms that these strains are not identical. However, they did not differ in their carbon source growth profile.

In contrast, significant differences were observed between the different black *Aspergillus* species, demonstrating that the interspecies variation with respect to carbon source utilisation is larger than the variation within a species. The absence of growth on D-galactose for all the black aspergilli has been reported before (de Vries *et al.* 2005), but our study demonstrate that *A. brasiliensis* is able to grow on this substrate. This suggests a significant difference between this species and the other black aspergilli. Whether the difference is at the level of sugar transport or metabolism is not clear at this point. Previous studies with an *A. niger* high affinity hexose transporter demonstrated that this protein could transport D-glucose, D-fructose and D-mannose, but not D-galactose (vanKuyk *et al.* 2004), indicating that D-galactose transport may be different from the other hexoses.

The absence of growth on plates of *A. ellipticus* can be explained by its temperature profile, as this strain is not able to grow above 27 °C and the experiment was performed at 30 °C. This appears to be a species characteristic, as a second *A. ellipticus* strain that was tested showed the same temperature profile. *Aspergillus ellipticus* did show slow growth at 30 °C in liquid shaken culture, indicating that the culture set-up affects its ability to cope with high temperatures. The culture conditions cannot explain the absence of growth on carbon source test plates for *A. piperus*, especially since

the same strain grew very well in liquid culture at 30 °C and also was able to grow on malt extract agar plates at temperatures up to 42 °C. Possibly, minimal medium lacks a specific component (*e.g.* an amino acid) that cannot be synthesised sufficiently by *A. piperus* itself, but that is present in both MEA and wheat bran.

These results suggest that growth profiles on defined media and at different temperatures can be used as a first step in the identification of different black *Aspergillus* species, as they do not differentiate between strains of the same species isolated from different environments.

No strong differences were observed in hydrolase production between the *A. niger* isolates during growth on wheat bran. Wheat bran was used as a substrate as it has been shown to induce the production of a large variety of hydrolases by *Aspergillus* (Yamane *et al.* 2002, Kang *et al.* 2004). Strain CBS 115989 overall had lower levels of activity than the other *A. niger* isolates, but this strain also grew significantly slower on all substrates than the other isolates. Based on the activity profile, CBS 101705 is the best producer of ABF, BXL, LAC and BGL, while CBS 242.93 is the best producer of AGL. These differences demonstrate that the variety among natural isolates with respect to enzyme production could be exploited for selection of novel production strains or for understanding the factors (*e.g.* regulators, metabolic differences) that affect production of specific enzymes.

Similar to the growth experiments, much larger differences in hydrolase production were observed between the *Aspergillus* species than between the *A. niger* isolates. Production of all hydrolases was particularly low in *A. ellipticus*, *A. acidus*, *A. heteromorphus*, *A. homomorphus* and *A. carbonarius* (except for BGL). For *A. ellipticus* this can be explained by poor growth at this temperature, while in the case of *A. acidus* this is partly caused by a high extracellular protein production, but a low absolute enzyme activity. Except for *A. acidus*, all species with low activity cluster together in the phylogeny of the black aspergilli (Samson *et al.* 2007), suggesting that this phenomenon can be traced back to the combined origin of these species. The strong similarity between *A. lacticoffeatus* and the *A. niger* isolates is easily explained as recent studies showed that *A. lacticoffeatus* is in fact the same as *A. niger* (Varga *et al.* 2011). This suggests that species identification can already largely be determined using SDS-PAGE profiles after growth on wheat bran for the black aspergilli, which would be a relative easy tool that could also be applied in low-tech facilities. SDS-PAGE profiles of intracellular samples have been used previously for species identification when comparing isolates of *A. niger*, *A. nidulans*, *A. flavus* and *A. fumigatus* (Rath 2001). However, these profiles are more complex and more sensitive to variation (de Vries *et al*., unpubl. results).

Identification of the proteins that are secreted by these species would be interesting as this may shed some light on their physiology in the presence of crude carbon sources. Polysaccharide hydrolases have mainly been purified from *A. niger* and *A. aculeatus*, while some have also been reported from *A. acidus*, *A. japonicus*, *A. tubingensis*, *A. carbonarius* and *A. brasiliensis* (Takada *et al.* 1999, Brumbauer *et al.* 2000, van Casteren *et al.* 2000, Decker *et al.* 2000, Ademark *et al.* 2001a, 2001b, de Vries & Visser 2001, Kiss *et al.* 2002, el-Gindy 2003, Liu *et al.* 2007, Pedersen *et al.* 2007). No papers about polysaccharide hydrolases have been reported for any of the other species. The data of the current study indicates that some of these species (*e.g. A. piperis*) could be interesting sources of hydrolytic enzymes, which may have different properties from those described previously.

In summary, this study demonstrates that *A. niger* isolates have a similar potential for growth on monomeric and polymeric sugars as well as their polysaccharide hydrolase profiles, even when they have been isolated from significantly different biotopes. In contrast, strong differences were found in growth and hydrolase profiles among closely related *Aspergillus* species, indicating that these parameters may be considered species characteristics.

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Analysis of regulation of pentose utilisation in *Aspergillus niger* reveals evolutionary adaptations in *Eurotiales*

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Abstract: Aspergilli are commonly found in soil and on decaying plant material. D-xylose and L-arabinose are highly abundant components of plant biomass. They are released from polysaccharides by fungi using a set of extracellular enzymes and subsequently converted intracellularly through the pentose catabolic pathway (PCP).

In this study, the L-arabinose responsive transcriptional activator (AraR) is identified in *Aspergillus niger* and was shown to control the L-arabinose catabolic pathway as well as expression of genes encoding extracellular L-arabinose releasing enzymes. AraR interacts with the D-xylose-responsive transcriptional activator XlnR in the regulation of the pentose catabolic pathway, but not with respect to release of L-arabinose and D-xylose.

AraR was only identified in the *Eurotiales*, more specifically in the family *Trichocomaceae* and appears to have originated from a gene duplication event (from XlnR) after this order or family split from the other filamentous ascomycetes. XlnR is present in all filamentous ascomycetes with the exception of members of the *Onygenales*. Since the *Onygenales* and *Eurotiales* are both part of the subclass *Eurotiomycetidae*, this indicates that strong adaptation of the regulation of pentose utilisation has occurred at this evolutionary node. In *Eurotiales* a unique two-component regulatory system for pentose release and metabolism has evolved, while the regulatory system was lost in the *Onygenales*. The observed evolutionary changes (in *Eurotiomycetidae*) mainly affect the regulatory system as in contrast, homologues for most genes of the L-arabinose/D-xylose catabolic pathway are present in all the filamentous fungi, irrespective of the presence of XlnR and/or AraR.

INTRODUCTION

The order *Eurotiales* consists of the families *Trichocomaceae* and *Elaphomycetaceae*. Most species belonging to the *Trichocomaceae* are saprobic filamentous ascomycetes, which in nature grow predominantly in soil or on decaying plant material. The *Elaphomycetaceae* entails a family of underground, saprobic or mycorrhiza-forming fungi. The family *Trichocomaceae* includes the well-known genera of *Penicillium* and *Aspergillus*. Aspergilli are found throughout the world in almost all ecosystems and are well-known for their ability to degrade different complex plant polymers. Despite the fact that some *Aspergillus* species have evolved additional lifestyles, for example as human or plant pathogens, there seems to be no restriction to a specific niche concerning their saprobic lifestyle.

Decaying plant material consists for a major part of plant cell wall polysaccharides which can be split into three major groups: cellulose, hemicellulose and pectin. L-arabinose and/or D-xylose are the main components of the hemicelluloses arabinoxylan and xyloglucan, and of pectin. Release of these sugars from polysaccharides as well as metabolic conversion of them through the pentose catabolic pathway (PCP) has been studied for many years, particularly in *Aspergillus* and the genus *Trichoderma* belonging to the order *Hypocreales* [reviewed in (de Vries & Visser 2001, de Vries 2003, Stricker *et al.* 2008)]. The PCP was first described in *Aspergillus niger* (Witteveen *et al.* 1989) and shown to consist of a series of reversible reductase/dehydrogenase steps followed by phosphorylation to D-xylulose-5-phosphate, which enters the pentose phosphate pathway (PPP). In *A. niger,* the gene encoding D-xylose reductase (*xyrA*) (Hasper *et al.* 2000), D-xylulokinase (*xkiA*) (vanKuyk *et al.* 2001), L-arabitol dehydrogenase (*ladA*) and xylitol dehydrogenase (*xdhA*) (Seiboth *et al.* 2003, de Groot *et al.* 2007) have been characterised. For *Trichoderma reesei,* genes encoding L-arabitol dehydrogenase (*lad1*) (Richard *et al.* 2001) and xylitol dehydrogenase (*xdh1*) (Seiboth *et al.* 2003) have been described. In *A. niger*, induction of pentose release and the PCP occurs in the presence of L-arabinose and/or D-xylose (Witteveen *et al.* 1989). In the presence of D-xylose, the xylanolytic transcriptional activator XlnR (van Peij *et al.* 1998b) regulates the expression of genes encoding extracellular polysaccharide degrading enzymes, as well as the expression of *xyrA* [reviewed in (de Vries 2003)]. L-arabinose induction of the PCP is not mediated via XlnR. The genes of the L-arabinose catabolic pathway are co-regulated with the genes encoding extracellular arabinanolytic enzymes (α-L-arabinofuranosidase and endoarabinanase) (Flipphi *et al.* 1994, de Vries *et al.* 1994) and L-arabitol is most likely the inducer (de Vries *et al.* 1994, vanKuyk *et al.* 2001). Analysis of *A. niger* arabinanolytic regulatory mutants, *araA* and *araB*, demonstrated an antagonistic effect between XlnR and the L-arabinose/L-arabitol responsive regulation (de Groot *et al.* 2003).

In this study, we report the identification and characterisation of the L-arabinose catabolic pathway specific regulator (AraR) in *A. niger* and demonstrate that this regulator is only present in the order *Eurotiales*. These fungi have evolved a fine-tuned two-regulator activating system for pentose release and catabolism compared to other filamentous ascomycetes that only contain XlnR or have neither of the regulators.

MATERIALS AND METHODS

Strains, media and growth conditions

The *A. niger* strains used in this study are listed in Table 1 and are all derived from *A. niger* CBS 120.49. *Aspergillus niger* strains were grown in Minimal Medium (MM) or Complete Medium (CM) with addition of a carbon source at 30 °C. MM contained (per liter): 6

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Table 1. Strains used in this study.

Strain	Genotype	Reference
N402	*cspA1*	Bos *et al.* (1988)
N572	*cspA1, fwnA1, pyrA6, xkiA1, nicA1*	vanKuyk *et al.* (2001)
NW249	*cspA1, ΔargB, pyrA6, nicA1, leuA1*	Jalving *et al.* (2000)
UU-A049.1	*cspA1, pyrA6, nicA1, leuA1, ΔargB::pIM2101(argB+)*	This study
UU-A033.21	*cspA1, pyrA6, nicA1, leuA1, ΔargB:: pIM2101(argB+), ΔaraR*	This study
UU-A054.4	*cspA1, pyrA6, nicA1, leuA1, ΔargB:: pIM2101(argB+), ΔaraR::araR*	This study
UU-A062.10	*cspA1, ΔargB, nicA1, leuA, pyrA6:: A. oryzae pyrA,ΔxlnR*	This study
UU-A063.22	*cspA1, nicA1, leuA1, ΔargB:: pIM2101(argB+), ΔaraR, pyrA6::A. oryzae pyrA, ΔxlnR*	This study

g $NaNO_3$, 1.5 g KH_2PO_4, 0.5 g KCL, 0.5 g $MgSO_4 \cdot 7\ H_2O$ and 200 µl trace elements solution (Vishniac & Santer 1957), pH 6.0. CM = MM supplemented with (/L): 2 g peptone, 1 g casamino acids, 1 g yeast extract and 0.5 g yeast ribonucleic acids, pH 6.0. For growth on solid media, 1.5 % agar was added to the medium. When necessary, the medium was supplemented with 0.2 g/L arginine, 0.2 g/L leucine, 0.2 g/L uridine and/or 1 mg/L nicotinamide.

In transfer experiments, all the strains were pre-grown in CM containing 2 % D-fructose. After 16 h of incubation, the mycelium was harvested without suction over a filter, washed twice with MM without a carbon source and transferred to 50 mL MM containing the appropriate carbon source and supplements. The mycelium was harvested with suction over a filter and culture samples were taken after 2 and 4 h of incubation. The mycelium samples were dried between tissue paper and directly frozen in liquid nitrogen.

Molecular biology methods

Molecular biology methods were performed according to standard procedures (Sambrook *et al.* 1989), unless stated otherwise. All PCR reactions were performed using Accutaq™ LA DNA Polymerase (Sigma-Aldrich) according to the manufacturer's instruction. The flanking regions of the *araR* gene were amplified with 5'primers and 3'-primers (see online Supplemental Table 1) by PCR to generate the 5' flank with the *Hind*III/*Sph*I site and 3' flank with a *Kpn*I/*BamH*I site, respectively, to enable deletion of the complete coding region of *araR* by replacing it with the *argB* selection marker. The functional construct was obtained using PCR with the extreme 5'- end 3'-primers (see online Supplemental Table 1) for complementation of *araR*. The *araR* disruption cassette (containing the *argB* gene for selection for arginine prototrophy) was transformed to the *A. niger* strain NW249 (*pyrA6*, *leuA1*, *nicA1*, Δ*argB*). The *xlnR* gene was amplified with the extreme 5'-primer and 3'-primer by PCR (see online Supplemental Table 1) The PCR fragment was ligated into pGEM-T-easy (Promega) from which the *Nsi*I/*Pst*I restriction sites were removed. The construct was digested with *Sal*I/*EcoR*I to remove most of the coding region including the DNA binding domain and ligated with the *A. oryzae pyrA* gene that was digested with *BamH*I (made blunt with Klenow fragment) and *SalI.* The *xlnR* disruption cassette was transformed to *A. niger* strains NW249 (*pyrA6*, *leuA1*, *nicA1*, Δ*argB*) and UU-A033.21 (*pyrA6*, *leuA1*, *nicA1*, Δ*araR*). All *A. niger* transformations were carried out as described previously (Kusters-van Someren *et al.* 1991).

The primers used to generate the probes for Southern and Northern analysis are listed in online Supplemental Table 1. The probes were DIG-labelled using the PCR DIG Probe Syntheses Kit (Roche Applied Science) according to the supplier's instructions. A cDNA library (de Groot *et al.* 2007) or genomic DNA (obtained from N402) was used as a template in the PCR reactions for synthesis of the probes.

Expression analysis

Total RNA was isolated from mycelium that was ground in a microdismembrator (B Braun) using a standard RNA isolation method with the TRIzol Reagent (Invitrogen). In the Northern analysis, 3 µg total RNA was transferred to a Hybond-N^+ membrane (Amersham Biosciences). The Minifold II slot blot apparatus (Schleicher & Schuell) was used for Slot blot analysis. Equal loading was determined by soaking the blot for 5 min in 0.04 % methylene blue, 0.5 M acetate pH 5.2 solution.

Hybridisation of the DIG-labeled probes to the blot was performed according to the DIG user's manual (www.roche-applied-science.com). All the blots were incubated overnight at 50 °C. The blots were exposed for 25 min up to 24 h to a Lumi-Film Chemiluminescent Detection Film (Roche Applied Science). Micro array analysis was performed as described previously (Levin *et al.* 2007).

Phylogenetic analysis

The amino acid sequences of AraR, XlnR, LadA, XdhA, XyrA and XkiA were used as queries in a local Blast against the protein files of 38 fungal genomes (see online Supplemental Table 2) with a expect value cut-off of 1E-10. The resulting ORFs were aligned using ClustalX and a Maximum Parsimony tree (1 000 bootstraps) was produced using MEGA (v. 4.0).

Enzyme assays

Extracellular enzyme activity was measured using 0.01 % *p*-nitrophenol linked substrates, 10 µL of the culture samples, 25 mM sodium acetate pH 5.0 in a total volume of 100 µL. Samples were incubated in microtiter plates for 120 min at 30 °C. Reactions were stopped by addition of 100 µL 0.25 M Na_2CO_3. Absorbance was measured at 405 nm in a microtiter platereader (Biorad Model 550). The extracellular enzyme activity was calculated using a standard curve ranging from 0 to 80 nmol *p*-nitrophenol per assay volume.

To measure intracellular enzyme activity, cell free extract was prepared by adding 1 mL extraction buffer (50 mM K_2HPO_4, 5 mM

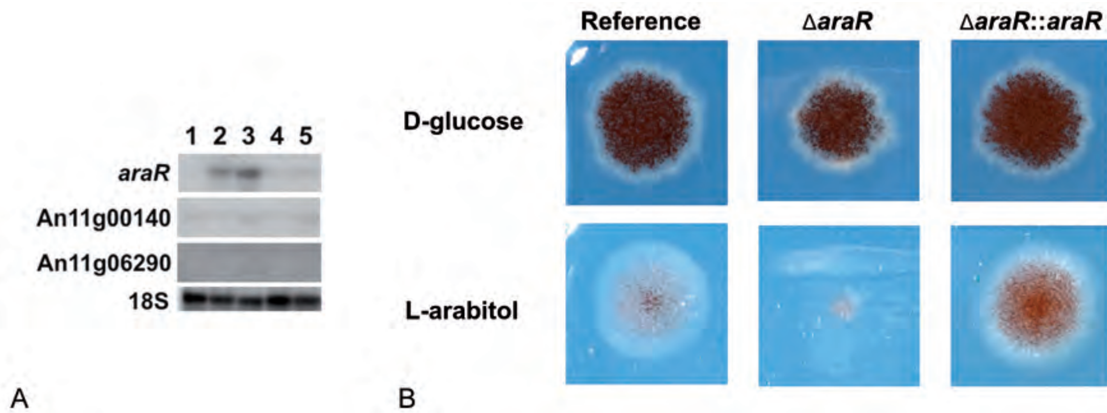

Fig. 1. A. Expression analysis of the three XlnR homologues (An04g08600 (*araR*), An11g00140 and An11g06290) on D-fructose (1), L-arabinose (2), L-arabitol (3), D-xylose (4) and xylitol (5). **B.** Growth of the reference (UU-A049.1), Δ*araR* (UU-A033.21) and Δ*araR::araR* (UU-A054.4) on D-glucose and L-arabitol.

MgCl2, 5 mM 2-mercaptoethanol, 0.5 mM EDTA) to powdered mycelium. The mixtures were centrifuged for 10 min at 12000 RPM at 4 °C. The L-arabitol and xylitol dehydrogenase activities were determined using 100 mM glycine pH 9.6, 0.4 mM NAD^+ and 1 M L-arabitol or xylitol, respectively. L-arabinose reductase and D-xylose reductase activities were determined using 50 mM Tris-HCL pH 7.8, 0.2 mM NADPH and 1 M L-arabinose or D-xylose, respectively. L-arabinose reductase (ArdA) and D-xylose reductase (XyrA) both convert D-xylose to xylitol and L-arabinose to L-arabitol, but have a higher activity on their primary substrate (de Groot *et al.* 2003). As a result, the measured activity is the sum of the two enzymes. To be able to discriminate between the two enzymes, the ratio of the activity on L-arabinose and on D-xylose was calculated that allows us to extrapolate the relative activities of ArdA and XyrA. An increase in the ratio indicates a relative increase in ArdA or decrease in XyrA, while a reduction in the ratio indicates a relative increase in XyrA or decrease in ArdA.

Absorbance changes were measured at 340 nm using a spectrometer (Spectronic Unicam UV1). L-arabinose and D-xylose reductase activity and L-arabitol and D-xylitol dehydrogenase activity was calculated using the molar coefficient for NADPH and NADH (both ε = 6.22 mM-1cm-1) and the following formula:

Activity (U/mL) = [(A/min-Abl/min)* d * v] / (l * a * ε).

Abl/min = decrease absorbance per minute before adding substrate. A/min = decrease absorbance per minute after adding substrate. a = sample volume (mL). d = sample dilution. v= total volume cuvet. l = lightpath (cm). Protein concentrations of intracellular and extracellular samples were determined using a BCA protein assay kit (Pierce).

RESULTS

Identification and analysis of araR

Blast analysis of XlnR against the *A. niger* genome (Pel *et al.* 2007) revealed 3 homologues with expect values smaller than e^{-30} (An04g08600, An11g00140, An11g06290). Expression analysis of these genes revealed that the closest *xlnR* homologue (An04g08600) was specifically induced in the presence of L-arabinose or L-arabitol, while only low constitutive expression was observed for An11g06290 and no expression for An11g00140 (Fig. 1A). In order to study its possible role in L-arabinose utilisation, a disruption strain for An04g08600 (referred to as *araR*) was constructed and verified by Southern analysis (data not shown). The disruption strain showed poor growth on L-arabitol, whereas complementation with *araR* restored growth again (Fig. 1B).

The araR gene consists of 2552 bp interrupted by a single intron of 53 bp. Within the 1000 bp promoter region of *araR* putative six binding sites for the carbon catabolite repressor protein CreA (Kulmburg *et al.* 1993) and two binding sites for the xylanolytic regulator XlnR (van Peij *et al.* 1998b, de Vries *et al.* 2002) can be found. The AraR protein contains a Zn(2)Cys(6) binuclear cluster domain (amino acids 36-73, Pfam00172) and a Fungal specific transcription factor domain (amino acids 386-532, Pfam04082). An amino acid motif Arg-Arg-Thr-Leu-Trp-Trp is found at position 493 to 498. This motif differs in only one amino acid from a conserved motif of unknown function found in Zn(2)Cys(6) family members (Arg-Arg-Arg-Leu-Trp-Trp), first described in the UaY regulator in *Aspergillus nidulans* (Suarez *et al.* 1995). AraR shows 32 % identity to XlnR, with the highest homology in the C-terminal part of the proteins. The sequence between the 2nd and the 3rd Cysteine in the Zn(2)Cys(6) region was previously shown to be important in DNA binding specificity of this class of regulators (Marmorstein *et al.* 1992, Marmorstein & Harrison 1994), but differs significantly between AraR ($C_2HSRRVRC_3$) and XlnR ($C_2NQLRTKC_3$). Between the third and the fourth Cysteine, the Proline residue can be found that is essential for correct folding of the DNA binding domain (Marmorstein *et al.* 1992) and is highly conserved in all the fungal zinc binuclear transcriptional regulators.

The presence of AraR in the genome is restricted to *Eurotiales* and possibly to *Trichocomaceae*

BlastP analysis of both AraR and XlnR against 38 fungal genome sequences (see online Supplemental Table 2) identified homologues for both proteins in all 11 analysed species of the family *Trichocomaceae* of the order *Eurotiales* (*Aspergillus clavatus*, *A. flavus*, *A. fumigates*, *A. nidulans*, *A. niger*, *A. oryzae*, *A. terreus*, *Neosartorya fischeri*, *Penicillium chrysogenum*, *P. marneffei*, *Talaromyces stipitatus*), but neither of them was found in three representatives of *Onygenales* (*Coccidioides immitis*, *Histoplasma capsulatum*, *Uncinocarpus reesei*) (Fig. 2). XlnR was also found in the genomes of all other filamentous ascomycetes used in this study. No XlnR and AraR homologues were found in ascomycete yeasts, basidiomycetes or zygomycetes.

In addition, a BlastP analysis was performed with the amino acid sequence of four genes of the *A. niger* pentose catabolic

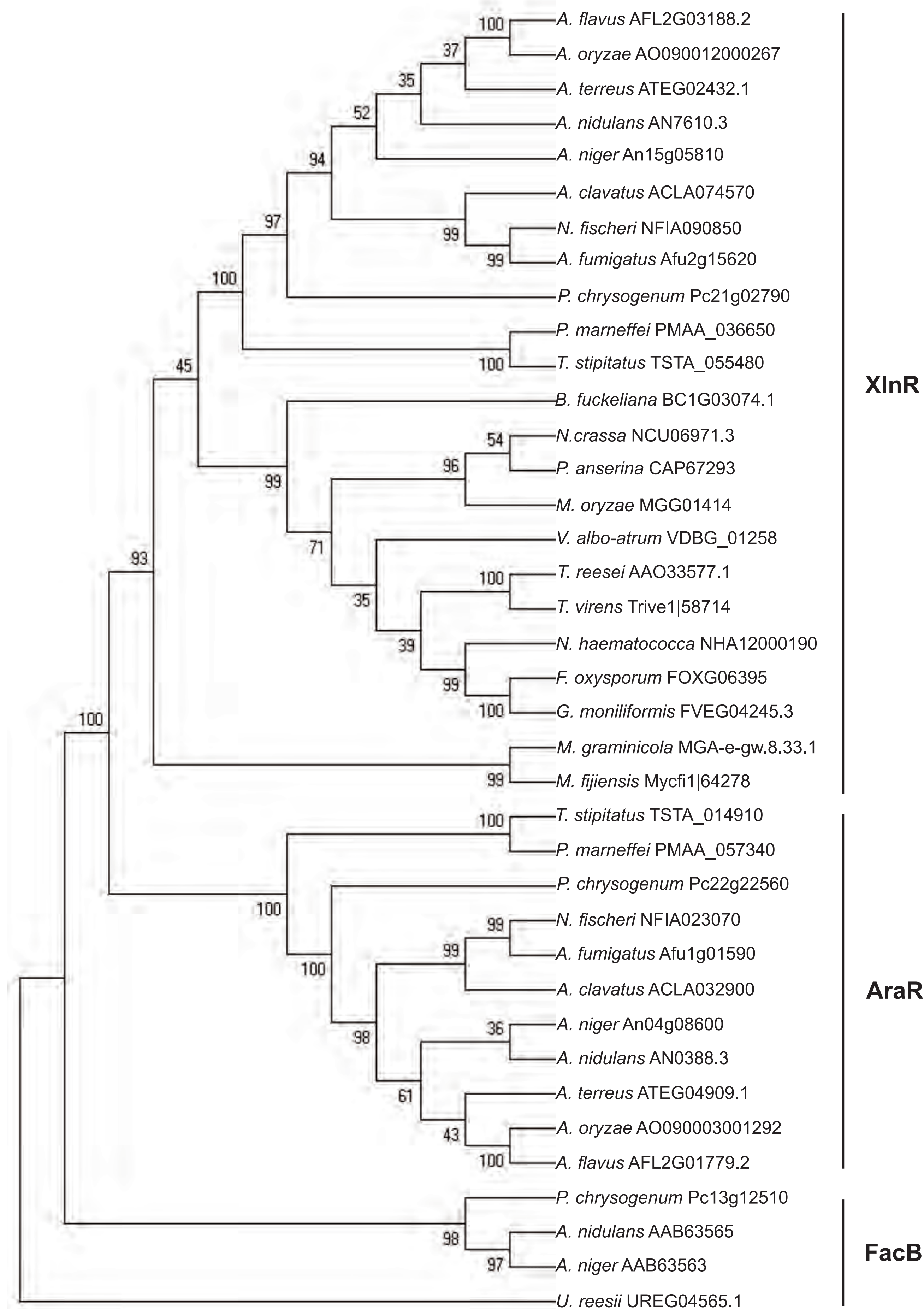

Fig. 2. Bootstrapped (1000 bs) Maximum Parsimony tree of of putative homologues of XlnR and AraR in fungi. Homologues of the *A. nidulans* acetate regulatory protein (FacB) were used as an outgroup.

pathway (*ladA*, *xyrA*, *xdhA* and *xkiA*) against the genomes of the fungal species that contain XlnR and/or AraR as well as the three *Onygenales* genomes used in this study (see online Supplemental Fig. 1). Phylogenetic analysis showed that all genomes contain homologues of three genes of the pentose catabolic pathway. Homologues for the 4th gene (*ladA*) were found in all species except for *Onygenales*.

Influence of AraR and XlnR on growth of *A. niger* on monomeric and polymeric carbon sources

In addition to the *araR* disruptant (UU-A033.21), an *xlnR* disruptant (UU-A062.10) and an *araR/xlnR* double disruptant (UU-A063.22) were generated, as described in Materials and Methods. The utilisation of several monomeric and polymeric carbon sources was analysed in all strains (including the reference) to determine the effect of the single disruption of the *araR* gene and the double disruption of *araR* and *xlnR* (Fig. 3). Polymeric sugars containing L-arabinose residues (arabinan, Arabic gum, arabinogalactan and apple pectin) and D-xylose residues (birchwood xylan) were included in the analysis. Guar gum was used as a control; it is a galactomannan and contains no L-arabinose or D-xylose residues.

Disruption of *araR* resulted in reduced growth on L-arabinose, xylitol, arabinan, Arabic gum, arabinogalactan and apple pectin and poor growth on L-arabitol (Fig. 3). Disruption of *xlnR* resulted in reduced growth on birchwood xylan, while growth was unaffected on D-xylose, xylitol and the other carbon sources. Disruption of both regulators resulted in a similar phenotype as disruption of *araR* for L-arabitol, Arabic gum, arabinan and arabinogalactan and a similar phenotype as disruption of *xlnR* for birchwood xylan. In contrast to the single disruptants, no growth was observed on D-xylose for the double disruptant, only residual growth on L-arabitol and L-arabinose, and reduced growth on xylitol.

AraR and XlnR control L-arabinose and D-xylose release and catabolism

The reference, Δ*araR*, Δ*xlnR* and Δ*araR/*Δ*xlnR* strains were pre-grown in complete medium containing D-fructose. After 16 h of growth, equal amounts of mycelium were transferred for 2 and 4 h to minimal medium containing 25 mM D-fructose, 25 mM L-arabinose or 25 mM D-xylose. Extracellular α-L-arabinofuranosidase (Abf) and intracellular PCP enzyme activities (Ard, Xyr, Lad, Xdh) were analysed. Activity of α-L-arabinofuranosidase (Abf), L-arabitol dehydrogenase (Lad) and xylitol dehydrogenase (Xdh) was strongly reduced in the Δ*araR* and Δ*araR/*Δ*xlnR* strain compared to the reference strain when grown on L-arabinose (Fig. 4A). On D-xylose, Lad and Xdh activity was reduced in Δ*araR* and Δ*araR/*Δ*xlnR.* For L-arabinose reductase (ArdA) and D-xylose reductase (XyrA), the ratio of the activity on L-arabinose and on D-xylose was calculated that allowed extrapolation of the relative activities of ArdA and XyrA (see Materials and Methods). The ratio in the Δ*araR* strain became less than 1.0 after 4 h growth in the presence of L-arabinose, while the ratio of the reference strain was around 1.5, which suggests that the ArdA activity was reduced in the Δ*araR* strain (Fig. 4A). The Ard/Xyr ratio in the wild type and Δ*araR* disruptant grown on D-xylose were both around 1. In the absence of both regulators, no Ard and Xyr activities were detected (data not shown). Xylitol dehydrogenase activity (Xdh) was reduced in the Δ*araR* strain on L-arabinose and to a lesser extent on D-xylose compared to the reference strain (Fig. 4). All the measured activities after 2 h of growth on L-arabinose and D-xylose in the Δ*xlnR* are similar to those published previously (de Groot *et al.* 2003). After 4 h, the difference in activity between the reference and Δ*xlnR* is similar to that observed after 2 h of growth, except for Xdh and Abf. Xdh activity in the Δ*xlnR* became similar to that in the reference strain after 4 h on D-xylose, whereas the Abf activity increased at this point. No activity for any of the enzymes was detected during growth of D-fructose.

In addition, expression levels were determined using micro array analysis for genes involved in release (*abfA, abfB*) and catabolism (*ladA, xdhA, xyrA, xkiA*) of L-arabinose and D-xylose. No gene expression was observed for any of the genes discussed in this section during growth on 25 mM D-fructose (data not shown). Expression profiles of all the genes in Table 2, except for *araR* and *xlnR,* were confirmed by Northern analysis (see online Supplemental Fig. 1). Expression of *araR* and *xlnR* was below detection levels for Northern analysis in these samples. Disruption of *araR* resulted in 74, 6, 10, 2 and 13-fold reduced expression levels of *abfA*, *abfB*, *ladA*, *xdhA* and *xkiA*, respectively, after 2 h of growth on L-arabinose (Table 2). Disruption of *xlnR* did not significantly reduce expression levels of any of the tested genes, except for *xyrA* for which expression reduced 2-fold after 2 h of growth on D-xylose. Disruption of *araR* did not affect *xdhA*, *xkiA* and *xyrA* expression on D-xylose, while none of the genes were affected on L-arabinose by disruption of *xlnR* (see online Supplemental Fig. 1). None of the tested genes were expressed in the Δ*araR/*Δ*xlnR* strain, except for *abfB* (see online Supplemental Fig. 1). Expression of *xlnR* was not affected in the Δ*araR* on L-arabinose, whereas *araR* expression showed a 3-fold increase in the Δ*xlnR* on D-xylose compared to the reference.

DISCUSSION

Previously, it has been shown that the pentose catabolic pathway is under control of the D-xylose specific transcriptional activator (XlnR) and a second, unidentified L-arabinose specific transcriptional activator regulator (de Groot *et al.* 2007). In this study, we identified the gene encoding the L-arabinose responsive regulator, AraR, and confirmed its role in the release and catabolism of L-arabinose and D-xylose. AraR is a member of the Zn(2)Cys(6) family of transcriptional regulators and a close homologue of the xylanolytic transcriptional activator XlnR from *A. niger*. Functional analysis of AraR and XlnR as described in this study confirm the previously published antagonistic relation of the two regulatory systems involved in pentose catabolism (de Groot *et al.* 2003).

Expression levels of *abfA*, *abfB*, *ladA* as well as the corresponding enzyme activities (Abf and Lad) were strongly reduced in the Δ*araR* strain on L-arabinose, indicating that they are only controlled by AraR. Gene expression levels of *xdhA* and *xkiA* are reduced in the Δ*araR* strain after 2 h of growth on L-arabinose. On D-xylose, *xdhA* expression is up-regulated in the Δ*xlnR* strain compared to the reference strain, which confirms data published previously (de Groot *et al.* 2007). An increase in *xkiA* expression was observed in the Δ*xlnR* strain on L-arabinose. These results indicate that both AraR and XlnR are involved in regulating the expression of *xdhA* and *xkiA*. The stronger effect in the Δ*araR* strain, suggests that AraR has a larger influence on *xdhA* and *xkiA* expression than XlnR.

Expression of the AraR regulated genes on D-xylose and reduction of the expression in the *araR* disruptant can be explained by the presence of a small amount of L-arabinose in the D-xylose

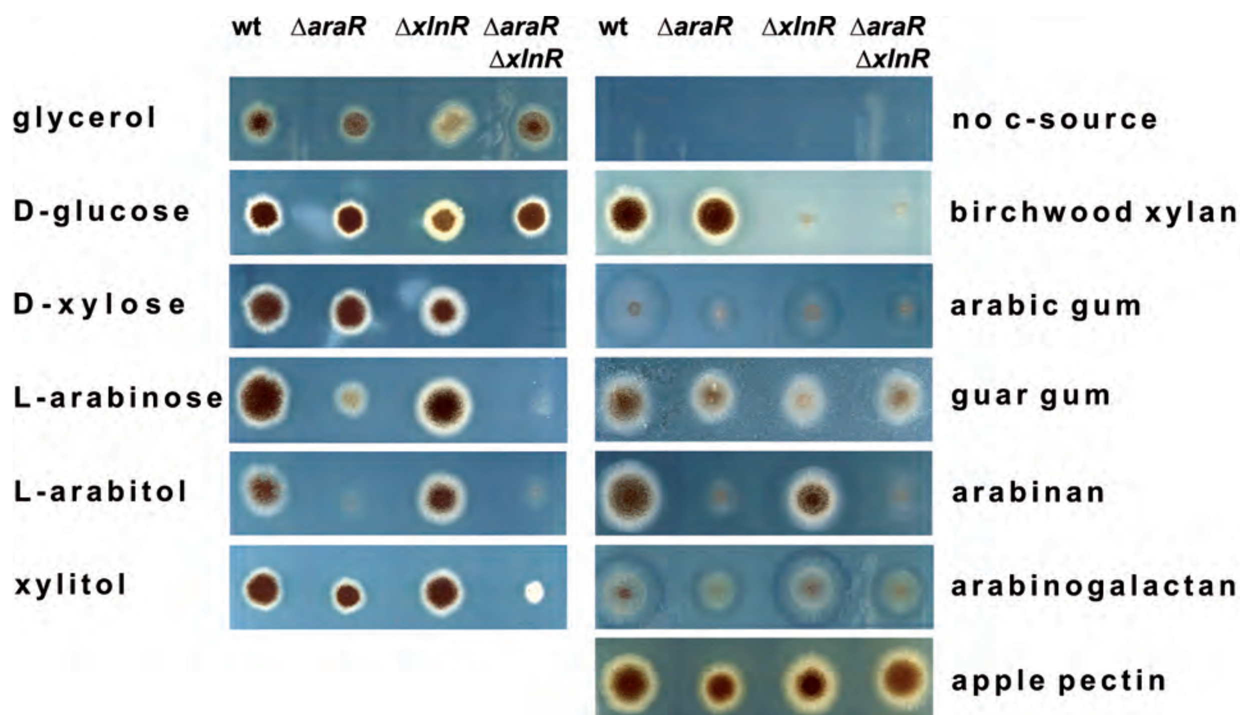

Fig. 3. Growth of the reference strain (Ref., UU-A049.1), and the Δ*araR* (UU-A033.21), Δ*xlnR* (UU-A062.10) and Δ*araR*/Δ*xlnR* (UU-A063.22) strains on a selection of mono- and polysaccharides. Concentrations of the substrates were 25 mM for D-glucose, D-xylose, L-arabinose, L-arabitol, xylitol and glycerol, and 1 % for birchwood xylan, Arabic gum, guar gum, arabinan, arabinogalactan and apple pectin.

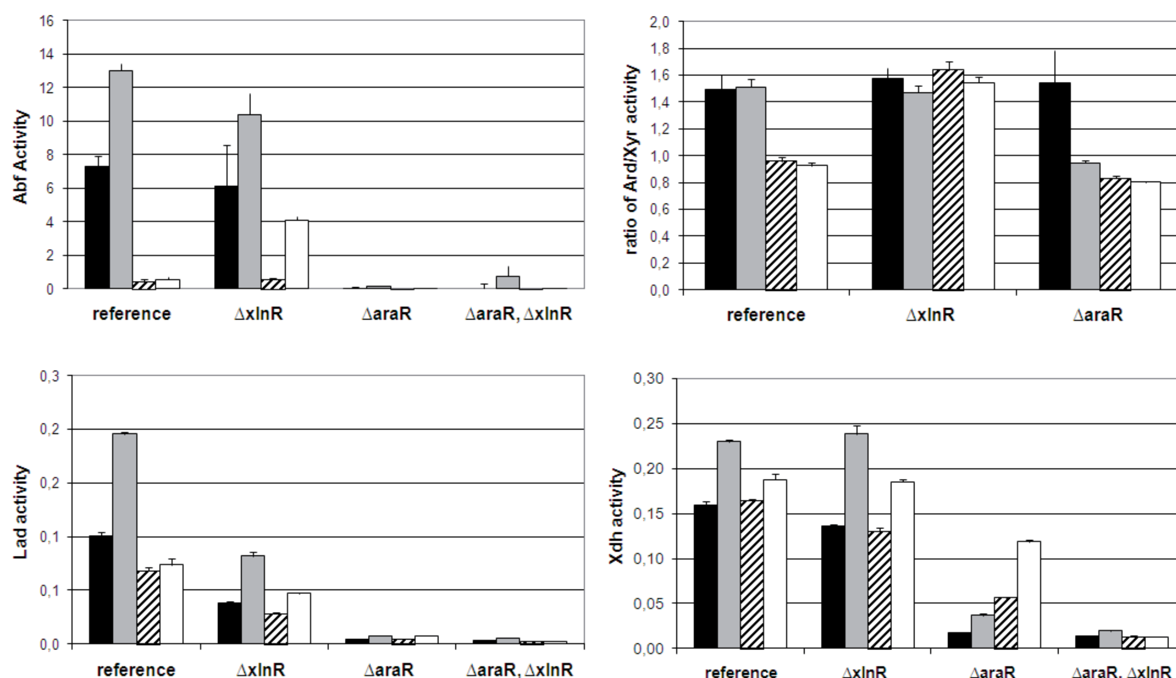

Fig. 4. Comparison of intracellular and extracellular enzyme activities in reference and disruption strains. The reference strains (UU-A049.1), Δ*araR* (UU-A033.21), Δ*xlnR* (UU-A062.10) and Δ*araR*/Δ*xlnR* (UU-A063.22) were transferred for 2 and 4 h on 25 mM L-arabinose or 25 mM D-xylose. Extracellular α-L-arabinofuranosidase (Abf), the ratio of intracellular L-arabinose reductase (ArdA) and D-xylose reductase (XyrA) activity, and the intracellular activities of xylitol dehydrogenase (Xdh) and L-arabitol dehydrogenase (Lad). Black bars: L-arabinose, 2 h; grey bars: L-arabinose, 4 h; dashed bars: D-xylose, 2 h; white bars: D-xylose, 4 h.

preparation from SIGMA (R.P. de Vries, unpubl. data). This is supported by a reduction in the expression of these genes on D-xylose at 4 h compared to 2 h.

The discrepancies between some of the expression and activity data can be explained by the substrate specificities of the enzymes. The L-arabinose and D-xylose reductases are both active on both pentoses, so under conditions where both are expressed, the measured activity is the result of the combined activity of the two enzymes. Although xylitol dehydrogenase is (almost) not active on L-arabitol, the L-arabitol dehydrogenase is active on xylitol (de Groot *et al.* 2007), indicating that the measured xylitol dehydrogenase can also consist of two components depending on the condition used.

Previously, it has been shown that the expression of *xyrA* was only reduced and not absent in the Δ*xlnR* on D-xylose (de Groot *et al.* 2003) and it was suggested that in addition to XlnR another unknown inducing factor is involved. Our results confirm this observation. The reason why there is no reduction in growth of the Δ*xlnR* strain on D-xylose can be explained by the fact

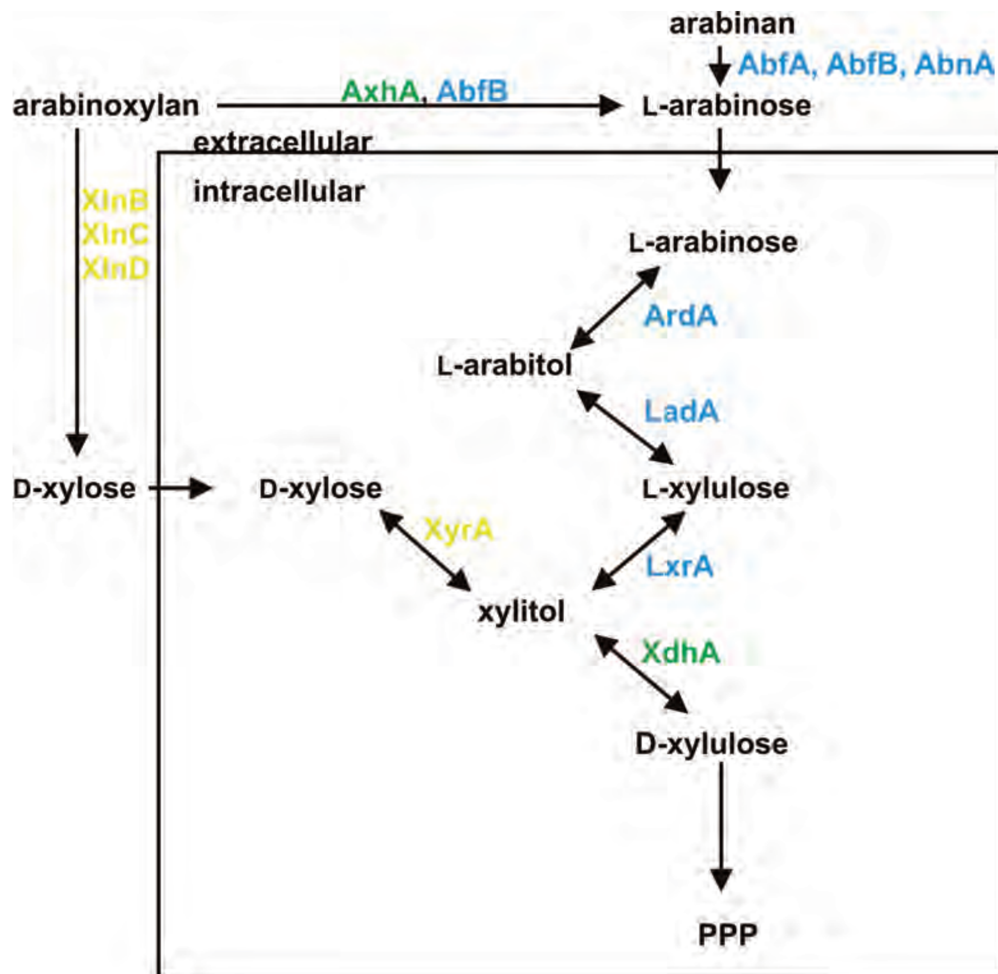

Fig. 5. Regulatory model for release and utilisation of D-xylose and L-arabinose in *A. niger*. ArdA = L-arabinose reductase; LadA = L-arabitol dehydrogenase; LxrA = L-xylulose reductase; XdhA = xylitol dehydrogenase; XyrA = D-xylose reductase; XkiA = D-xylulose kinase; AbfA, AbfB = α-L-arabinofuranosidase A and B; AbnA= endo-1,5-alpha-L-arabinanase; AxhA = arabinoxylan arabinofuranohydrolase; XlnB, XlnC = endoxylanases B and C; XlnD = β-xylosidase. The square depicts the fungal cell wall. AraR regulated genes are in blue. XlnR regulated genes are in yellow. Genes regulated by AraR and XlnR are in green. Inclusion of *axhA*, *abnA*, *xlnB*, *xlnC*, *xlnD* was based on co-regulation with the other genes as reported previously (Gielkens *et al.* 1997, van Peij *et al.* 1998a, de Groot *et al.* 2003).

that *xyrA* expression/activity was not absent combined with the compensatory regulation by AraR for *xkiA* and *xdhA* expression. No growth was observed for the double disruptant on D-xylose, suggesting both regulators are necessary for growth on D-xylose. The strong growth reduction of the Δ*xlnR* strain on xylan, similar to growth of the double disruptant, indicates that D-xylose release is mainly dependent on XlnR.

Only residual growth was observed for the double disruptant on L-arabinose and L-arabitol, demonstrating the importance of AraR and XlnR for growth on these substrates. Strongly reduced growth was observed for the Δ*araR* strain and the Δ*araR*/Δ*xlnR* strain on arabinan, indicating that release of L-arabinose residues depends only on AraR.

The absence of AraR orthologues in fungal genomes except for those of the aspergilli and penicillia and its similarity to XlnR suggests that this regulator has originated by a gene duplication of *xlnR* after *Eurotiales* split from the other filamentous ascomycetes. All genomes available from *Eurotiales* are of the family *Trichocomaceae*, while currently none are available for the other family of this order, *Elaphomycetaceae*. At this point we can therefore not determine whether this gene duplication may have occurred even later, when *Elaphomycetaceae* and *Trichocomaceae* split into two different families.

The regulatory system controlling pentose release and utilisation in this group of fungi likely evolved to become a highly interactive two-regulator system. Whether this implies that in the other ascomycete fungi XlnR is responsible for L-arabinose and D-xylose induced expression remains to be studied. It suggests there are large evolutionary differences in regulation of the pentose catabolic pathway. Afterthe *Onygenales* split from *Eurotiales* it seems to have lost both XlnR and AraR regulators. Homologues for three of the *A. niger* genes of the pentose catabolic pathway (*xdhA*, *xyrA* and *xkiA*) are present in the other fungal genomes. The L-arabitol dehydrogenase encoding gene (*ladA*) appears to have been lost in *Onygenales*, but is present in all species that contain XlnR. This may suggest that loss of L-arabinose utilisation has proceded further in *Onygenales* than just loss of the regulatory systems.

Data from our study was combined with the previously reported data on XlnR (van Peij *et al.* 1998a, de Groot *et al.* 2003) to construct a regulatory model for release and utilisation of L-arabinose and D-xylose in the *A. niger* (Fig. 5). This model correlates not only well with the expression profiles of the pentose-related genes but also with the growth comparison of the disruptant strains and the reference. It indicates that XlnR and AraR control distinct sets of genes in response to the presence of D-xylose and L-arabinose, respectively. However, in the absence of one of the regulators the other can partially compensate for this loss. Although the data supporting this model comes from *A. niger*, we postulate that this model applies to all *Eurotiales*, since we have demonstrated in this study that the presence of AraR is conserved among all species of *Eurotiales* studied so far.

Table 2. Expression analysis of genes encoding extracellular L-arabinose releasing enzymes and PCP enzymes. *abfA*, *abfB* = α -L-arabinofuranosidase A and B, *ladA* = L-arabitol dehydrogenase, *xdhA* = xylitol dehydrogenase, *xyrA* = D-xylose reductase, *xkiA* = D-xylulose kinase, *araR* = arabinanolytic regulator, *xlnR* = xylanolytic regulator. The expression levels are mean values of duplicate samples. The ratio was calculated of the expression levels of the reference strain and disruption strain.

	Reference 2 h L-ara	Δ*araR* 2 h L-ara	Ratio ref/ Δ*araR*	Reference 2 h D-xyl	Δ*xlnR* 2 h D-xyl	Ratio ref/ Δ*xlnR*
abfA	6622 ± 919	89 ± 9	74.4	5827 ± 545	7578 ± 748	0.8
abfB	4985 ± 516	901 ± 143	5.5	869 ± 4	2176 ± 150	0.4
ladA	4224 ± 417	414 ± 12	10.1	2229 ± 24	3482 ± 8	0.6
xdhA	5013 ± 661	2281 ± 417	2.2	4344 ± 315	6567 ± 377	0.7
xyrA	4808 ± 641	4048 ± 685	1.2	6248 ± 587	3655 ± 66	1.7
xkiA	2690 ± 402	211 ± 18	12.7	2588 ± 34	1843 ± 102	1.4
araR	100 ± 28	1 ± 0	100	20 ± 1	65 ± 5	0.3
xlnR	145 ± 21	185 ± 41	0.8	157 ± 9	3 ± 0	52.3

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New species in *Aspergillus* section *Terrei*

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Abstract: Section *Terrei* of *Aspergillus* was studied using 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 to describe three new species in this section. Based on phylogenetic analysis of calmodulin and β-tubulin sequences seven lineages were observed among isolates that have previously been treated as *A. terreus* and its subspecies by Raper & Fennell (1965) and others. *Aspergillus alabamensis, A. terreus* var. *floccosus*, *A. terreus* var. *africanus*, *A. terreus* var. *aureus*, *A. hortai* and *A. terreus* NRRL 4017 all represent distinct lineages from the *A. terreus* clade. Among them, *A. terreus* var. *floccosus*, *A. terreus* NRRL 4017 and *A. terreus* var. *aureus* could also be distinguished from *A. terreus* by using ITS sequence data. New names are proposed for *A. terreus* var. *floccosus*, *A. terreus* var. *africanus*, *A. terreus* var. *aureus,* while *Aspergillus hortai* is recognised at species level. *Aspergillus terreus* NRRL 4017 is described as the new species *A. pseudoterreus*. Also included in section *Terrei* are some species formerly placed in sections *Flavipedes* and *Versicolores*. A. clade including the type isolate of *A. niveus* (CBS 115.27) constitutes a lineage closely related to *A. carneus*. *Fennellia nivea*, the hypothesized teleomorph is not related to this clade. *Aspergillus allahabadii, A. niveus* var. *indicus*, and two species originally placed in section *Versicolores*, *A. ambiguus* and *A. microcysticus*, also form well-defined lineages on all trees. Species in *Aspergillus* section *Terrei* are producers of a diverse array of secondary metabolites. However, many of the species in the section produce different combinations of the following metabolites: acetylaranotin, asperphenamate, aspochalamins, aspulvinones, asteltoxin, asterric acid, asterriquinones, aszonalenins, atrovenetins, butyrolactones, citreoisocoumarins, citreoviridins, citrinins, decaturins, fulvic acid, geodins, gregatins, mevinolins, serantrypinone, terreic acid (only the precursor 3,6-dihydroxytoluquinone found), terreins, terrequinones, terretonins and territrems. The cholesterol-lowering agent mevinolin was found in *A. terreus* and *A. neoafricanus* only. The hepatotoxic extrolite citrinin was found in eight species: *A. alabamensis, A. allahabadii, A. carneus, A. floccosus, A. hortai, A. neoindicus, A. niveus* and *A. pseudoterreus*. The neurotoxic extrolite citreoviridin was found in five species: *A. neoafricanus, A. aureoterreus, A. pseudoterreus, A. terreus* and *A. neoniveus*. Territrems, tremorgenic extrolites, were found in some strains of *A. alabamensis* and *A. terreus*.

Key words: Ascomycetes, *Aspergillus* section *Terrei,* β-tubulin, calmodulin, citreoviridin, *Eurotiales*, extrolites, ITS, polyphasic taxonomy.
Taxonomic novelties: *Aspergillus aureoterreus* stat. et nom. nov., *Aspergillus floccosus* comb. et stat. nov., *Aspergillus neoafricanus* stat. et nom. nov., *Aspergillus neoindicus* stat. et nom. nov., *Aspergillus neoniveus* nom. nov., *Aspergillus pseudoterreus* sp. nov.

INTRODUCTION

Aspergillus section *Terrei* (Gams *et al.* 1985; *A. terreus* species group according to Raper & Fennell 1965) includes species with columnar conidial heads in shades of buff to brown. The most important species of this section is *A. terreus,* which is an ubiquitous fungus in our environment. Strains of this cosmopolitan species are frequently isolated from desert and grassland soils and compost heaps, and as contaminants of plant products like stored corn, barley and peanuts (Kozakiewicz 1989). *Aspergillus terreus* is an economically important species from a number of aspects. *Aspergillus terreus* isolates are used in the fermentation industry for the production of itaconic acid and itatartaric acid and for enzyme production (Bigelis & Arora 1992, Lowe 1992). *Aspergillus terreus* isolates produce a range of secondary metabolites, some of which have properties valuable for mankind, including lovastatin, a cholesterol lowering drug (Alberts *et al.* 1980), the antitumor metabolites terrein (Arakawa *et al.* 2008, Demasi *et al.* 2010), quadrone (Carlton *et al.* 1978) and asterriquinone (Kaji *et al.* 1998), acetylcholinesterase inhibitors like territrem B (Chen *et al.* 1999) and terreulactone, butyrolactones (Schimmel *et al.* 1998), and cyclosporine A (Sallam *et al.* 2003). Antiviral compounds such as acetylaranotin has also been reported from *Aspergillus terreus* (Miller *et al.* 1968, Kamata *et al.* 1983). Other secondary metabolites reported to be produced by *A. terreus* isolates are considered as mycotoxins, including citreoviridin (Franck & Gehrken, 1980), patulin (Kent & Heatley 1945, Draughon & Ayres 1980, Reddy & Reddy 1988), citrinin (Sankawa *et al.* 1983), emodin (Fujii *et al.* 1982), terretonin (Springer *et al.* 1979, Li *et al.* 2005), geodin (Kiriyama *et al.* 1977, Rønnest *et al.* 2011), territrems (Ling *et al.* 1979), gliotoxin (Lewis *et al.* 2005, Kupfahl *et al.* 2008), and cytochalasin E (Fujishima *et al.* 1979). *Aspergillus terreus* is also an important human pathogen, and often causes disseminated infection with increased lethality compared to other *Aspergillus* spp. (Tracy *et al.* 1983, Iwen *et al.* 1998, Lass-Florl *et al.* 2000, Walsh *et al.* 2003, Baddley *et al.* 2003, Steinbach *et al.* 2004, Balajee 2009). Recent data indicate that the accessory conidia produced by *A. terreus* can induce elevated inflammatory responses in a pulmonary model of aspergillosis (Deak *et al.* 2009, 2011). The importance of *A. terreus* to human health and industry is underlined by the fact that annotation of the full genome sequence of *A. terreus* isolate NIH 2624 is in progress (Birren *et al.* 2004, http://fungi.ensembl.org/Aspergillus_terreus/Info/Index), while whole-genome shotgun sequencing of isolate ATCC 20542 has also been carried out (Askenazi *et al.* 2003). Additionally, transcriptional and metabolite profiles (Askenazi *et al.* 2003) and the extracellular proteome of *A. terreus* have also been examined recently (Han *et al.* 2010).

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Table 1. *Aspergillus* strains examined in this study.

Taxon	Strain No.	Origin
A. neoafricanus	CBS 130.55ᵀ = NRRL 2399	*Aspergillus terreus* var. *africanus*; soil, Tafo, Ghana
	NRRL 4609	*Aspergillus terreus* var. *africanus*; soil, Panama
	IBT 13121	*Aspergillus terreus* var. *africanus*; soil, Japan
A. alabamensis	IBT 12702	Soil, New Mexico
	WB 1920 = IBT 22563	Soil, Cuba
	DTO 15-F8 = IBT 29084	Soil, Argentina
	DTO 15-F9 = IBT 29086	Soil, Argentina
	UAB 18	Sputum, Alabama, USA
	UAB 15	Sputum, Alabama, USA
	UAB 20T	Wound, Alabama, USA
	NRRL 29810 = IBT 29081	Soil, Florida, USA
A. allahabadii	CBS 164.63ᵀ = NRRL 4539	Soil, India
	CCRC 32133 = IBT 21128	Soil, Taipei, Taiwan
A. ambiguus	CBS 117.58ᵀ = NRRL 4737	Savannah soil, Somalia
A. aureoterreus	CBS 265.81	Wheat flour, India
	CBS 503.65ᵀ = NRRL 1923	Soil, Texas, USA
A. carneus	CBS 494.65ᵀ = NRRL 527 = IBT 13986	Culture contaminant, District of Columbia, USA
	NRRL 1928	Soil, Kansas, USA
	NRRL 298 = IBT 22569	Soil, Kansas, USA
A. floccosus	CBS 116.37ᵀ = WB 4872 = IBT 22556	*Aspergillus terreus* var. *floccosus*; Waste cloth, Wuchang, China, isolated by Y.K. Shih as No. A 369
A. hortai	CBS 124230ᵀ = NRRL 274 = IBT 26384	Clinical isolate, from ear, Brazil
	IBT 16744	Soil, Galapagos Islands
	IBT 16745	Soil, Galapagos Islands
	IBT 6271	Soil, Florida, USA
A. microcysticus	CBS 120.58ᵀ = NRRL 4749	Savannah soil, Somalia
A. neoindicus	CBS 444.75ᵀ = NRRL 6134	*Aspergillus niveus* var. *indicus*; Soil, Maharashta, India
A. neoniveus	CBS 261.73ᵀ = NRRL 5299	Forest soil, Thailand
	CBS 262.73 = NRRL 5502	Forest soil, Thailand
	CBS 114.33 = NRRL 515	P. Biourge
	CBS 471.91 = NRRL 1955	Soil, Ontario, Canada
A. niveus	CBS 115.27ᵀ = NRRL 5505	A. Blochwitz
	NRRL 4751ᵀ	*Fennellia nivea* var. *bifida*; unknown
A. pseudoterreus	CBS 123890 = NRRL 4017	Soil, Argentina
A. terreus	IBT 26915	Capybara droppings, Gamboa, Panama
	CBS 601.65ᵀ = NRRL 255	Soil, Connecticut, USA
	NRRL 260	Soil, College Station, Texas, USA
	NRRL 1913	Lung of pocket mouse, Arizona, USA
	IBT 6450	Corn, India
	IBT 14590 = UAMH 4733	Soil, Golf course, Japan
	IBT 24859	Saltern, Slovenia
	NRRL 680 = CBS 594.65 = IBT 6252	Soil, G. Ledingham
	CBS 117.37 = WB 4873	*Aspergillus terreus* var. *subfloccosus*; Air, Wuchang, China

Aspergillus terreus was the only species assigned to the *A. terreus* species group by Raper & Fennell (1965). Molecular studies have since indicated that this section should be expanded to include a number of other species (Peterson 2000, 2008, Varga *et al.* 2005). Besides *A. terreus* and its varieties, section *Terrei* also includes *A. niveus* (teleomorph: *Fennellia nivea*), *A. carneus*, *A. niveus* var. *indicus*, *A. allahabadii*, *A. ambiguus* and *A. microcysticus* (Peterson 2000, 2008, Varga *et al.* 2005). The first three species have previously been placed in section *Flavipedes* and the last three species were placed in section *Versicolores* (Raper & Fennell 1965, Samson 1979). *Aspergillus niveus* has been reported to cause pulmonary aspergillosis (Auberger *et al.* 2008), and recent data indicate that several isolates previously assigned to *A. terreus,* including clinical isolates causing aspergillosis, actually represent a separate species, *A. alabamensis* (Balajee *et al.* 2009). The last authors also indicated that *A. terreus* var. *aureus* should be recognised as distinct species, but they did not provide a formal description (Balajee *et al.* 2009).

In this study, we examined available isolates, which morphologically belong to section *Terrei,* to clarify the taxonomic

status of this section. We used the 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 clarify their taxonomic identity.

MATERIALS AND METHODS

Isolates

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

Morphological analysis

For macromorphological observations, Czapek Yeast Autolysate (CYA), Malt Extract (MEA) Agar, Yeast Extract Sucrose Agar (YES), Creatine Agar (CREA), and Oatmeal Agar (OA) were used (Samson *et al.* 2010). 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, 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, CA, USA) fitted with a 2 x 2 mm guard column.

Genotypic analysis

The cultures used for the molecular studies were grown on malt peptone (MP) broth using 1 % (w/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). Sequences have been deposited in GenBank under accession numbers FJ491703–FJ491731, and FJ531192–FJ531243.

Data analysis

The sequence data was optimised using the software package Seqman from DNAStar Inc. Sequence alignments were performed by MEGA v. 4.0 (Tamura *et al.* 2007) and improved manually. For parsimony analysis, the PAUP v. 4.0 software was used (Swofford 2002). Alignment gaps were treated as a fifth character state and all characters were unordered and of equal weight. Maximum parsimony analysis was performed for all data sets using the heuristic search option with 100 random taxa additions and tree bisection and reconstruction (TBR) as the branch-swapping algorithm. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. The robustness of the trees obtained was evaluated by 1 000 bootstrap replications (Hillis & Bull 1993). *Fennellia flavipes* NRRL 5504^T was chosen as outgroup in these analyses on the basis of prior studies (Peterson 2008).

RESULTS AND DISCUSSION

Phylogeny

Of the aligned β-tubulin sequences, a portion of 569 positions including 244 parsimony informative characters was selected for the analysis; MP analysis of the sequence data resulted in 46 679 similar, equally most parsimonious trees (tree length = 569 steps, consistency index = 0.7232, retention index = 0.9204), one of which is shown in Fig. 1. The calmodulin data set consisted of 764 characters including 299 parsimony informative sites; MP analysis resulted in 12 most parsimonious trees (length = 806, consistency index = 0.7122, retention index = 0.8704), one of which is presented in Fig. 2. The ITS data set consisted of 499 characters including 55 parsimony informative sites; MP analysis resulted in 36 equally most parsimonious trees (length = 102, consistency index = 0.8529, retention index = 0.9600), one of which is presented in Fig. 3.

Seven lineages were observed among isolates that have previously been treated as *A. terreus* and its subspecies by Raper & Fennell (1965) and others. *Aspergillus alabamensis, A. terreus* var. *floccosus*, *A. terreus* var. *africanus*, *A. terreus* var. *aureus* (*A. aureoterreus* according to Balajee *et al.* 2009), *A. hortai* and *A. terreus* NRRL 4017 all represent distinct lineages from the *A. terreus* clade based on phylogenetic analysis of calmodulin and β-tubulin sequences (Figs 1, 2). Among them, *A. terreus* var. *floccosus*, *A. terreus* NRRL 4017 and *A. aureoterreus* could also be distinguished from *A. terreus* by using ITS sequence data (Fig. 3). The *A. terreus* clade includes some other isolates which form well-defined subclades on the trees based on both β-tubulin and calmodulin sequence data. Further studies are needed to clarify if they represent separate species.

Also included in section *Terrei* are some species formerly placed in sections *Flavipedes* and *Versicolores*. A clade including the type isolate of *A. niveus* (CBS 115.27) constitutes a lineage closely related to *A. carneus*. *Fennellia nivea*, the hypothesised teleomorph is not related to this clade. *Aspergillus allahabadii, A. niveus* var. *indicus*, and two species originally placed in section *Versicolores*, *A. ambiguus* and *A. microcysticus* also form well-defined lineages on all trees (Figs 1–3).

Extrolites

Species in *Aspergillus* section *Terrei* are producers of a diverse array of secondary metabolites (Table 2). However, many of the species in the section produce different combinations of the following metabolites: acetylaranotin, asperphenamate, aspochalamins, aspulvinones, asteltoxin, asterric acid, asterriquinones, aszonalenins, atrovenetins, butyrolactones, citreoisocoumarins, citreoviridins, citrinins, decaturins, fulvic acid, geodins, gregatins, mevinolins, serantrypinone, terreic acid (only the precursor 3,6-dihydroxytoluquinone found), terreins,

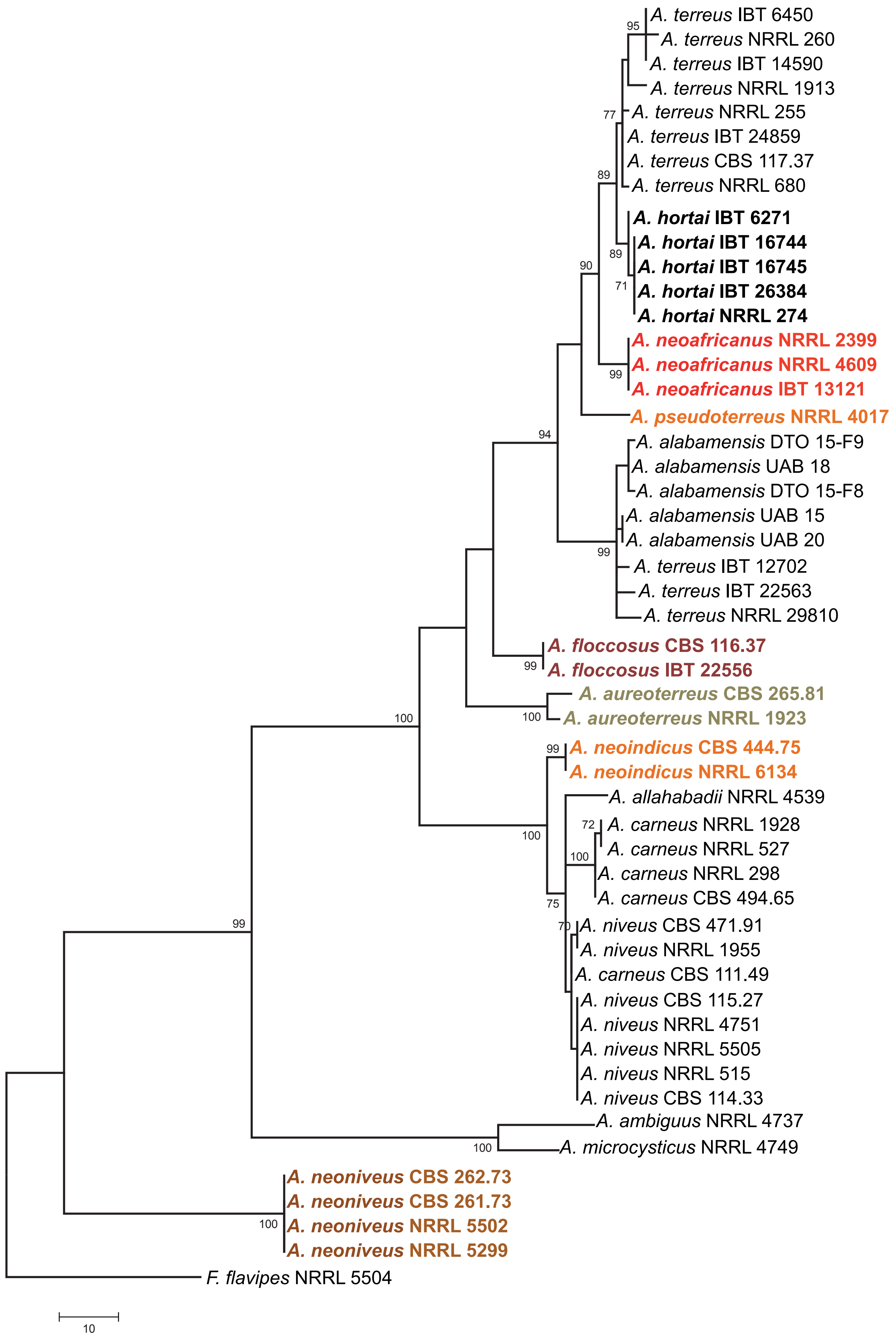

Fig. 1. The single MP tree obtained based on phylogenetic analysis of β-tubulin sequence data of *Aspergillus* section *Terrei*. Numbers above branches are bootstrap values. Only values above 70 % are indicated. *F.* = *Fennellia.*

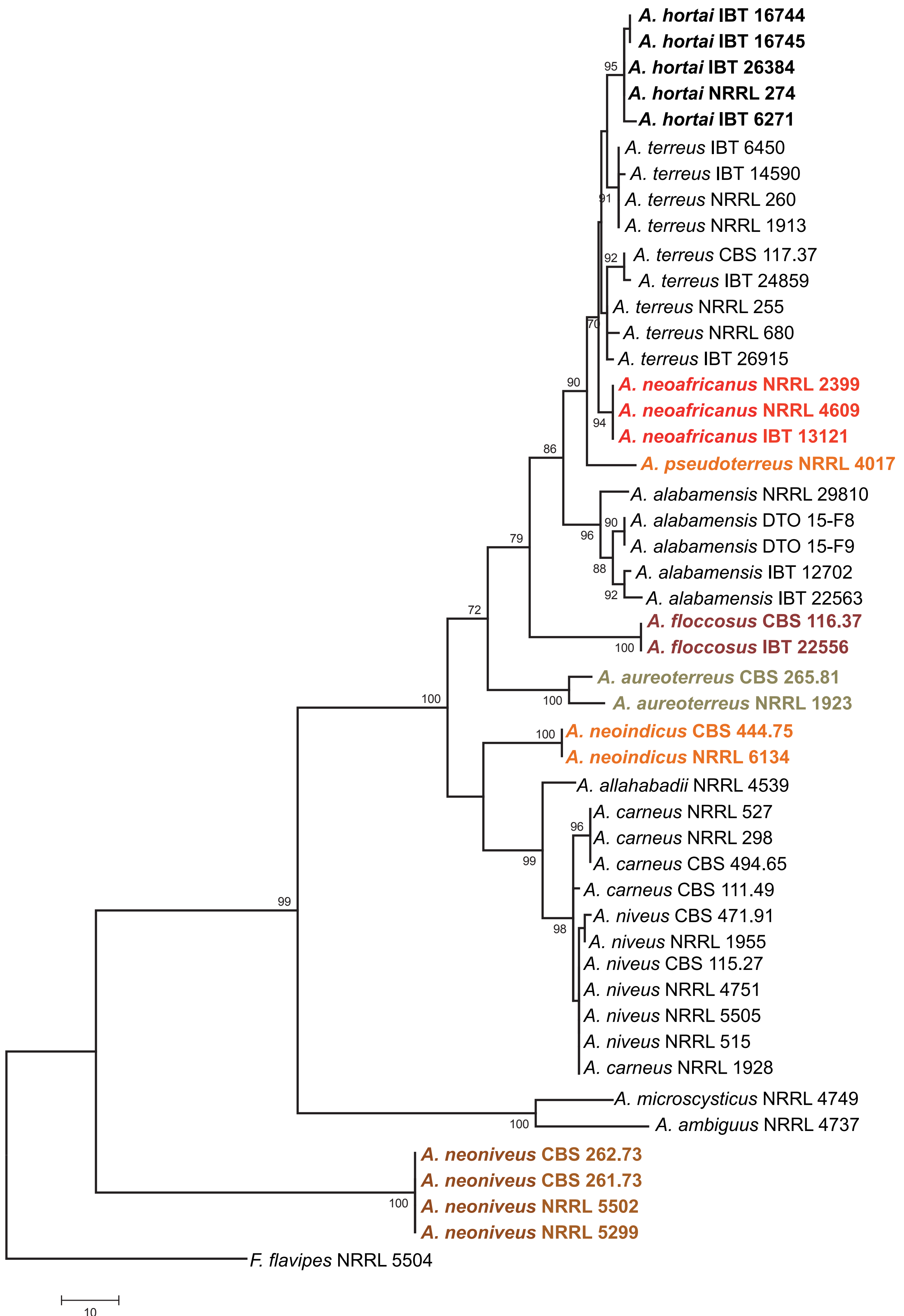

Fig. 2. One of the MP trees obtained based on phylogenetic analysis of calmodulin sequence data of *Aspergillus* section *Terrei*. Numbers above branches are bootstrap values. Only values above 70 % are indicated. *F.* = *Fennellia*.

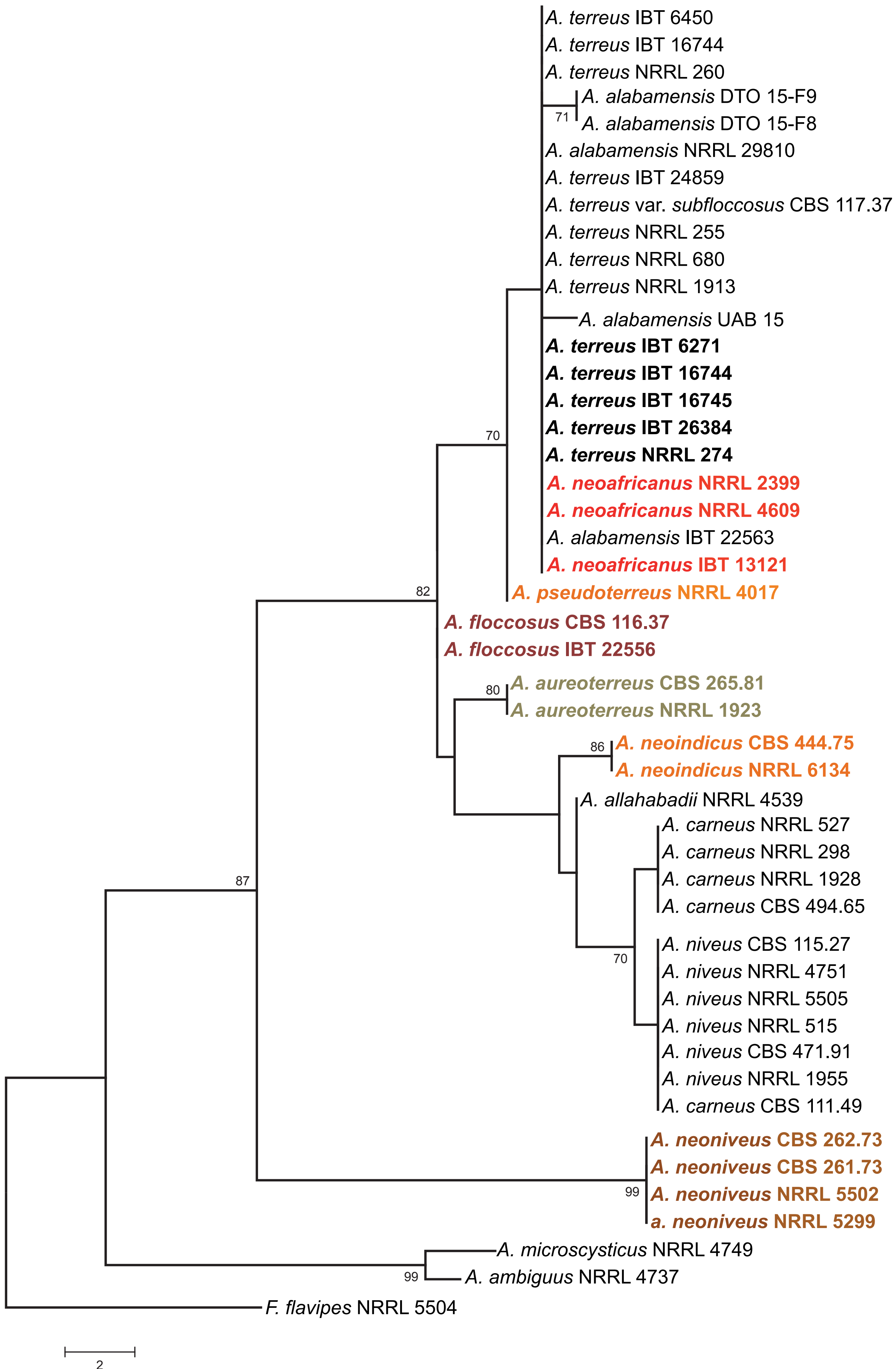

Fig. 3. One of the MP trees obtained based on phylogenetic analysis of ITS sequence data of *Aspergillus* section *Terrei*. Numbers above branches are bootstrap values. Only values above 70 % are indicated. *F.* = *Fennellia.*

Table 2. Extrolites produced by species in *Aspergillus* section *Terrei*.

Species	Strains examined	Extrolites
A. neoafricanus	CBS 130.55 = IBT 6266; ATCC 46560 = IFO 8835 = IBT 13121	Aspulvinone, asterriquinone, butyrolactones, citreoviridin, mevinolin, terrein, terrequinone A, (OSTO*). ATCC 46560 was reported to produce aspulvinones, asterriquinones, 3,6-dihydroxytoluquinone, emodin, α-oxo-β-(p-hydroxyphenyl)-γ-(p-hydroxy-m-3,3-dimethylallyl-benzyl)-γ-methoxycarbonyl-γ-butyrolactone = butyrolactone I, terrein (Kiriyama *et al.* 1977).
A. alabamensis	WB 1920 = IBT 22563; CDC UAB 28 = IBT 29083; IBT 29085 = CDC UAB1; 15-F8 = IBT 29084; 15-F9 = IBT 29086; IBT 12702; NRRL 28910 = IBT 29081	Asterriquinones, citrinin, terrein, terrequinone A, (NO2*) (in IBT 29084 = DTO 15-F8 additionally citreoviridin and a territrem). IBT 12702 produces asteltoxin, asterriquinones citrinin, fulvic acid, terrein, and NO2 and several unique extrolites only seen in this particular isolate.
A. allahabadii	IMI 139273 = IBT 23179; IBT 21128 = CCRC 32133	Asperphenamate, atrovenetins, butyrolactones, citrinin, gregatins, (SILKO*, ASPERGU*).
A. ambiguus	CBS 117.58ᵀ	a butyrolactone, (ATROV*), terrequinone A.
A. auroterreus	CBS 503.6.5 = IBT 22090; IBT 23362	Citreoviridin, PR-toxin?, terrequinone A (AU*, AR*, AE*, SPA*).
A. carneus	NRRL 527. = IBT 13986; WB 298 = IBT 22569	Aszonalenin, asperphenamate, citrinin, dihydrocitrinone, gregatins.
		Reported in literature: the depsipeptides aspergillicins A-E, marcfortine A, acyl aszonalenin (Capon *et al.* 2003), citrinin, dihydrocitrinone and sclerin (Chien *et al.* 1977).
A. floccosus	CBS 116.37ᵀ = IBT 10846 = WB 4872 = IBT 22556	Aszonalenin, austalides?, butyrolactones, citrinin, a decaturin, dihydrocitrinone, an isocoumarin, serantrypinone (NB*, NO22*, OSTO*, SOFL*).
A. hortai	NRRL 274ᵀ = IBT 26384; IBT 16745; IBT 16744	Acetylaranotin, butyrolactones, citrinin, 3-methylorsellinic acid, terrein, terrequinone A.
A. microcysticus	IMI 139275 = IBT 23270 = CBS 120.58	Asperphenamate, butyrolactones, terrequinone A, (FUSI*, SORBA*, SOSTAI*, OLKA-1,2,3*); asposterol (Heberle *et al.* 1974), aspochalasins A-D (Kelle-Schierlein & Kupfer 1979).
A. neoindicus	CBS 444.75ᵀ	Citrinin, naphthalic anhydride, atrovenetins, (SILKO).
A. niveus	CBS 115.27ᵀ = IBT 10831, CBS 114.33 = IBT 19832; IMI 165060 = NRRL 1955 = IBT 13985 = CBS 471.91; IBT 16747; IBT 28598; IBT 28597; IBT 18418	Aszonalenine, butyrolactones, citrinin and gregatins, (SILKO*, SNOP*); CBS 115.27ᵀ and CBS 114.33 only produce gregatins.
A. pseudoterreus	NRRL 4017 = IBT 29082 = 47-E6	Aspulvinones, asterriquinones, butyrolactones, citreoisocoumarin, citreoviridin, citrinin, 3-methylorsellinic acid, terrein, terrequinone A, (XANT*, AQ-1460*).
A. terreus	WB 255 = IBT 22562; CBS 601.65 = IBT 22089; IBT 24859; IBT 14590 = UAMH 4733; ATCC 20542; IBT 26974; IBT 6450; NRRL 1913 = IBT 26385; NRRL 260; CBS 117.37 = WB 4873 = IBT 22565	Acetylaranotin, aspulvinones, asterric acid, asterriquinones, aszonalenin (few strains), benzomalvins or related compounds, butyrolactones, citreoisocoumarin (in few strains), citreoviridin, 3,6-dihydroxytoluquinone (in some strains), erdin, geodin, geodoxin, 3-methylorsellinic acid, mevinolin, terrein, terrequinone A, terretonin (in some strains), territrems (in few strains), (GNOC*, GYAL*, SNIR*).
F. neonivea	IMI 171878 = NRRL 5299 = CBS 261.73ᵀ	phenylahistin ?, paspalicine, an aspochalamin, many indol-alkaloids, (NB*); the cytochalasan derivatives aspochalamins A-D, aspochalasins D & Z, and citreoviridin A & B were isolated by Holtzel *et al.* (2004) and Gebhardt *et al.* (2004) from *A. niveus* LU 9575 (latter strain not available).
var. *curvatus*	CBS 265.81 = IBT 29947	Acyl aszonalenin, asperphenamate, terreic acid?

*Metabolites annotated with capitals had characteristic UV spectra, but their structure has not been elucidated as yet.

terrequinones, terretonins and territrems. The cholesterol-lowering agent mevinolin was found in *A. terreus* and *A. neoafricanus* only. The hepatotoxic extrolite citrinin was found in eight species: *A. alabamensis, A. allahabadii, A. carneus, A. floccosus, A. hortai, A. neoindicus, A. niveus* and *A. pseudoterreus*. The neurotoxic extrolite citreoviridin was found in five species: *A. neoafricanus, A. aureoterreus, A. pseudoterreus, A. terreus* and *Fennellia neonivea*. Territrems, tremorgenic extrolites, were found in some strains of *A. alabamensis* and *A. terreus*.

Species descriptions

Aspergillus aureoterreus Samson, S.W.Peterson, Frisvad & Varga, **stat. et nom. nov.** MycoBank MB560392. Fig. 5.
Basionym: *Aspergillus terreus* Thom var. *aureus* Thom & Raper – In A Manual of the Aspergilli: 198. 1945.

Type of *Aspergillus terreus* var. *aureus* from soil, Texas, USA.

This variety was proposed by Thom & Raper (1945) based on the slow growing colonies, which are bright yellow. It produces conidiophores tardily. As with the variety *africanus,* the ex-type isolate can be clearly distinguished based on our phylogenetic analysis. Therefore we propose to raise the taxon to species level.

Aspergillus floccosus Samson, S.W. Peterson, Frisvad & Varga, **comb. et stat. nov.** MycoBank MB560393. Fig. 6.
= *Aspergillus terreus* var. *floccosus* Shih, Lignan Sci Journal 15: 372. 1936.

Type of *Aspergillus terreus* var. *floccosus* from waste cloth, Wuchang, China, isolated by Y.K. Shih as No. A 369.

The ex-type culture shows white floccose colonies with some hyaline exudate on Czapek yeast agar. On MEA colonies are white with a light brown centre. Isolates of *A. terreus* may vary in colony morphology sometimes showing floccose colonies. The ex-type isolate of *A. terreus* var. *floccosus* CBS 116.37, which CBS directly received from Dr Shih, grouped in a distinct lineage, and therefore we propose to raise the variety to species level. Thom & Raper (1945) accepted the variety to accommodate colonies of *A. terreus* with strongly floccose colonies and conidial heads which are less compact and lighter in colour. Raper & Fennell (1965) however, synonymised var. *floccosus* under *A. terreus*, because

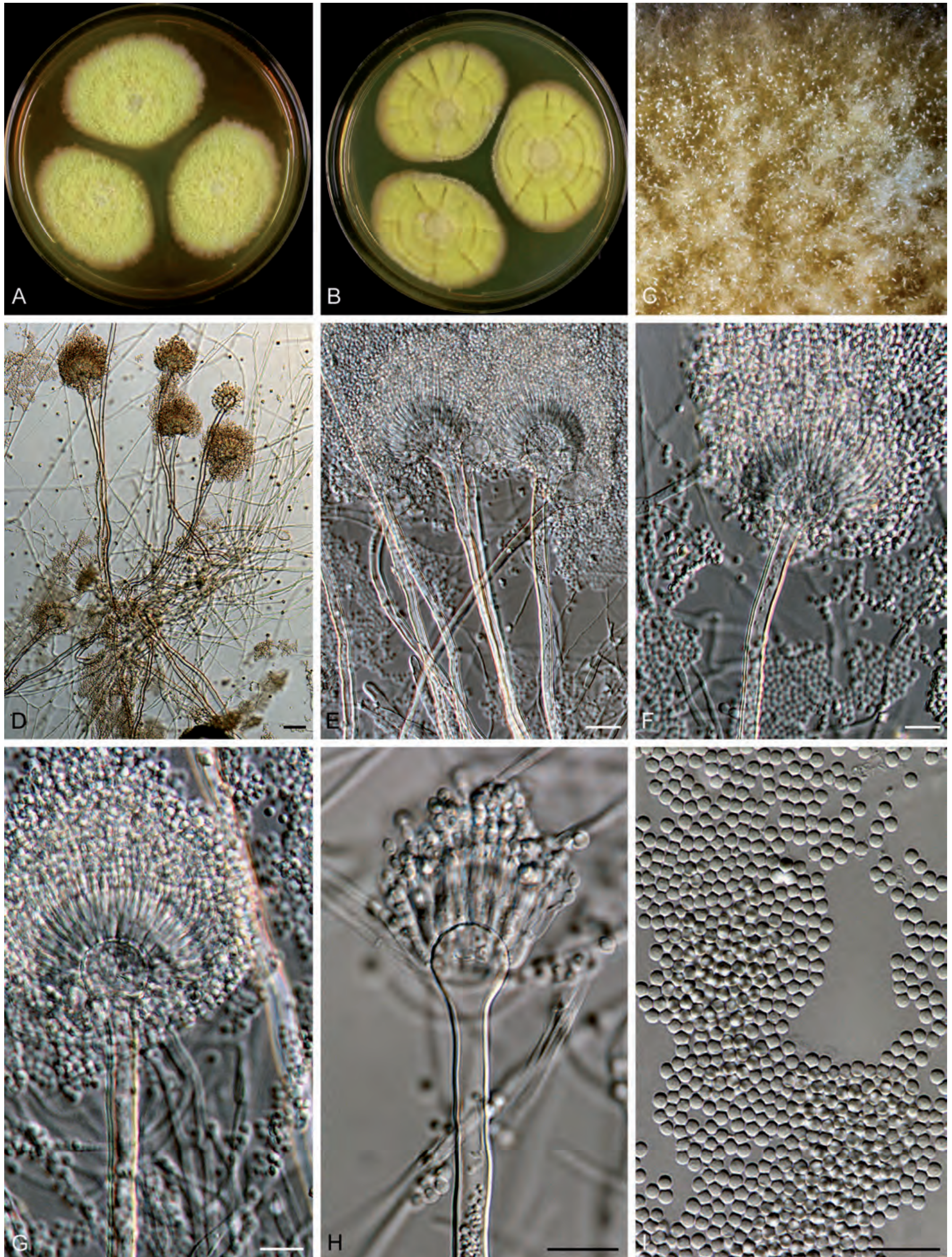

Fig. 4. *Aspergillus aureoterreus*. A–C. Colonies incubated at 25 °C for 7 d, A. CYA, B. MEA, C. CREA, D–I. Conidiophores and conidia. Scale bars = 10 µm.

Fig. 5. *Aspergillus floccosus*. 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.

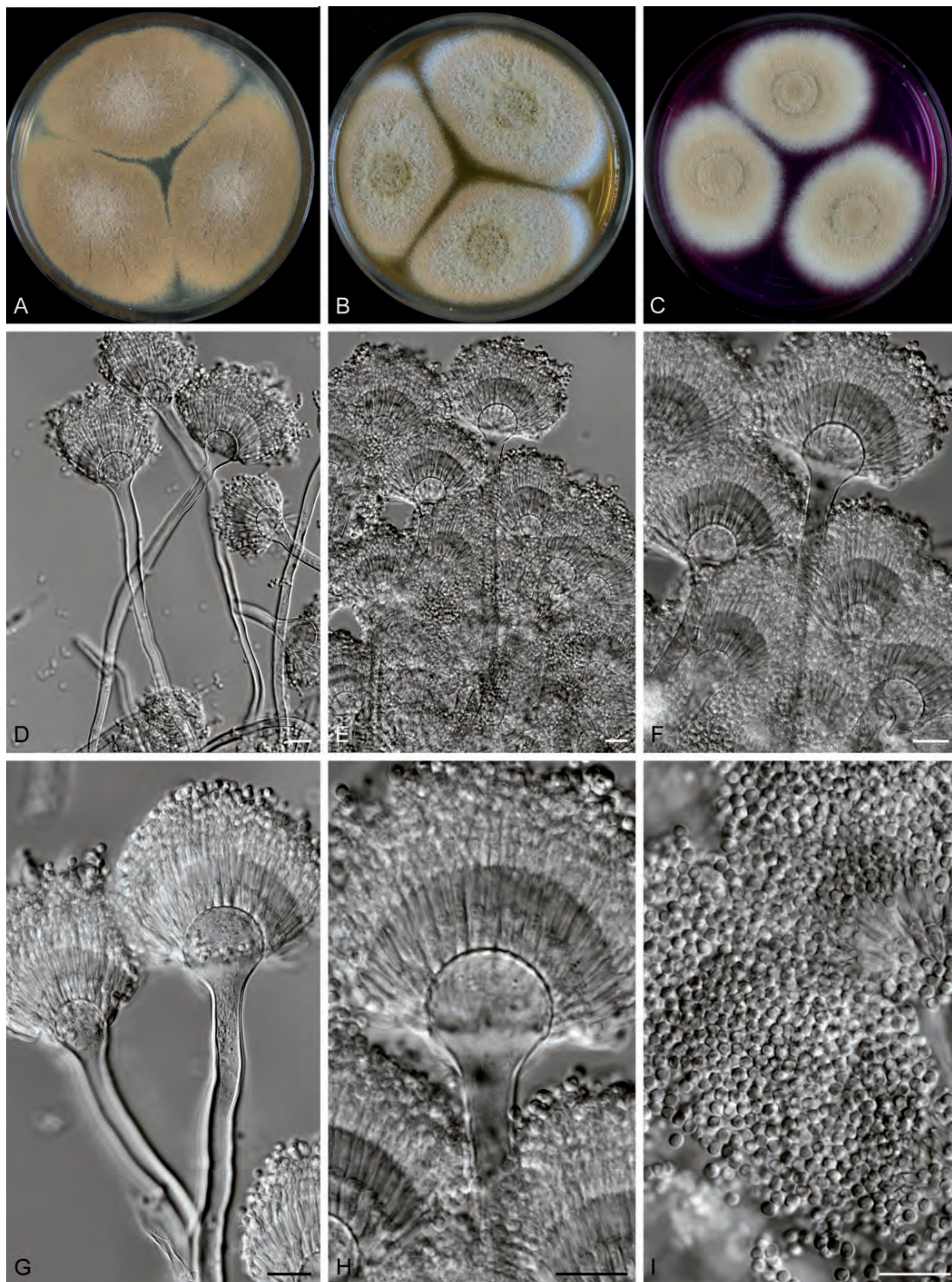

Fig. 6. *Aspergillus hortai*. A–C. Colonies incubated at 25 °C for 7 d, A. CYA, B. MEA, C. CREA, D–I. Conidiophores and conidia. Scale bars = 10 µm.

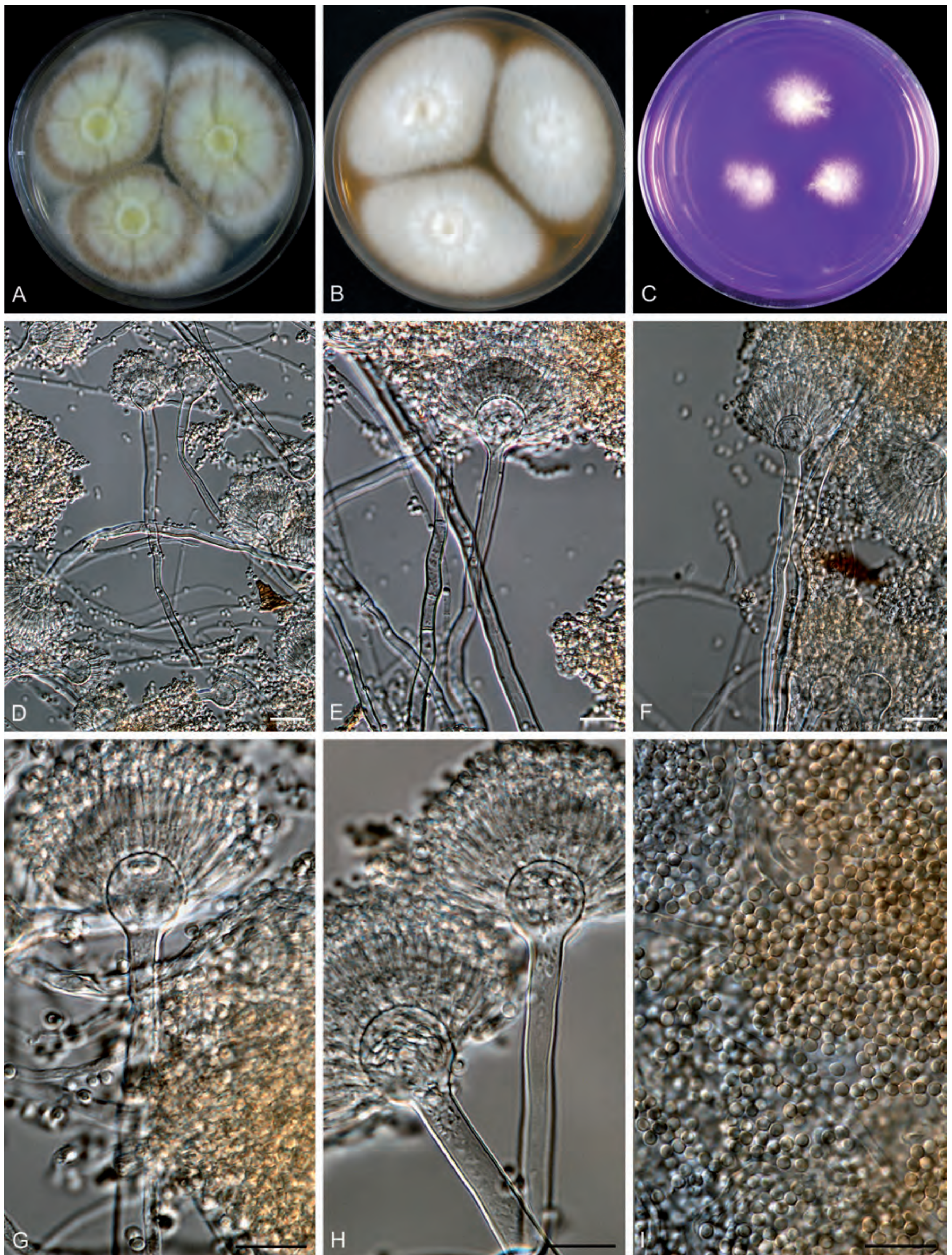

Fig. 7. *Aspergillus neoafricanus*. A–C. Colonies incubated at 25 °C for 7 d, A. CYA, B. MEA, C. CREA, D–I. Conidiophores and conidia. Scale bars = 10 µm.

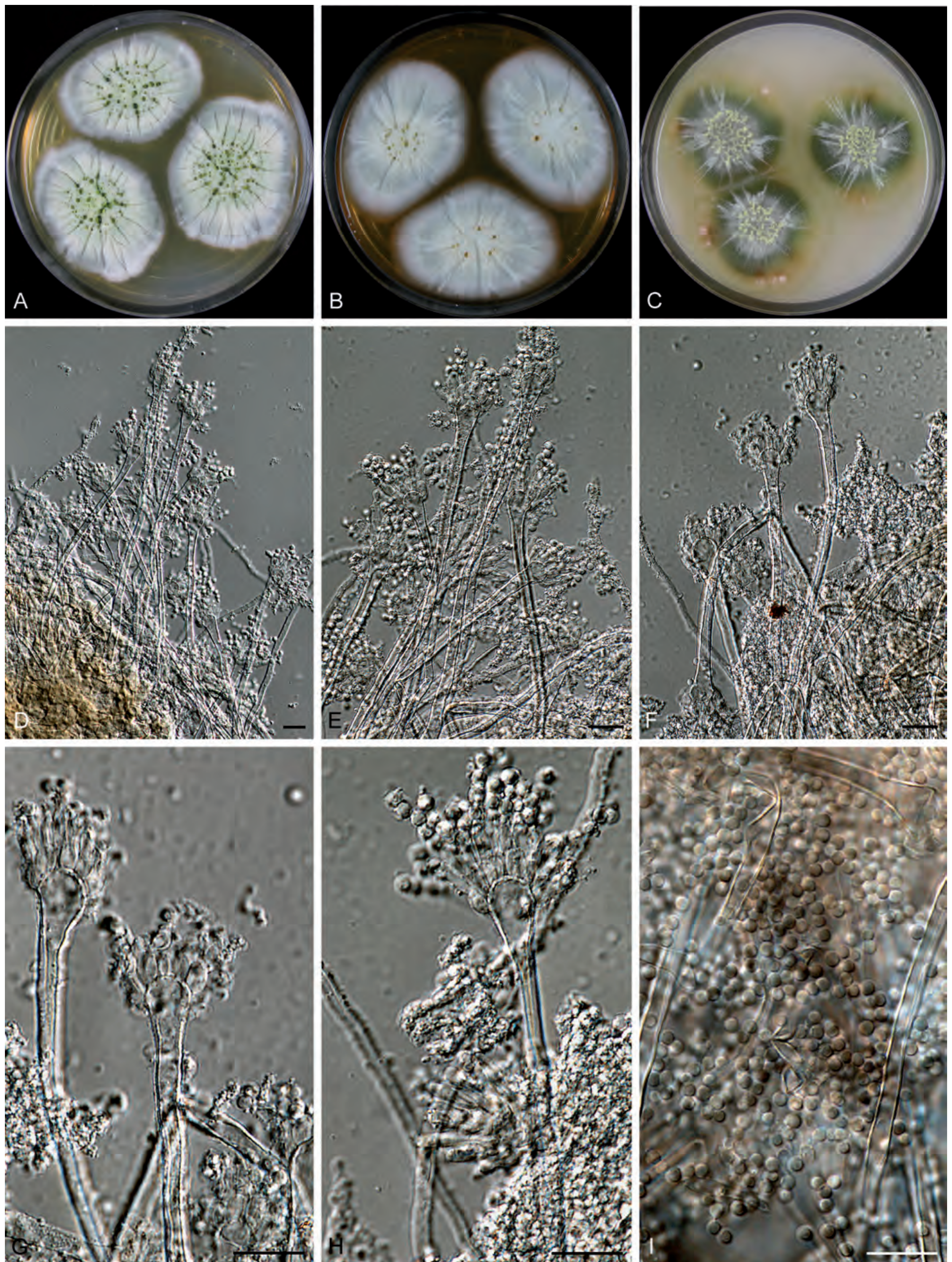

Fig. 8. *Aspergillus neoindicus*. A–C. Colonies incubated at 25 °C for 7 d, A. CYA, B. MEA, C. CREA, D–I. Conidiophores and conidia. Scale bars = 10 µm.

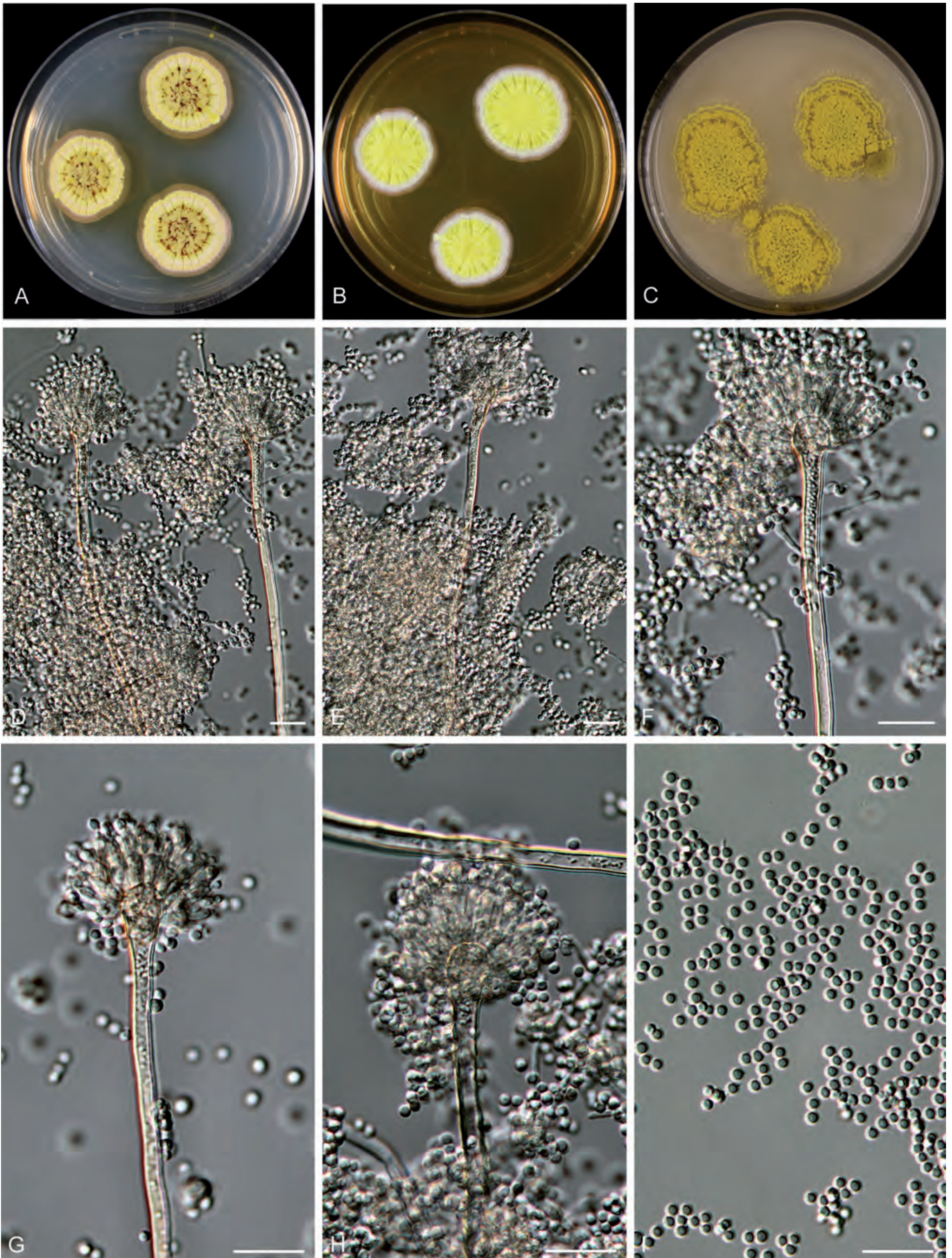

Fig. 9. *Aspergillus neoniveus* sp. nov. A–B. Colonies incubated at 25 °C for 7 d, A. CYA, B. MEA, C. Crusts of Hülle cells, D–E, G–I. Conidiophores and conidia. F. Hülle cells. Scale bars = 10 µm, except F = 100 µm.

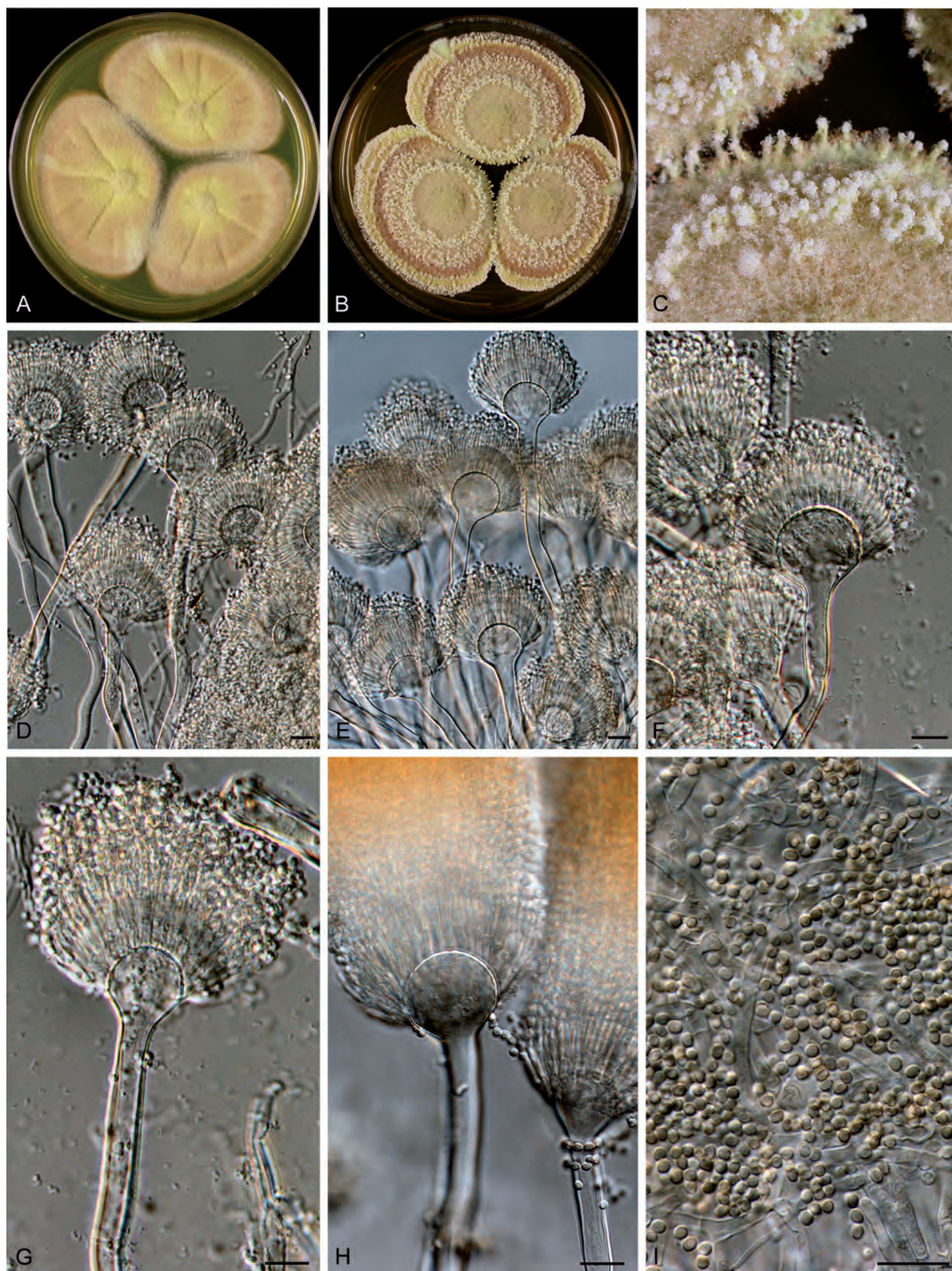

Fig. 10. *Aspergillus pseudoterreus*. A–C. Colonies incubated at 25 °C for 7 d, A. CYA, B. MEA, C. CREA, D–I. Conidiophores and conidia. Scale bars = 10 µm.

they examined the ex-type culture and believed that it belonged to *A. flavipes*. Interestingly they did not treat it as a synonym of this species, but left the name under *A. terreus.*

Aspergillus hortai (Langeron) Dodge, in Medical Mycology: 628. 1935. Fig. 7.
Basionym: *Sterigmatocystis hortai* Langeron, Bulletin Societé de Pathologie Exotique 15: 383. 1922.

Type of *Sterigmatocystis hortai* as clinical isolate, from ear, Rio de Janeiro, Brazil.

Langeron (1922) described this fungus from a human ear in Rio de Janeiro. Dodge (1935) noticed the resemblance of Langeron's fungus and transferred it to *Aspergillus*. However, Raper & Fennell (1965) considered it as a synonym of *Aspergillus terreus,* but our phylogenetic analysis clearly shows that it is distinct from *A. terreus* and therefore we accept it as a distinct species. *Aspergillus hortai* is known from the ex-type isolate and soil isolates from the Galapagos Islands and Florida (USA). The species show a strong morphological resemblance to *A. terreus*, but have a distinct extrolites profile.

Aspergillus neoafricanus Samson, S.W. Peterson, Frisvad & Varga, **comb. et stat. nov.** MycoBank MB560391. Fig. 4.
Basionym: *Aspergillus terreus* Thom var. *africanus* Fennell and Raper, Mycologia 47: 86. 1955.

Type of *Aspergillus terreus* var. *africanus* from soil, Tafo, Ghana.

The ex-type strain of *Aspergillus terreus* var. *africanus* is grouped in a distinct lineage from *Aspergillus terreus* and therefore we propose to raise the taxon to species level. Raper & Fennell (1965) considered this as a variety because they observed slow growing colonies on Czapek agar bright yellow vegetative mycelium. CBS 130.55 = NRRL 2399 derived from the type is now more or less floccose, with a yellow centre. Sporulation with yellow brown conidia occurs at the edges of the colonies. The degenerated condition of the culture also explains why we did not observe the sclerotium-like structures on malt extract agar + 20 % sucrose, which were reported by Raper & Fennell (1965).

Aspergillus neoindicus Samson, S.W. Peterson, Frisvad & Varga, **stat. et nom. nov.** MycoBank MB560394. Fig. 8.
Basionym: *Aspergillus niveus* var. *indicus* Lal & Sarbhoy, Indian Phytopathology 25: 309. 1973.

Type of *Aspergillus niveus* var. *indicus* from soil, Maharashta, India.

This species was described as a variety from soil in Maharastra by Lal & Sarbhoy (1973) and was considered by Samson (1979) as a synonym of *A. flavipes*. The species is phylogenetically distinct from *A. flavipes*, and is characterised by yellow green mycelial tufts. On OA a dark green pigment is diffusing into agar. The discrete masses of ellipsoidal and elongate Hülle cells described by Lal & Sarbhoy (1973) and Samson (1979) have not been observed in our current study.

Aspergillus neoniveus Samson, S.W. Peterson, Frisvad & Varga, **nom. nov.** MycoBank MB5603945. Fig. 9.
Basionym: *Emericella nivea* Wiley & Simmons, Mycologia 65: 934. 1973 (non *Aspergillus niveus* Blochwitz, 1929).
≡ *Fennellia nivea* (Wiley & Simmons) Samson, Stud. Mycol. 18: 5. 1979

Type from forest soil in Thailand.

Samson (1979) considered *Emericella nivea* distinct from the other *Emericella* species by the hyaline to pale yellow ascospores and the anamorph belonging to the *A. flavipes* group. The species is similar to *Fennellia flavipes* Wiley & Simmons and could be classified as the second species of *Fennellia.* However our phylogenetic analysis and those by Peterson (2008) and Peterson *et al*. (2008) showed that the isolates of *Emericella nivea* clustered separately from the isolates of *Aspergillus niveus* and hence it represents a different taxon. Following the need for an orderly transitition to a single-name nomenclatural system (Hawksworth *et al*. 2011) we have chosen to name this species in *Aspergillus* and not in its teleomorph genus, *Fennellia*.

Aspergillus pseudoterreus **S.W.** Peterson, Samson & Varga, **sp. nov.** MycoBank MB560396. Fig. 10.

Coloniis in agaro CYA aurantiaco-brunneis, caespitulis flavis usque ad 4 cm diam. Coloniis in agaro MEA cum marginibus clare aurantiacis. Conidiophoris aggregatis in synnematibus laxis, namque in coloniis vetustioribus (ad 14 d). Conidiophoris plerumque biseriatis, capitulis columnaribus efferentibus. Vesiculis globosis, 16–23 µm diam, stipitibus levibus, hyalinis, 4.5–7 µm latis. Conidiis levibus, hyalinis, globosis vel ellipsoideis, 1.5–2.2 × 1.8–2.5 µm.

Typus: from soil Argentina (CBS H-20631 -- holotypus, culture ex-type NRRL 4017).

Colonies on CYA orange brown with yellow tufts reaching a diameter of 4 cm. On MEA colonies are bright yellow with a clear orange edge. In older cultures of up to 14 d conidiophores are bundled in loose synnemata. Conidiophores typically biseriate, producing columnar heads. Vesicles globose 16–23 µm, stipe smooth, hyaline, 4.5–7 µm, conidia smooth, hyaline, globose to ellipsoidal, 1.5–2.2 × 1.8–2.5 µm.

This species was isolated from soil in Argentina and is characterised by a pronounced synnematal growth on MEA. The colony colour is reddish brown with biseriate conidiophores producing globose to ellipsoidal conidia.

ACKNOWLEDGEMENTS

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Two new aflatoxin producing species, and an overview of *Aspergillus* section *Flavi*

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Abstract: *Aspergillus* subgenus *Circumdati* section *Flavi* includes species with usually biseriate conidial heads, in shades of yellow-green to brown, and dark sclerotia. Several species assigned to this section are either important mycotoxin producers including aflatoxins, cyclopiazonic acid, ochratoxins and kojic acid, or are used in oriental food fermentation processes and as hosts for heterologous gene expression. A polyphasic approach was applied using morphological characters, extrolite data and partial calmodulin, β-tubulin and ITS sequences to examine the evolutionary relationships within this section. The data indicate that *Aspergillus* section *Flavi* involves 22 species, which can be grouped into seven clades. Two new species, *A. pseudocaelatus* sp. nov. and *A. pseudonomius* sp. nov. have been discovered, and can be distinguished from other species in this section based on sequence data and extrolite profiles. *Aspergillus pseudocaelatus* is represented by a single isolate collected from *Arachis burkartii* leaf in Argentina, is closely related to the non-aflatoxin producing *A. caelatus*, and produces aflatoxins B & G, cyclopiazonic acid and kojic acid, while *A. pseudonomius* was isolated from insects and soil in the USA. This species is related to *A. nomius*, and produces aflatoxin B_1 (but not G-type aflatoxins), chrysogine and kojic acid. In order to prove the aflatoxin producing abilities of the isolates, phylogenetic analysis of three genes taking part in aflatoxin biosynthesis, including the transcriptional regulator *aflR*, norsolonic acid reductase and O-methyltransferase were also carried out. A detailed overview of the species accepted in *Aspergillus* section *Flavi* is presented.

Key words: aflatoxin, Ascomycetes, *Aspergillus* section *Flavi,* β-tubulin, calmodulin, extrolites, ITS, polyphasic taxonomy.
Taxonomic novelties: *Aspergillus pseudocaelatus* Varga, Samson & Frisvad sp. nov., *Aspergillus pseudonomius* Varga, Samson & Frisvad sp. nov.

INTRODUCTION

Aspergillus section *Flavi* historically includes species with conidial heads in shades of yellow-green to brown and dark sclerotia. Isolates of the so-called domesticated species, such as *A. oryzae, A. sojae* and *A. tamarii* are used in oriental food fermentation processes and as hosts for heterologous gene expression (Campbell-Platt & Cook 1989). Genetically modified *A. oryzae* strains are used for the production of enzymes including lactase, pectin esterase, lipase, protease and xylanase (Pariza & Johnson 2001). Several species of section *Flavi* produce aflatoxins, among which aflatoxin B_1 is the most toxic of the many naturally occurring secondary metabolites produced by fungi. Aflatoxins are mainly produced by *A. flavus* and *A. parasiticus*, which coexist with and grow on almost any crop or food.

Several species have been described in the past which were assigned to *Aspergillus* section *Flavi* mainly based on traditional methods (morphological parameters, including colony diameter, colour and texture, size and texture of conidia and conidiophore structure; Klich 2002). However, species classification may be difficult due to extensive divergence of morphological characters produced by a high level of genetic variability (Kumeda & Asao 1996). Despite intense investigation, the taxonomy of this group of fungi is still highly complex. Recent data indicate that several of the species assigned to section *Flavi* cannot be distinguished based on morphological features alone (Frisvad *et al.* 2005, Pildain *et al.* 2008). Recently, a six-step molecular strategy using real-time PCR, RAPD and SmaI digestion of the nuclear DNA has been worked out to distinguish nine species of the section (Godet & Munaut 2010). In this study, we examined available isolates of the species proposed to belong to this section to clarify its taxonomic status. The methods used include sequence analysis of the ITS region (including intergenic spacer regions 1 and 2, and the 5.8 S rRNA gene of the rRNA gene cluster), and parts of the β-tubulin and calmodulin genes, macro- and micromorphological analysis, and analysis of extrolite profiles of the isolates. We also examined the presence of three aflatoxin biosynthetic genes in some aflatoxin-producing and non-producing isolates.

MATERIALS AND METHODS

Isolates

The strains used in this study are listed in Table 1. Sequence data of several other isolates available from GenBank database have also been used for constructing phylogenetic trees.

Morphological analysis

For macromorphological observations, Czapek Yeast Autolysate (CYA), Malt Extract Autolysate (MEA) agar, Yeast Extract Sucrose Agar (YES), Creatine Agar (CREA), and *Aspergillus flavus/ parasiticus* Agar (AFPA) 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, microscopic mounts were made in lactic acid with cotton blue from MEA colonies and a drop of alcohol was added to remove air bubbles and excess conidia.

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Table 1. *Aspergillus* isolates examined.

Name	Isolate	Source
A. albertensis	NRRL 20602^T = ATCC 58745	Human ear, Alberta, Canada
A. alliaceus	CBS 542.65^T = NRRL 4181	Soil, Australia
	CBS 536.65	Dead blister beetle *Macrobasis albida*, Washington, CO, USA
	CBS 612.78 = NRRL 5181	Buenos Aires, Argentina
A. arachidicola	CBS 117610^T = IBT 25020	*Arachis glabrata* leaf, CO, Argentina
	CBS 117615 = IBT 27178	*Arachis glabrata* leaf, CO, Argentina
A. avenaceus	CBS 109.46^T = IBT 4376	*Pisum sativum* seed, UK
	CBS 102.45	NCTC 6548
A. bombycis	CBS 117187 = NRRL 26010^T	Frass in a silkworm rearing house, Japan
A. caelatus	CBS 763.97^T = NRRL 25528	Soil, USA
	CBS 764.97 = NRRL 25404	Soil, USA
A. coremiiformis	CBS 553.77^T = NRRL 13756	Soil, Ivory Coast
A. fasciculatus	CBS 110.55^T	Air contaminant, Brazil
A. flavofurcatIs	CBS 484.65^T	Air contaminant, Brazil
A. flavus	CBS 100927^T	Cellophane, South Pacific Islands
	CBS 116.48	Unknown source, the Netherlands
	CBS 616.94	Man, orbital tumor, Germany
A. flavus var. *columnaris*	CBS 485.65^T	Butter, Japan
	CBS 117731	*Dipodomys spectabilis* cheek pouch, New Mexico, USA
A. kambarensis	CBS 542.69^T	Stratigraphic core sample, Japan
A. lanosus	CBS 650.74^T	Soil under *Tectona grandis*, Gorakhpur, India
A. leporis	CBS 151.66^T	Dung of *Lepus townsendii*, USA
	CBS 349.81	Soil, Wyoming, USA
A. minisclerotigenes	CBS 117633	*Arachis hypogaea* seed, FO, Argentina
	CBS 117635^T = IBT 27196	*Arachis hypogaea* seed, CD, Argentina
A. nomius	CBS 260.88^T = NRRL 13137	Wheat, USA
A. oryzae	CBS 100925^T	Unknown source, Japan
A. parasiticus	CBS 100926^T	*Pseudococcus calceolariae*, sugar cane mealy bug, Hawaii, USA
A. parasiticus var. *globosus*	CBS 260.67^T	Unknown source, Japan
A. parvisclerotigenus	CBS 121.62^T	*Arachis hypogaea*, Nigeria
A. pseudocaelatus	CBS 117616	*Arachis burkartii* leaf, CO, Argentina
A. pseudonomius	CBS 119388 = NRRL 3353	Diseased alkali bees, USA
A. pseudotamarii	CBS 766.97^T = NRRL 25517	Soil, USA
	CBS 765.97	Soil, USA
A. sojae	CBS 100928^T	Soy sauce, Japan
A. subolivaceus	CBS 501.65^T	Cotton, Lintafelt, UK
A. tamarii	CBS 104.13^T	Activated carbon
A. terricola	CBS 620.95	WB4858
	CBS 579.65^T	USA
A. terricola var. *americanus*	CBS 580.65^T	Soil, USA
	CBS 119.51	Japan
A. terricola var. *indicus*	CBS 167.63^T	Mouldy bread, Allahabad, India
A. thomii	CBS 120.51^T	Culture contaminant
A. togoensis	CBS 272.89^T	Seed, Central African Republic
A. toxicarius	CBS 822.72^T	*Arachis hypogaea*, Uganda
	CBS 561.82	Löss deposit, Nebraska, USA
A. zhaoqingensis	CBS 399.93^T	Soil, China

CBS = CBS-KNAW Fungal Biodiversity Centre, Utrecht, the Netherlands. IBT = IBT Culture Collection of Fungi, Lyngby, Denmark. NRRL = USDA ARS Culture Collection, Peoria, USA. ATCC = American Type Culture Collection, Manassas, USA.

Extrolite analysis

The cultures were analysed according to the HPLC-diode array detection method of Frisvad & Thrane (1987, 1993) as modified by Smedsgaard (1997). The isolates were analysed on CYA and YES agar using three agar plugs (Smedsgaard 1997). 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), with minor modifications as described by Smedsgaard (1997).

Genotypic analysis

The cultures used for the molecular studies were grown on malt peptone (MP) broth using 1 % (w/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).

The presence of three genes taking part in aflatoxin biosynthesis has also been examined in some isolates. Part of the transcriptional regulator of aflatoxin biosynthesis, *aflR*, was amplified using the primers aflr-F (5'-GGGATAGCTGTACGAGTTGTGCCAG-3') and aflR-R (5'-TGGKGCCGACTCGAGGAAYGGGT-3') developed based on previously identified *aflR* sequences in the GenBank database. Part of the norsolonic acid reductase (*norA*, *aflE*; Yu *et al.* 2004) gene was amplified using the primers nor1 (5'-ACCGCTACGCCGGCACTCTCGGCA-3') and nor2 (5'-GTTGGCCGCCAGCTTCGACACAGC-3') developed by Geisen (1996). Part of the O-methyltransferase gene (*omtA*, *aflP*; Yu *et al.* 2004) was amplified using the primers omt1 (5'-GTGGACGGACCTAGTCCGACATCAC-3') and omt2 (5'-GTCGGCGCCACGCACTGGGTTGGGG-3') (Geisen 1996). Sequence analysis of the amplified products was carried out as described previously (Varga *et al.* 2007a).

DNA sequences were edited with the DNASTAR computer package. Alignments of the sequences were performed using MEGA v. 4 (Tamura *et al.* 2007). Phylogenetic analysis of sequence data was 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, 1000 bootstrap replicates were run by maximum parsimony (Hillis & Bull 1993). Other measures including tree length, consistency index, retention index and rescaled consistency index (CI, RI and RC, respectively) were also calculated. *Neopetromyces muricatus* CBS 112808^T was used as outgroup in the analyses of calmodulin, ITS and β-tubulin data sets, while *A. versicolor* SSRC 108 sequences were used as outgroups during analysis of *aflR* and *norA* sequences. No outgroup was used during the analysis of the *omtA* dataset, as sequences were not available from any other aflatoxin producing species outside *Aspergillus* section *Flavi*. Sequences were deposited at GenBank under accession numbers indicated on the figures.

RESULTS

Phylogenetic analysis

We examined the genetic relatedness of section *Flavi* isolates using sequence analysis of the ITS region of the ribosomal RNA gene cluster, and parts of the calmodulin and β-tubulin genes. During analysis of part of the β-tubulin gene, 561 characters were analysed, among which 223 were found to be phylogenetically informative. One of the 57 MP trees based on partial β-tubulin genes sequences is shown in Fig. 1 (tree length: 544 steps, consistency index: 0.7279, retention index: 0.9051). The calmodulin data set included 583 characters, with 221 parsimony informative characters. One of the 485 MP trees based on partial calmodulin gene sequences is shown in Fig. 2 (tree length: 557, consistency index: 0.7181, retention index: 0.9026). The ITS data set included 496 characters with 58 parsimony informative characters. One of the 235 MP trees is shown in Fig. 3 (tree length: 193, consistency index: 0.8446, retention index: 0.8592).

Phylogenetic analysis of ITS, calmodulin and β-tubulin sequence data indicated that the "*A. caelatus*" isolate CBS 117616 is closely related to, but phylogenetically distinct from *A. caelatus* (Figs 1–3). While all *A. caelatus* isolates known have came from soil, peanuts or tea fields located in Japan or USA, this isolate came from an *Arachis burkartii* leaf from Corrientes province, Argentina. This isolate also produces a set of different extrolites including aflatoxins B_1, B_2, G_1, G_2, kojic acid and cyclopazonic acid, while *A. caelatus* isolates produce kojic acid and aspirochlorin. Another isolate, "*A. nomius*" CBS 119388 (= NRRL 3353) was found to form a distinct clade on the trees based on calmodulin and β-tubulin sequence data (Fig. 1, 2). This isolate was also found to be different from *A. nomius* and *A. arachidicola* by physiological means; it produces chrysogine, kojic acid and aflatoxin B_1, similarly to *A. arachidicola*, which also produces aflatoxin G_1. In addition, *A. arachidicola* produces parasiticolide, ditryptophenaline and metabolite "NO2", the last one also being produced by isolate CBS 119388. *Aspergillus nomius* produces both B- and G-type aflatoxins, kojic acid, but not chrysogine. Based on phylogenetic analysis of calmodulin, β-tubulin, ITS and norsolonic acid reductase gene sequences, this new species includes several other isolates from insects and soil in Louisiana, Texas, Wyoming and Wisconsin in the USA (Peterson *et al.* 2001). Unfortunately, these isolates were not available for this study. The late C.W. Hesseltine (NRRL, Peoria USA) indicated in a personal communication to J.C. Frisvad, that he considered NRRL 3353 morphologically different from other *A. nomius*, which was backed up by differences in tolerance to low water activity. These observations should be further investigated.

The presence of 3 genes taking part in aflatoxin biosynthesis has also been examined in a set of isolates, including isolate CBS 117616 and several *A. caelatus* isolates. While isolate CBS 117616 carried homologs of all three examined genes, the *A. caelatus* isolates did not carry homologs of *aflR* and *norA* (Fig. 4). During analysis of the *aflR* dataset, 514 characters were analised, among which 113 were found to be phylogenetically informative. One of the 5 MP trees based on partial *aflR* genes sequences is shown in Fig. 5 (tree length: 464 steps, consistency index: 0.8836, retention index: 0.9339). The *norA* data set included 348 characters, with 40 parsimony informative characters. One of the 2 MP trees based on partial *norA* gene sequences is shown in Fig. 6 (tree length: 174, consistency index: 0.9138, retention index: 0.9032). The *omtA*

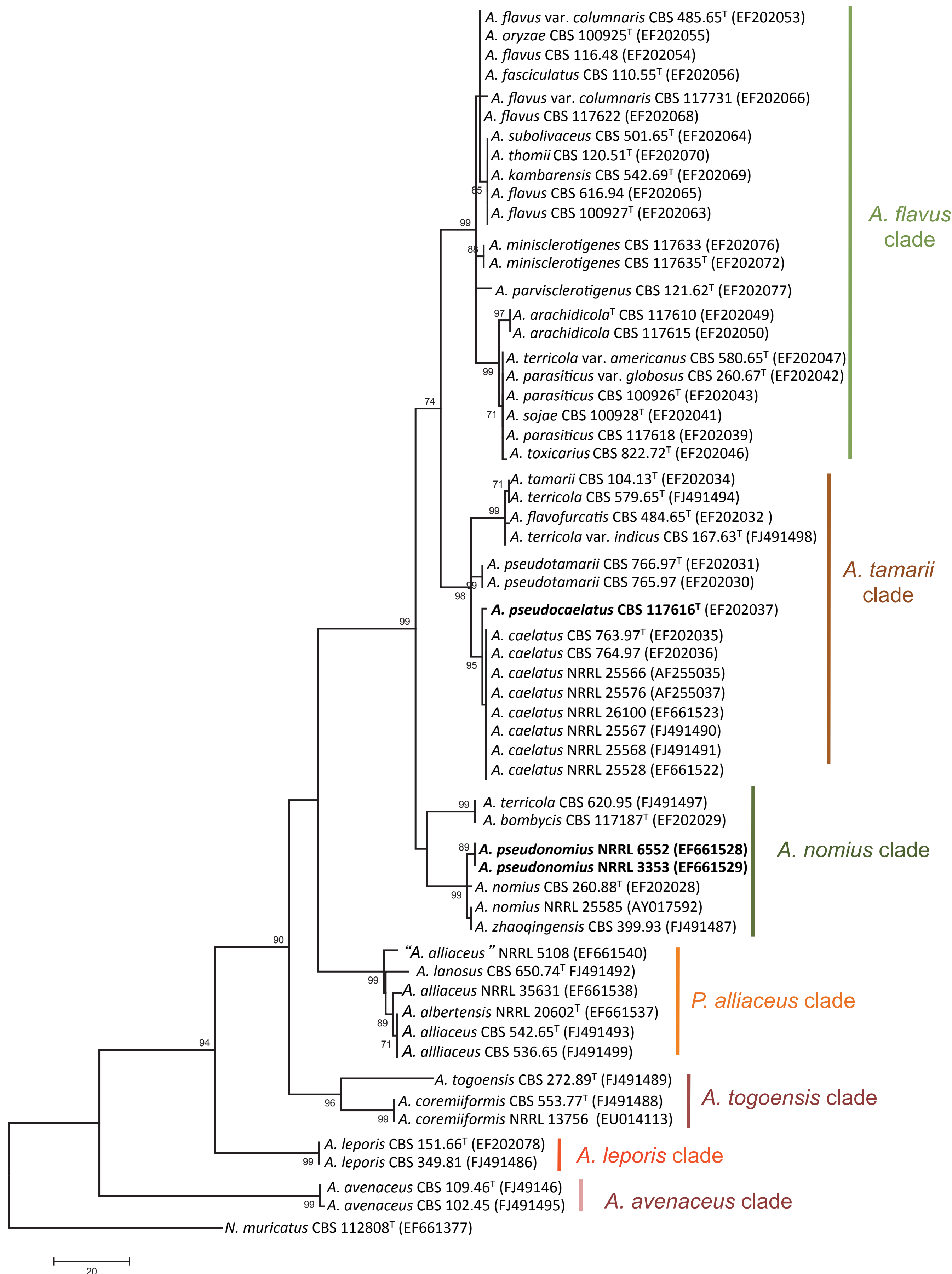

Fig. 1. Maximum parsimony tree based on β-tubulin sequence data of *Aspergillus* section *Flavi*. Numbers above branches are bootstrap values; only values above 70 % are indicated. *P.* = *Petromyces*. *N.* = *Neopetromyces*.

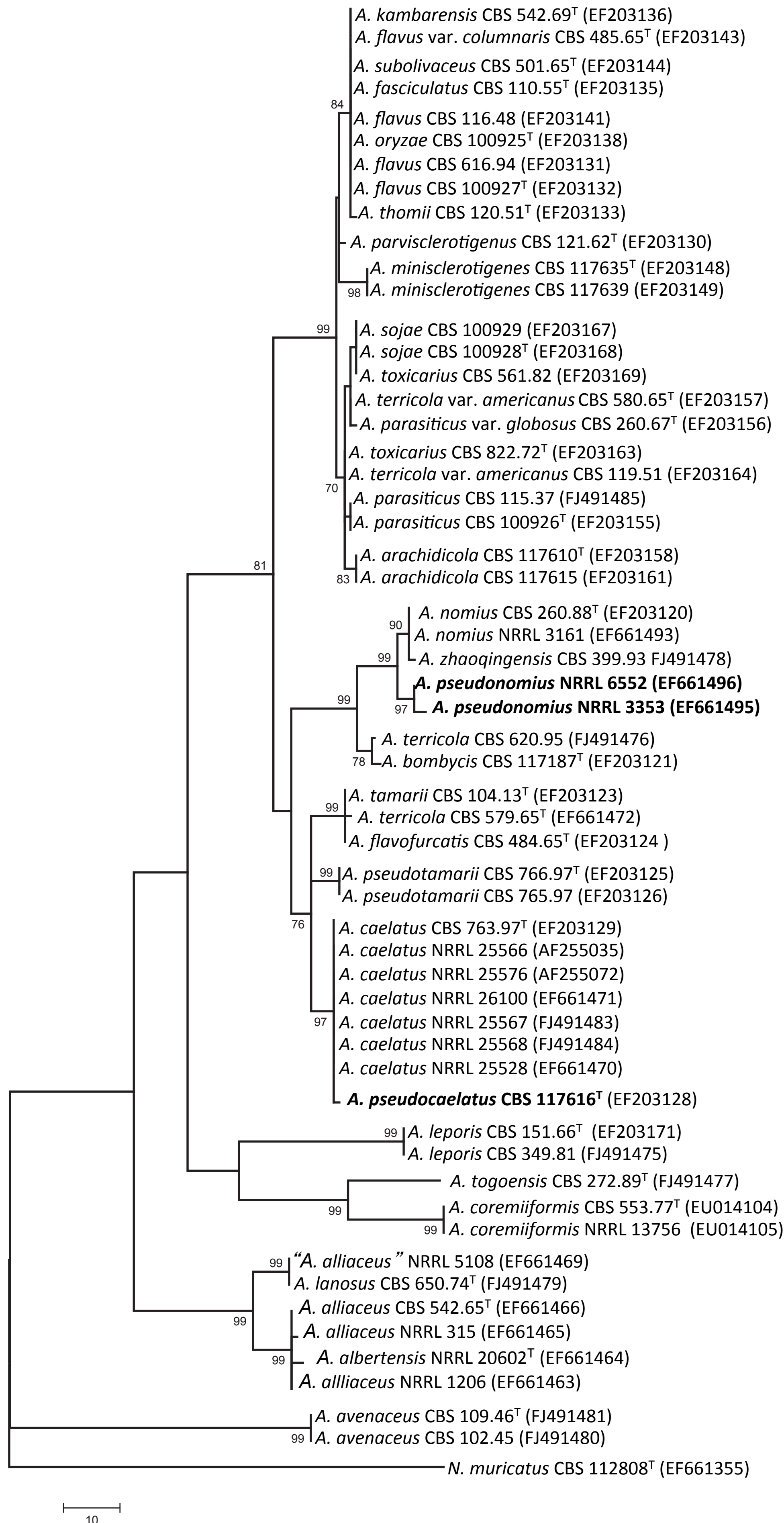

Fig. 2. Maximum parsimony tree based on calmodulin sequence data of *Aspergillus* section *Flavi*. Numbers above branches are bootstrap values; only values above 70 % are indicated. *N.* = *Neopetromyces*.

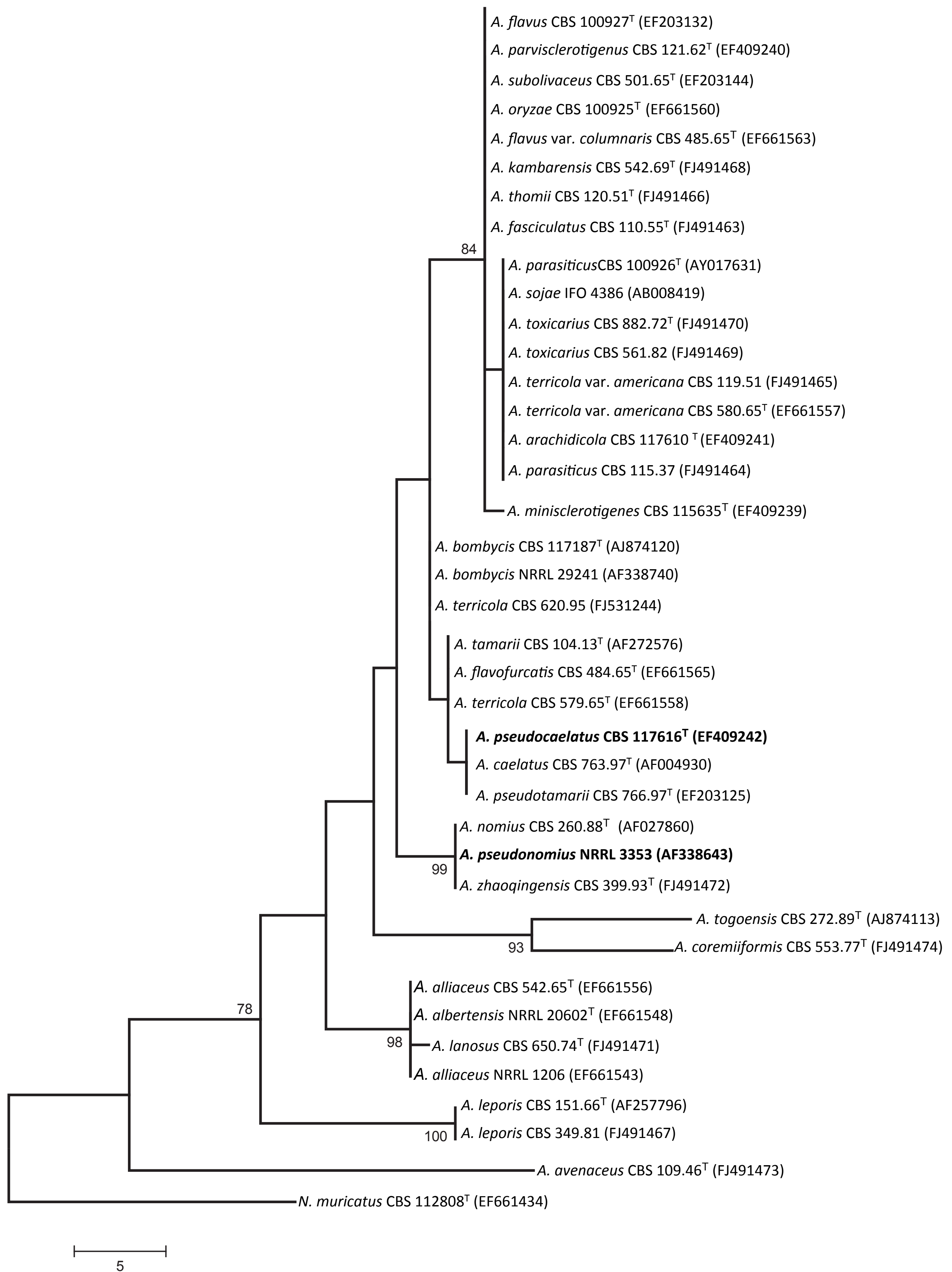

Fig. 3. Maximum parsimony tree based on ITS sequence data of *Aspergillus* section *Flavi*. Numbers above branches are bootstrap values. Only values above 70 % are indicated. *N.* = *Neopetromyces*.

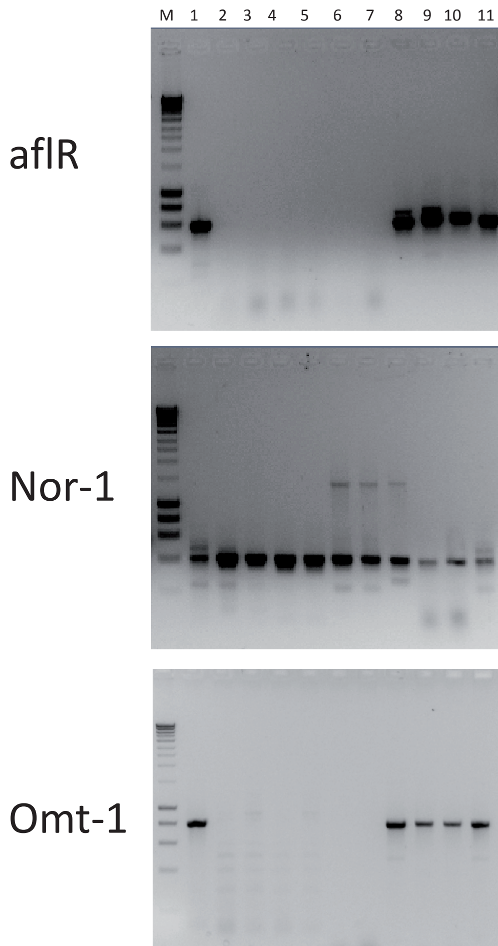

Fig. 4. PCR amplicons obtained using primer pairs developed for the *aflR*, *norA* and *omtA* genes in some isolates. M. 1 kb DNA ladder; 1. *A. pseudocaelatus* CBS 117616; 2–7. *A. caelatus* isolates (CBS 763.97, CBS 764.97, NRRL 25566, NRRL 25567, NRRL 25568 and NRRL 25569); 8. *A. minisclerotigenes* CBS 117633; 9. *A. arachidicola* CBS 117610; 10. *A. parvisclerotigenus* CBS 121.62; 11. *A. bombycis* NRRL 29236.

data set included 731 characters, with 136 parsimony informative characters. One of the 12 MP trees based on partial *omtA* gene sequences is shown in Fig. 7 (tree length: 386, consistency index: 0.7876, retention index: 0.8019). Isolate CBS 117616 was related to *A. pseudotamarii* based on *aflR* and *omtA* sequence data (Figs 5, 7), while the *norA* data set revealed that it is more closely related to *A. caelatus* (Fig. 6). Isolate CBS 119388 was related to, but distinct from *A. nomius* based on all trees. We propose the names *Aspergillus pseudocaelatus* and *A. pseudonomius* for these two new species.

Aspergillus pseudocaelatus Varga, Samson & Frisvad, **sp. nov.** MycoBank MB560397. Fig. 8.

Aspergillo caelato morphologice valde similis, sed aflatoxina (B & G), acor cyclopiazonicus et acor kojicus formantur.

Colonies on YES, MEA, OA and CYA attain a diam of 6–6.5 cm in 7 d at 25 °C; growing rapidly on CYA at 37 °C, with a diam of 6–7 cm. On CREA a typical acid production. Colony surface velvety with abundant conidial heads, olive to olive brown en masse. Reverse greenish yellow without diffusible pigments. Sclerotia not observed. Conidial heads uniseriate or biseriate. Stipes hyaline, smooth-walled, 5–8 µm wide variable in length, mostly (250–)400–600(21000) µm;

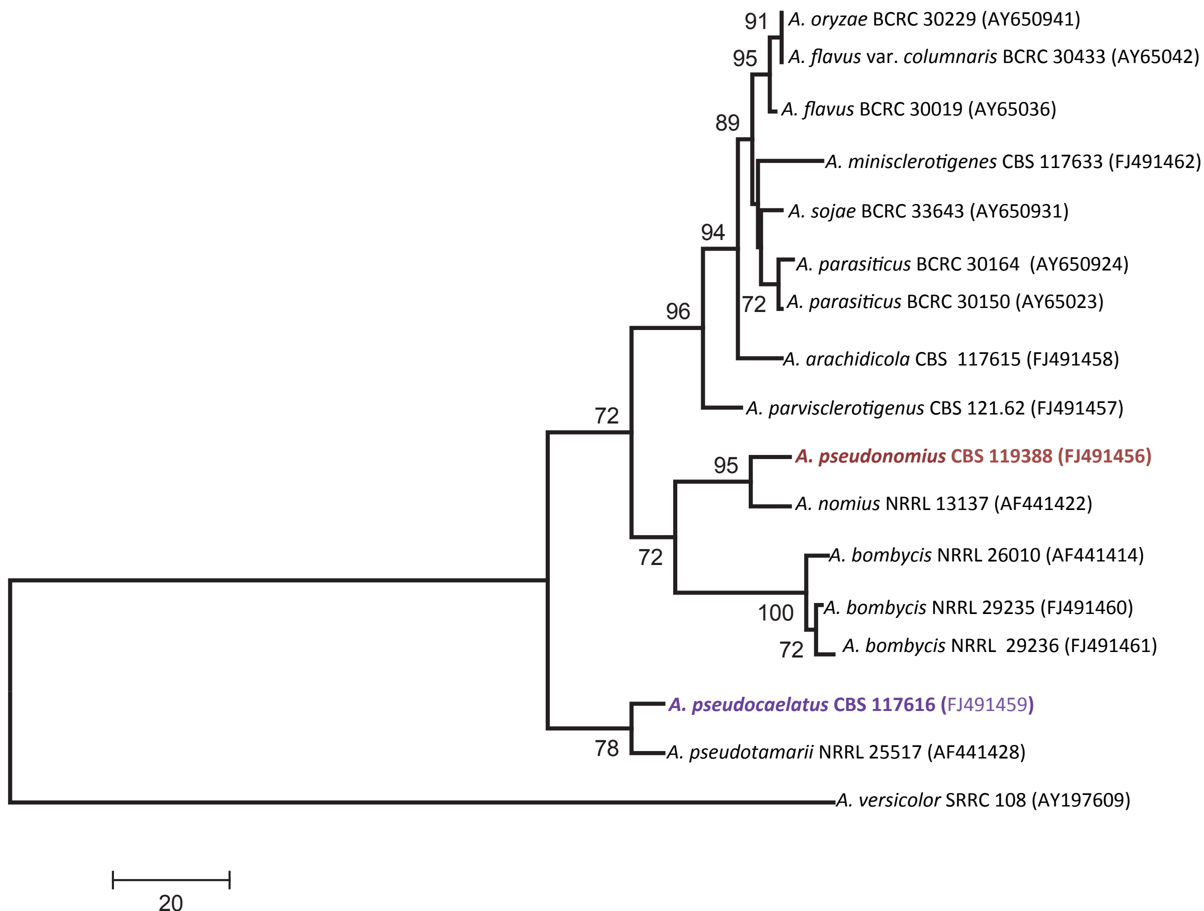

Fig. 5. Maximum parsimony tree based on *aflR* sequence data of *Aspergillus* section *Flavi*. Numbers above branches are bootstrap values; only values above 70 % are indicated.

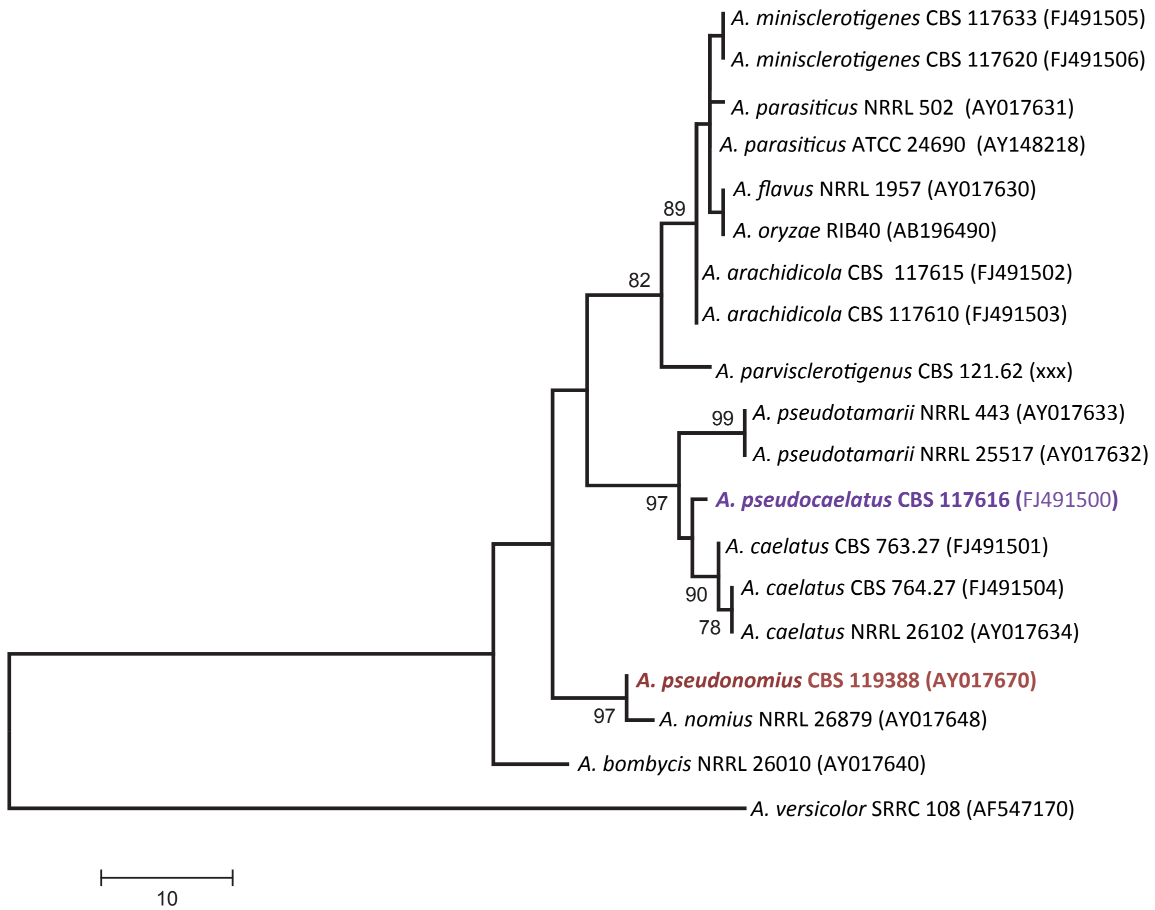

Fig. 6. Maximum parsimony tree based on *norA* sequence data of *Aspergillus* section *Flavi*. Numbers above branches are bootstrap values; only values above 70 % are indicated.

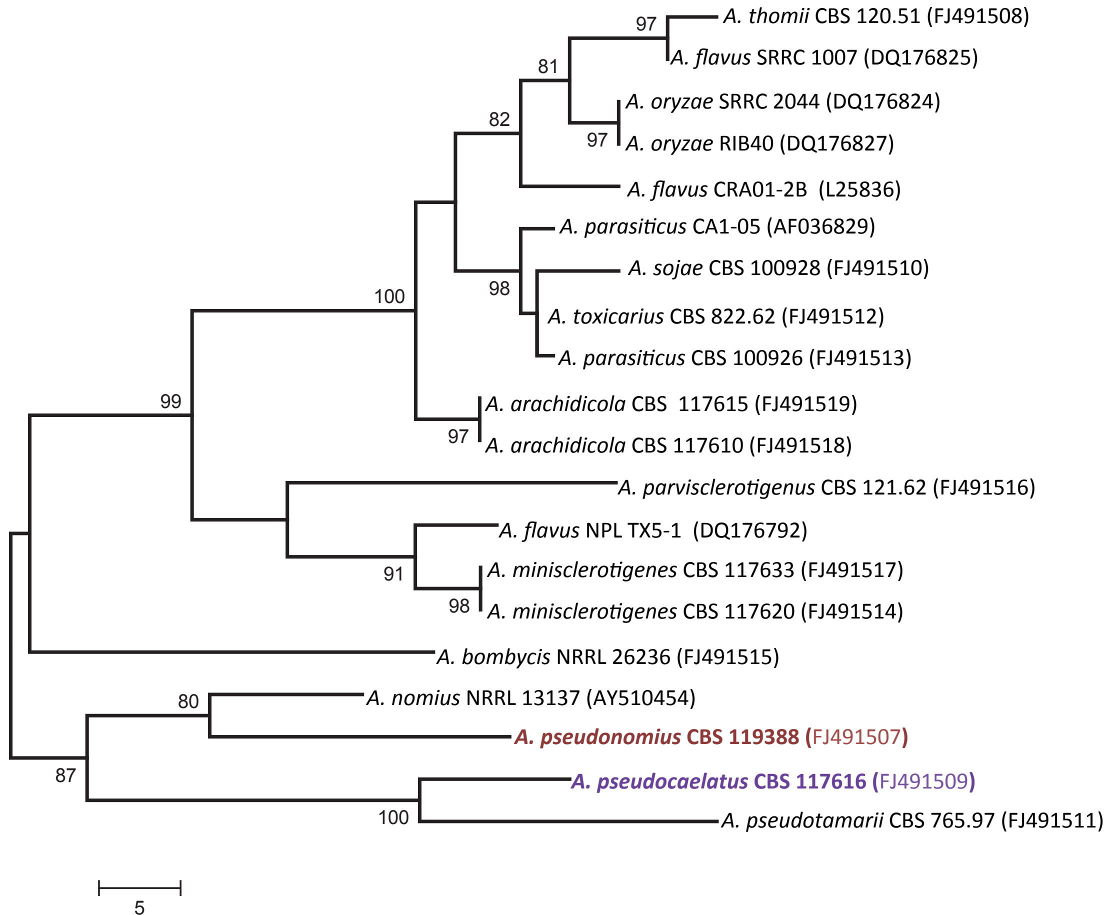

Fig. 7. Maximum parsimony tree based on *omtA* sequence data of *Aspergillus* section *Flavi*. Numbers above branches are bootstrap values; only values above 70 % are indicated.

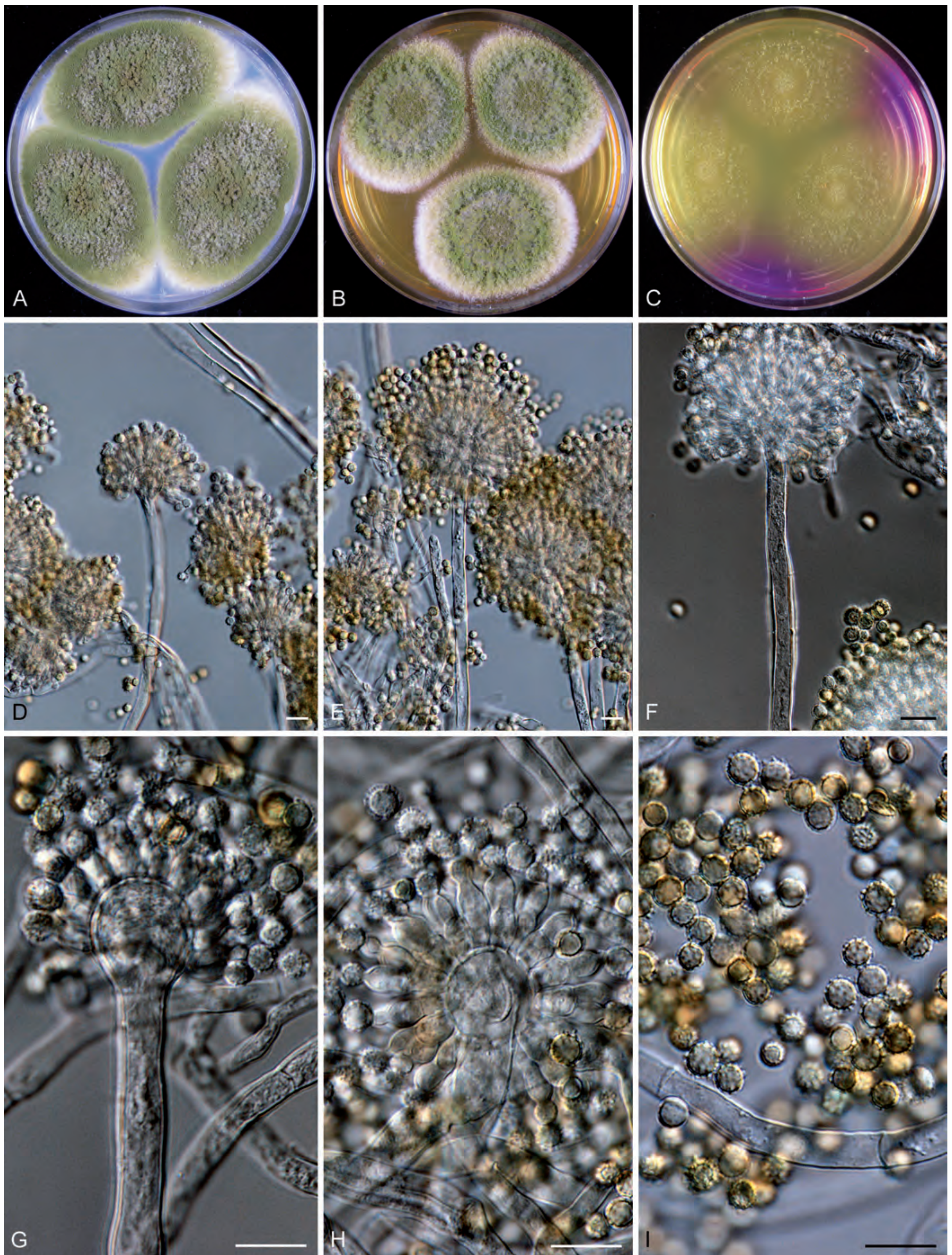

Fig. 8. *Aspergillus pseudocaelatus* sp. nov. A–C. Colonies incubated at 25 °C for 7 d, A. CYA, B. MEA, C. CREA, D–I. Conidiophores and conidia. Scale bars = 10 µm.

Vesicles globose to subglobose, 17–22 mm in diam. Conidia globose to subglobose, echinulate, greenish, 4.5–5 µm. Isolates grow well at 25, 37 and 42 °C.

Extrolites: strains of *A. pseudocaelatus* produce aflatoxins B_1,B_2 & G_1,G_2, cyclopiazonic acid and kojic acid.

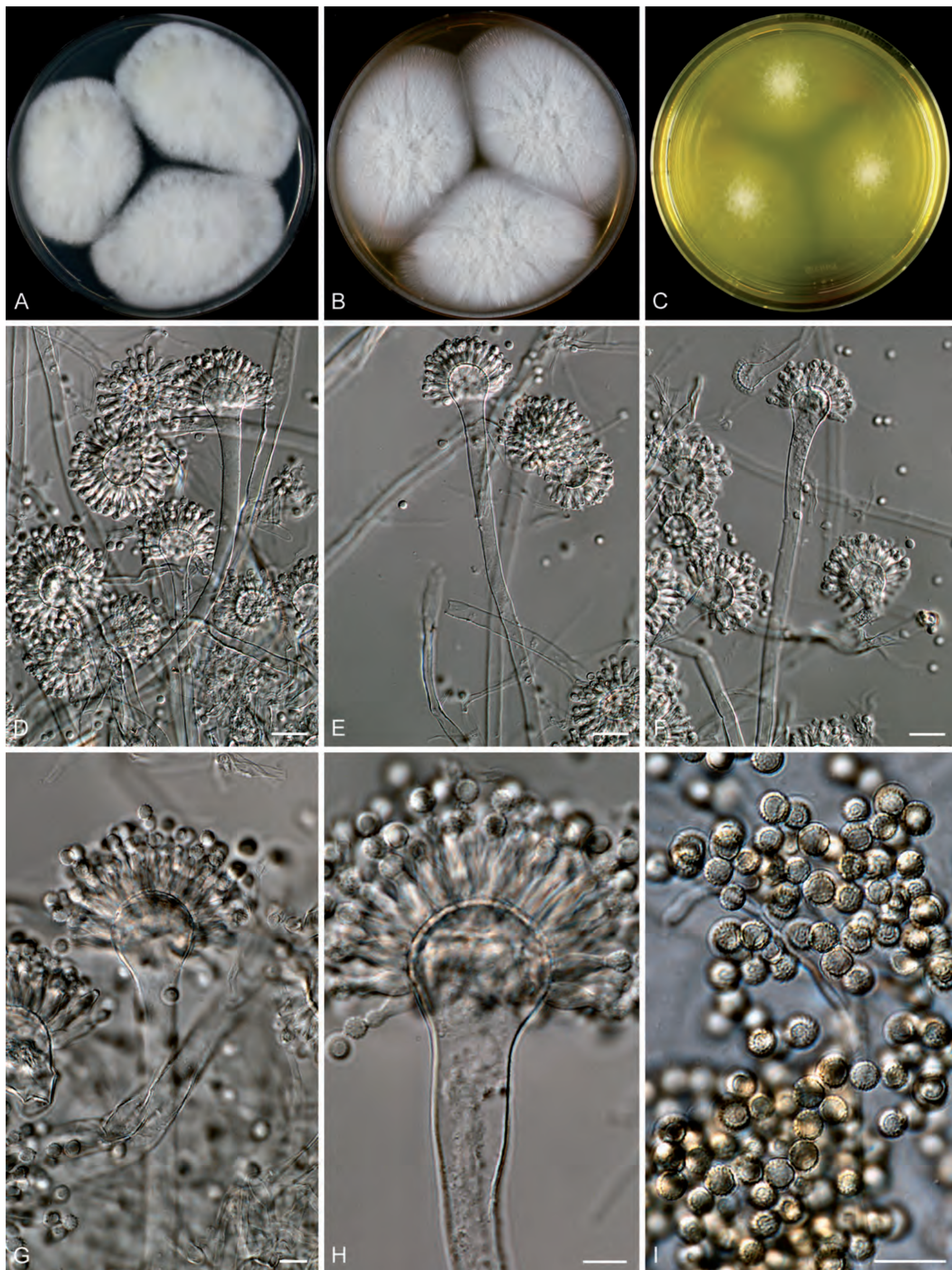

Fig. 9. *Aspergillus pseudonomius* sp. nov. A–C. Colonies incubated at 25 °C for 7 d, A. CYA, B. MEA, C. CREA, D–I. Conidiophores and conidia. Scale bars = 10 µm.

Typus: **Argentina**, Corrientes province; isolated from an *Arachis burkartii* leaf, Isolated by B. Pildain (CBS H-20632 -- holotypus, culture ex-type CBS 117616).

Aspergillus pseudocaelatus is represented by a single isolate collected from an *Arachis burkartii* leaf in Argentina. It is closely related to the non-aflatoxin producing *A. caelatus,* and produces aflatoxins B & G, cyclopiazonic acid and kojic acid. *Aspergillus caelatus* isolates produce kojic acid and aspirochlorin

Aspergillus pseudonomius Varga, Samson & Frisvad, **sp. nov.** MycoBank MB560398. Fig. 9.

Aspergillo nomio morphologice valde similis, sed aflatoxinum B1 (neque aflatoxina typi G), chrysoginum et acor kojicus formantur.

Colonies on YES, MEA, OA and CYA attain a diam of 6–6.5 cm in 7 d at 25 °C; growing rapidly on CYA at 37 °C, with a diam of 6–7 cm. On CREA a typical acid production. Colony surface floccose with dominant aerial mycelium with poor sporulation. Reverse not coloured. Sclerotia not observed. Conidial heads uniseriate. Stipes hyaline, smooth, variable in length, mostly (250–)400–600(21000) µm; diam just below vesicles 5–8 mm. Vesicles globose to subglobose, 15–30 µm in diam, fertile upper 75 % of their surface; Conidia globose to subglobose, echinulate, greenish, 4–5 µm. Isolates grow well at 25, 37 and 42 °C.

Extrolites: strains of *A. pseudonomius* produce aflatoxin B_1, chrysogine and kojic acid.

Typus: **USA**, was isolated diseased alkali bees (CBS H-20633 -- holotypus, culture ex-type CBS 119388^T = NRRL 3353).

Aspergillus pseudonomius was isolated from insects and soil in the USA. It is related to *A. nomius,* and produces aflatoxin B_1 (but not G-type aflatoxins), chrysogine and kojic acid.

An overview of *Aspergillus* section *Flavi*

In this study, we used sequence data from three loci to clarify the taxonomy of this section. Based on our phylogenetic analysis of calmodulin and ITS sequence data, *Aspergillus* section *Flavi* includes 7 main clades (Figs 1–3) with 20 or more taxa. The main clades isolates form well-defined subclades on the trees based on both β-tubulin and calmodulin sequence data. However, they are represented mostly by a single isolate *e.g. A. coremiiformis, A. togoensis.* Further collections and studies are needed to clarify if they represent separate species.

Figures 10–12 show the colonies of the accepted species on CYA, MEA and YES which are growing all well on these media, mostly reaching a diam of 6 cm within 7 d. However the colony colour differences are distinct allowing to recognise the less common species from the typical yellow-green colonies of *A. flavus* (Fig. 10 A)*, A. arachdicicola* (Fig. 10D)*, A. caelatus* (Fig. 10E)*, A. pseudocaelatus* (Fig. 11C) and *A. parasiticus* (Fig. 11H). Other species are brown (*A. tamarii* Fig. 12E) or have a less pronounced colony colours due to poor sporulation or the presence of dark sclerotia. Conidial shape and ornamentation of the species are depicted in Figs 13, 14. Conidia of species in section *Flavi* are mostly globose and rough to echinulate. The conidial shape of most species is globose with rough to distinct ornamentation. The conidial shape of *A. togoensis* and *A. coremiiformis* is irregularly shaped, smooth-walled and larger than those produced by other taxa in section *Flavi*. The conidia of *A. leporis*, and *Petromyces alliaceus* and *P. albertensis* are globose but relatively small.

Aspergillus avenaceus is the most basal member of the section. Isolates of this species produce very long black sclerotia and long conidiophores (Kozakiewicz 1989), and have Q-10 as their main ubiquinones (Kuraishi *et al.* 1990). Samson (1979) and Kozakiewicz (1989) suggested that *A. avenaceus* might be related to *A. alliaceus* based on morphological features; however, sequence data do not support this view. *Aspergillus avenaceus* has been found to produce avenaciolide, a water-insoluble bis-g-lactone antibiotic which possesses antifungal activity, and is a specific inhibitor of glutamate transport in rat liver mitochondria (Brookes *et al.* 1963, McGivan & Chapell 1970).

Another clade includes *A. leporis* isolates. This species is characterised by a Q-10 ubiquinone system, conidial heads in shades of olive, and white-tipped cinnamon coloured sclerotia (Christensen 1981, Kuraishi *et al.* 1990). Interestingly, isolates of this species produce sclerotia on rabbit dung, but not on CYA or MEA plates (Wicklow 1985). The sclerotia of *A. leporis* contain the antiinsectan N-alkoxypyridone metabolite, leporin A (Tepaske *et al.* 1991), which has been found to be effective in controlling Lepidopteran insect pests (Dowd *et al.* 1994).

Aspergillus coremiiformis and *A. togoensis* are related based on all sequence data. The species are characterised by the formation of synnemata as illustrated by the ex-type strain of *A. togoensis* (CBS 272.89) (Fig. 15). The close relationship of *A. coremiiformis* to species of section *Flavi* was also suggested by Samson (1979), Christensen (1981), and Roquebert & Nicot (1985) based on morphological features. The latter authors stated that "*Stilbothamnium nudipes* (= *A. coremiiformis*) differs from *A. tamarii* only by having septate phialides" (Roquebert & Nicot 1984). Molecular data also indicated previously that these species have affinities to section *Flavi* (Dupont *et al.* 1990, Rigó *et al.* 2002, Frisvad *et al.* 2005). The observation that an *A. togoensis* isolate produces sterigmatocystin, an intermediate of the aflatoxin biosynthetic pathway also indicates that this species is a member of *Aspergillus* section *Flavi* (Wicklow *et al.* 1989). Recently, *A. togoensis* was also found to be able to produce aflatoxin B_1 and O-methyl-sterigmatocystin (Rank *et al.* 2011). There are only a few isolates of *A. togoensis* and *A. coremiiformis* known and more strains should be made available to elucidate the relationship between these two taxa.

Aspergillus alliaceus together with *A. lanosus* and *A. albertensis* form another clade on all trees. Thom & Raper (1945) and Kozakiewicz (1989) assigned the *A. alliaceus* species to the *A. wentii* species group (*Aspergillus* section *Wentii*) based mainly on morphological features, while later the teleomorphic *Petromyces* genus was assigned to *Aspergillus* section *Circumdati* (Gams *et al.* 1985, Samson 1994). Varga *et al.* (2000a, b) and Frisvad & Samson (2000) found that *A. lanosus*, and anamorphs of *Petromyces alliaceus* and *P. albertensis* are closely related to *Aspergillus* section *Flavi*. *Aspergillus alliaceus* is of world-wide distribution. This species was first identified as a wound parasite of onion bulbs (Raper & Fennell 1965), and is mainly isolated from grassland soils, nuts, and from air (Christensen & Tuthill 1985, Kozakiewicz 1989). *Aspergillus albertensis* was isolated from a man's ear swab in Canada (Tewari 1985). While *A. alliaceus* produces determinate ellipsoidal black stromata, *A. albertensis* produces indeterminate irregularly shaped grey stromata (Tewari 1985). Both *A. alliaceus* and *A. albertensis* are homothallic, and produce ascospores in ascocarps embedded in stromata after relatively long incubation period (after about 8 wk in *A.*

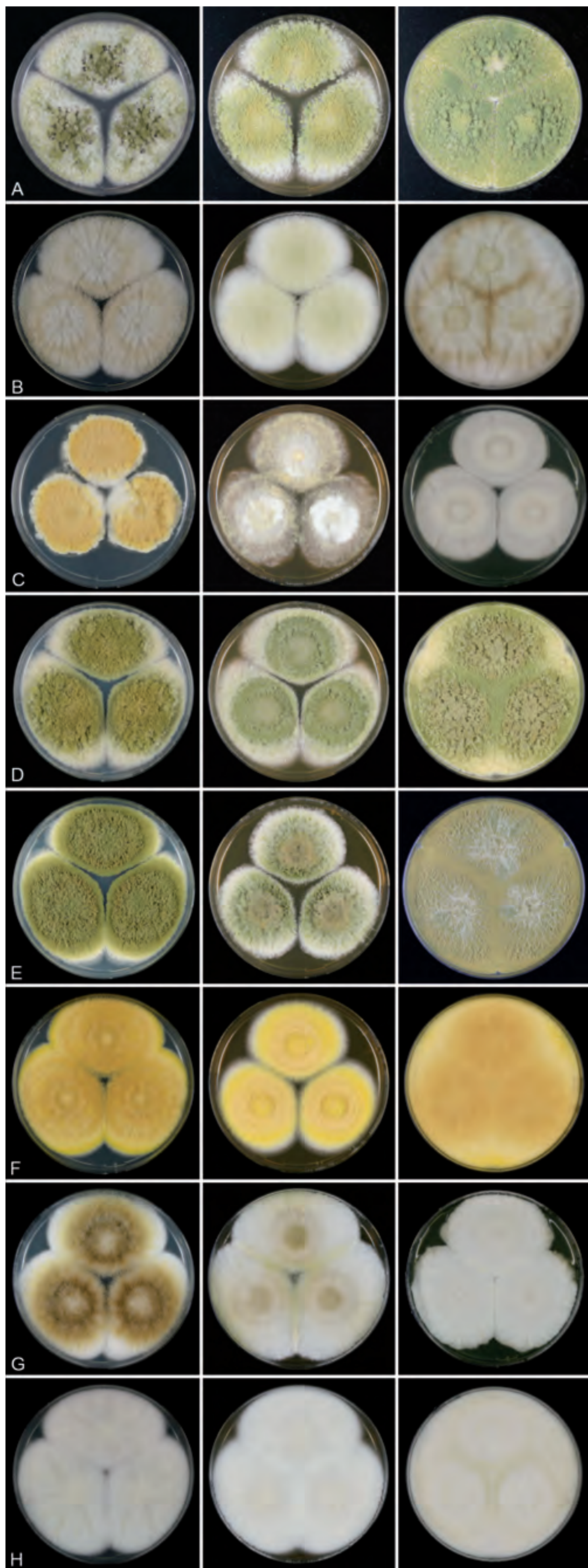

Fig. 10 Colonies of the various species of section *Flavi* on CYA. MEA and YES (7 d at 25 °C), A. *Aspergillus flavus* 100927, B. *A. avenaceus* 109.46, C. *A. coremiiformis* 553.77, D. *A. arachidicola* 117610, E. *A. caelatus* 763.27, F. *A. lanosus* 650.74, G. *A. bombycis* 117187, H. *A. leporis* 151.66.

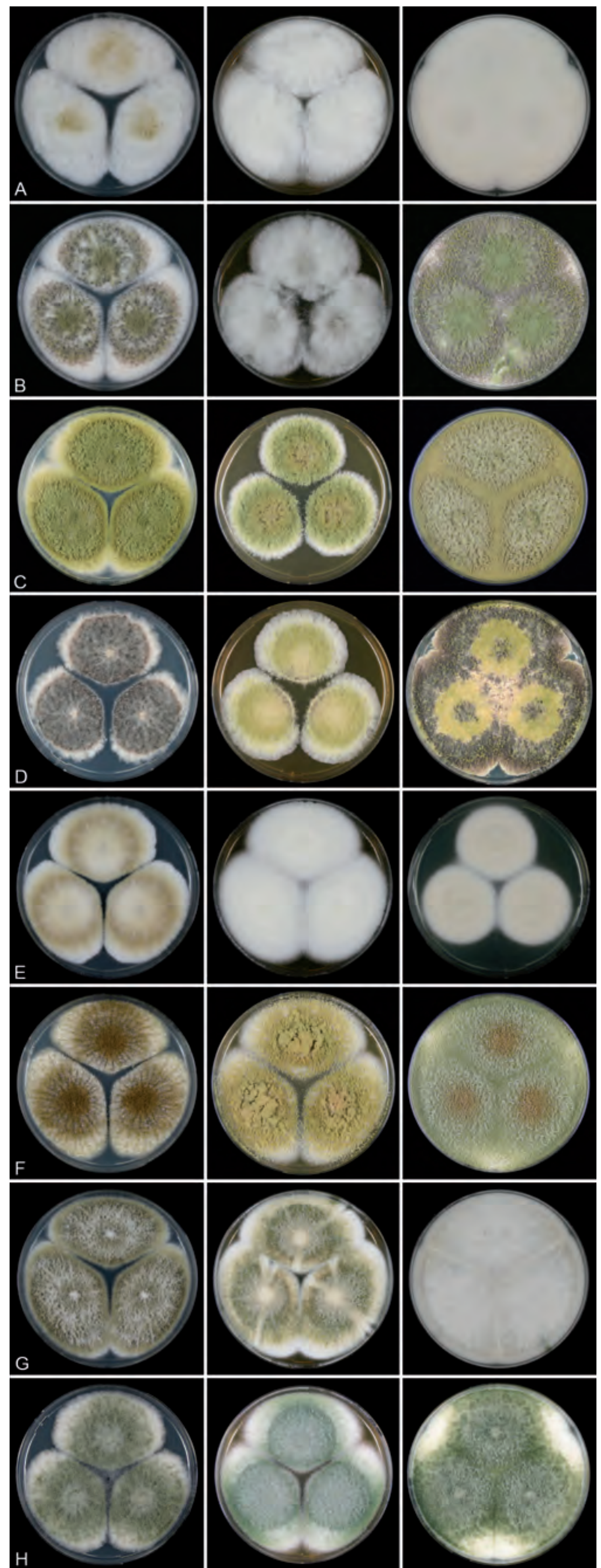

Fig. 11. Colonies of the various species of section *Flavi* on CYA. MEA and YES (7 d at 25 °C). A. *Aspergillus nomius* 119388, B. *A. minisclerotium* 117635, C. *A. pseudocaelatus* 117616, D. *A. parvisclerotigenus* 121.62, E. *A. oryzae* 100925, F. *A. pseudotamarii* 766.97, G. *A. sojae* 100928, H. *A. parasiticus* 100926.

albertensis, and after 3–4 mo in *A. alliaceus*; Fennell & Warcup 1959, Tewari 1985). Ascospores were found to be smooth with a fine ridge (Tewari 1985). Sequence analyses of multiple loci indicate that *A. albertensis* is a synonym of *A. alliaceus* (Figs 1–3; Varga *et al.* 2000, Peterson 2000, McAlpin & Wicklow 2005, Peterson 2008). Several isolates of these species are able to produce ochratoxin A & B, and are considered to be responsible for ochratoxin contamination of figs (Varga *et al.* 1996, Bayman *et al.* 2002). *Aspergillus alliaceus* isolates

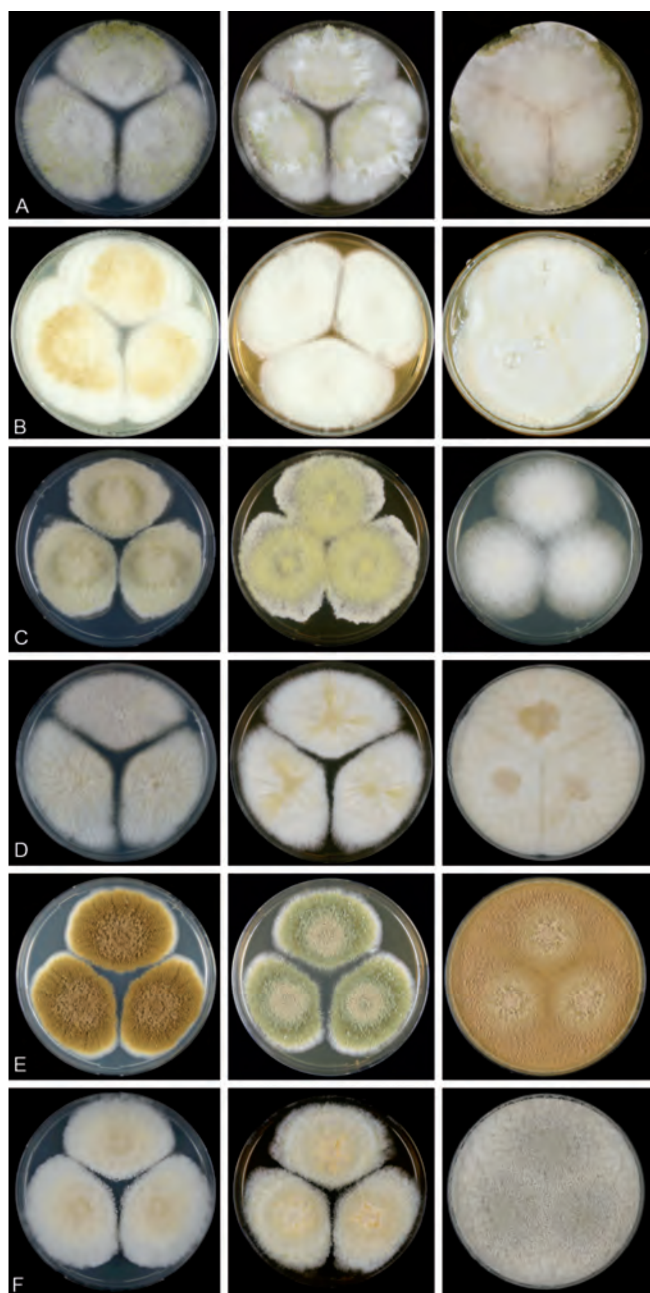

Fig. 12. Colonies of the various species of section *Flavi* on CYA. MEA and YES (7 d at 25 °C). A. *Aspergillus nomius* 260.88, B. *A. pseudonomius* C. *A. togoensis* 272.89, D. *Petromyces alliaceus* 110.26, E. *A. tamarii* 104.13, F. *P. albertensis* ATCC 58745.

are also able to produce ochratoxins under "*ex vivo*" conditions (Klich *et al.* 2009). Consequently, ochratoxins were suggested to act as potential virulence factors during pathogenesis. *Aspergillus alliaceus* has also been encountered in human infections including otorrhea (Koenig *et al.* 1985), invasive aspergillosis (Balajee *et al.* 2007) and pulmonary infection (Ozhak-Baysan *et al.* 2010). *Aspergillus alliaceus* was shown to exhibit reduced *in vitro* susceptibilities to amphotericin B and caspofungin (Balajee *et al.* 2007). Stromata of *A. alliaceus* strains contain compounds exhibiting insecticidal properties (Laakso *et al.* 1994, Nozawa *et al.* 1994), and asperlicins, potent cyclic peptide antagonists of cholecystokinin (Liesch *et al.* 1988). *Aspergillus alliaceus* strains are also used for steroid and alkaloid transformations (Burkhead *et al.* 1994, Sanchez-Gonzalez & Rosazza 2004), and for the production of pectin degrading enzyme preparations (Mikhailova *et al.* 1995).

Another clade includes *A. nomius, A. pseudonomius* and *A. bombycis* isolates. *Aspergillus nomius* and *A. bombycis* produce both aflatoxins B and G, *A. pseudonomius* produces only aflatoxin B_1, while none of them produce cyclopiazonic acid (Peterson *et al.* 2001, Table 2). *Aspergillus bombycis* was isolated from silkworm-rearing houses in Japan and Indonesia, while *A. nomius* is more widespread: it was originally isolated from mouldy wheat in the USA, and later from various substrates in India, Japan and Thailand. *Aspergillus nomius* is often associated with insects such as alkali bees (Hesseltine *et al.* 1970, Kurtzman *et al.* 1987) and termites (Rojas *et al.* 2001) and is frequently isolated from insect frass in silkworm-rearing houses in eastern Asia (Ito *et al.* 1998, Peterson *et al.* 2001). In addition soil populations in agricultural fields (Horn & Dorner 1998, Ehrlich *et al.* 2007) suggest that *A. nomius* might contribute to aflatoxin contamination of crops. *Aspergillus nomius* has been reported from tree nuts (Olsen *et al.* 2008, Doster *et al.* 2009), sugarcane (Kumeda *et al.* 2003) and an assortment of seeds and grain (Kurtzman *et al.* 1987, Pitt *et al.* 1993, Kumeda *et al.* 2003).

A recent study of soil samples from Thailand demonstrated that *A. nomius* is more widespread than may be commonly thought; it can be the predominant aflatoxin-producing *Aspergillus* species at certain geographic locations and must be considered a potential etiological agent of aflatoxin contamination events due to its ability to produce large quantities of aflatoxins (Ehrlich *et al.* 2007). For example, *A. nomius* accounted for > 9 % of section *Flavi* isolates from cornfield soils Iran (Razzaghi-Abyaneh *et al.* 2006). Recently, Olsen *et al.* (2008) have observed that *A. nomius* is an important producer of aflatoxins in Brazil nuts. *Aspergillus nomius* was recently identified from keratitis cases in India (Manikandan *et al.* 2009). Peterson *et al.* (2001) observed cryptic recombination in *A. nomius* populations using multilocus sequence data. Recently, Horn *et al.* (2010) identified the sexual state of *A. nomius* and named it as *Petromyces nomius*. An incubation period of 5 to 10 mo was needed for the formation of ascocarps within stromata. Ascocarp and ascospore morphology in *A. nomius* were similar to that of A. *flavus* and *A. parasiticus* and differences between teleomorphs were insufficient for species separation. The majority of *A. nomius* strains were either MAT1-1 or MAT1-2, but several strains contained both genes. MAT1-1/MAT1-2 strains were self sterile and capable of mating with both MAT1-1 and MAT1-2 strains; hence, *A. nomius* appears to be functionally heterothallic (Horn *et al*. 2010).

Aspergillus pseudonomius has so far only been isolated from insects and soil in the USA. *Aspergillus terricola* isolate CBS 620.95 (=WB4858), which was Blochwitz's strain of *A. luteo-virescens* (Raper & Fennell 1965), belongs to the *A. bombycis* species. *Aspergillus zhaoqingensis* was isolated from soil in China (Sun & Qi 1991), and found to be able to produce kojic acid, aspergillic acid, aflatoxin B_2 and tenuazonic acid, like most strains of *A. nomius* (unpubl. data). Molecular data indicate that *A. zhaoqingensis* is a synonym of *A. nomius* (Figs 1–3). Recent data indicate that *A. nomius* is a paraphyletic group likely to contain several other species (Egel *et al.* 1994, Cotty & Cardwell 1999, Kumeda *et al.* 2003, Ehrlich *et al.* 2003, Peterson 2008, Doster *et al.* 2009). Based on sequence alignments for three DNA regions the *A. nomius* isolates could be separated into three well-supported clades (Ehrlich *et al.* 2007). Further studies on these clades are in progress.

The "*A. tamarii*" clade contains species with ubiquinone system Q-10(H_2), and conidia in shades of olive to brown (Kuraishi *et al.* 1990, Rigó *et al.* 2002). This clade includes *A. tamarii* and its synonyms *A. terricola, A. terricola* var. *indicus* and *A. flavofurcatis, A. caelatus*, and two aflatoxin producing species: *A. pseudotamarii* and *A. pseudocaelatus*. *Aspergillus tamarii* isolates are widely used

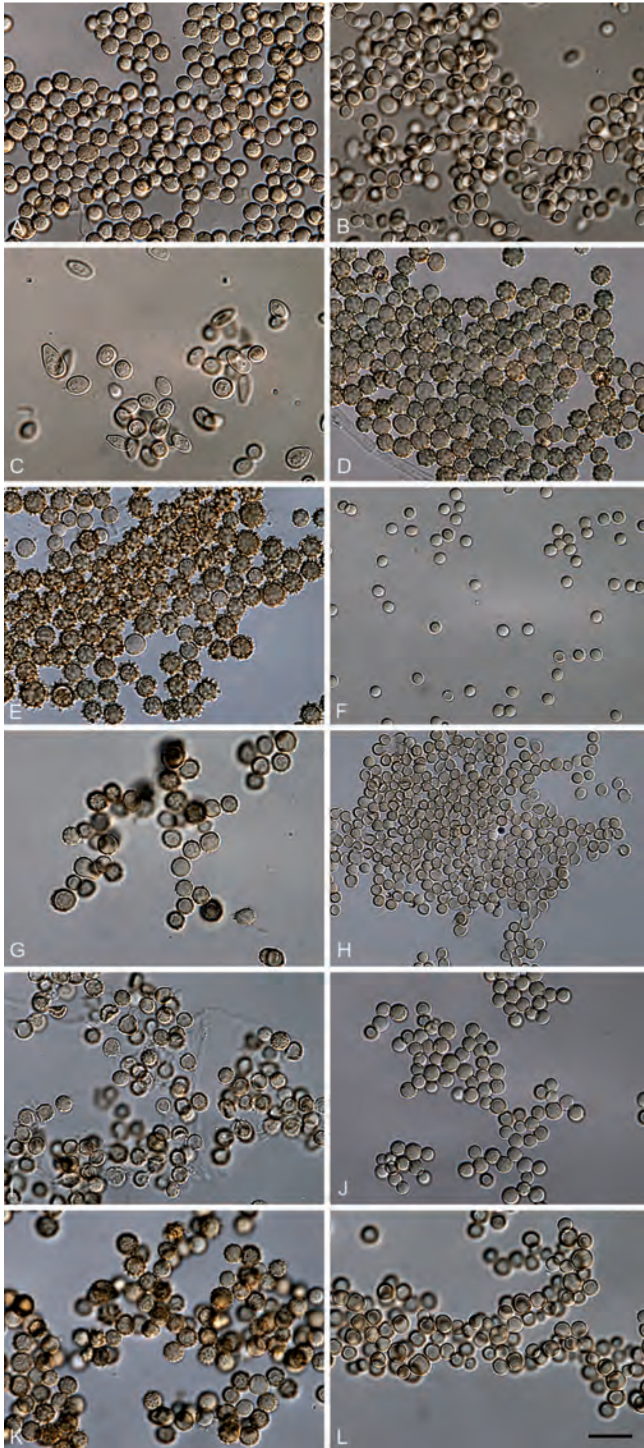

Fig. 13. Conidia of the various species of section *Flavi*. A. *Aspergillus flavus* 100927, B. *A. avenaceus* 109.46, C. *A. coremiiformis* 553.77, D. *A. arachidicola* 117610, E. *A. caelatus* 763.27, F. *A. lanosus* 650.74, G. *A. bombycis* 117187, H. *A. leporis* 151.66, I. *A. nomius* 119388, J. *A. minisclerotium* 117635, K. *A. pseudocaelatus* 117616, L. *A. parvisclerotigenus* 121.62.

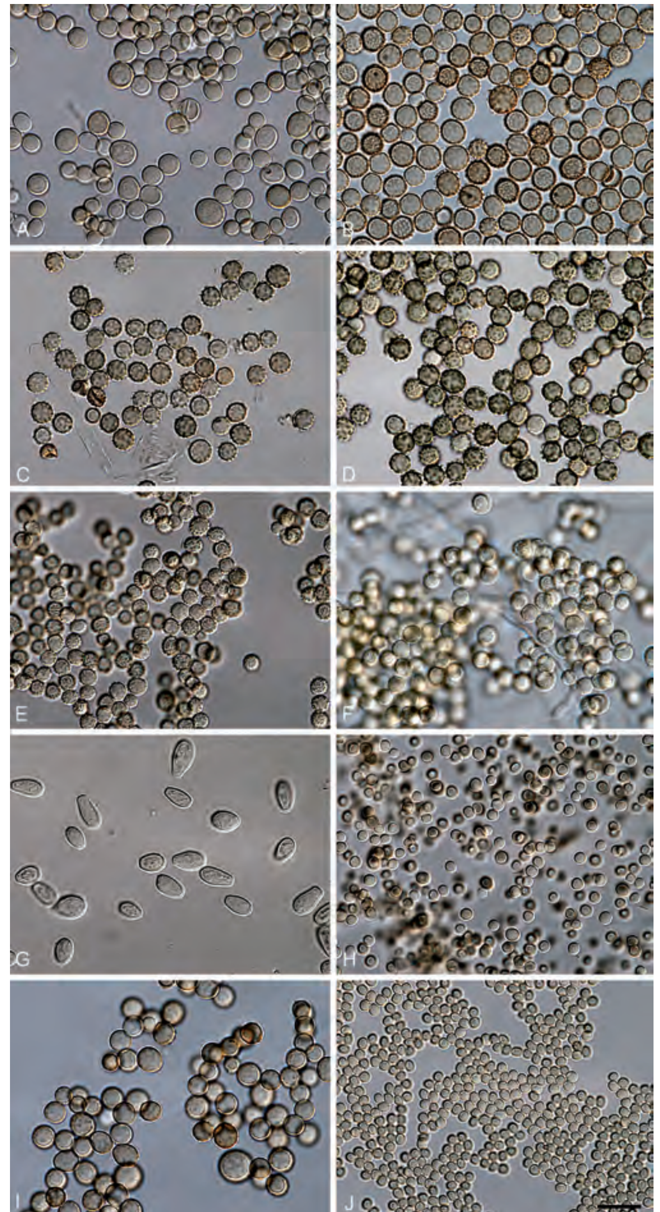

Fig. 14. Conidia of the various species of *section Flavi*. A. *Aspergillus oryzae* 100925, B. *A. pseudotamarii* 766.97, C. *A. sojae* 100928, D. *A. parasiticus* 100926, E. *A. nomius* 260.88, F. *A. pseudonomius* 119388, G. *A. togoensis* 272.89, H. *Petromyces alliaceus* 110.26, I. *A. tamarii* 104.13, J. *P. albertensis* ATCC 58745.

in the food industry for the production of soy sauce (known as red Awamori koji) (Jong & Birmingham 1992) and in the fermentation industry for the production of various enzymes, including amylases, proteases, and xylanolytic enzymes (Ferreira *et al.* 1999, Moreira *et al.* 2004). Recently, *A. tamarii* has also been identified as a cause of human keratitis in Southern India (Kredics *et al.* 2007), and *A. tamarii* spores were suggested as important sources of allergens present in the air (Vermani *et al.* 2010). Although *A. caelatus* was found to be very similar to *A. tamarii* morphologically, *A. caelatus* isolates were found not to produce cyclopiazonic acid, in contrast with *A. tamarii* isolates (Horn 1997, Ito *et al.* 1999). *Aspergillus terricola* and its subspecies were originally placed into section *Wentii* by Raper & Fennell (1965). Later *A. terricola* together with *A. flavofurcatis* and *A. tamarii* were placed into an "*A. tamarii* species group" by Kozakiewicz (1989). Sequence data indicate that these isolates belong to the same species. *Aspergillus pseudotamarii* (Ito *et al.* 2001) is an effective producer of B-type aflatoxins but the importance for mycotoxin occurrence in foods is unknown. The closely related species *A. tamarii* is not able to produce aflatoxins, despite several reports claiming this (Goto *et al.* 1996, Klich *et al.* 2000). *Aspergillus pseudocaelatus* is represented by a single isolate that came from a *Arachis burkartii* leaf from Argentina. This species produces both G- and B-type aflatoxins, and cyclopiazonic acid.

The "*A. flavus*" clade includes species characterised with Q-10(H_2) as their main ubiquinone, and conidial colours in shades of green, and several isolates produce dark sclerotia. *Aspergillus flavus* is the most common species producing aflatoxins (Sargeant *et al.* 1961), occurring in most kinds of foods in tropical countries. This species is very common on maize, peanuts and cottonseed,

Fig. 15. *Aspergillus togoensis* (CBS 272.89). A–B. Synnemata. C–E. Conidiophores, F. Conidia. Scale bars = 10 µm.

and produces only B-type aflatoxins. It has been estimated that only about 30–40 % of known isolates produce aflatoxin. Because of its small spores and its ability to grow at 37 °C, it can also be pathogenic to animals and humans. Infection by *A. flavus* has become the second leading cause of various forms of human aspergillosis (Hedayati *et al.* 2007, Pasqualotto & Denning 2008, Krishnan *et al.* 2009). *Aspergillus flavus* populations are genetically and phenotypically diverse (Geiser *et al.* 2000) with some isolates producing conidia abundantly, produce large (L) sclerotia, and variable amounts of aflatoxins, while another type produces abundant, small (S) sclerotia, fewer conidia and high levels of aflatoxins (Cotty 1989). The S-type isolates predominated in both soil and maize samples within aflatoxicosis outbreak regions, while the L strain was dominant in non-outbreak regions of Kenya (Probst *et al.* 2010). A related type, *A. oryzae* is atoxigenic and has been used as a source of industrial enzymes and as a koji (starter) mold for Asian fermented foods, such as sake, miso, and soy sauce (van den Broek *et al.* 2001). Although several lines of evidence suggest that *A. oryzae* and *A. sojae* are morphological variants of *A. flavus* and *A. parasiticus*, respectively, it was suggested that these taxa should be retained as separate species because of the regulatory confusion that conspecificity might generate in the food industry (Geiser *et al.* 1998b). *Aspergillus oryzae* isolates carry various mutations in the aflatoxin biosynthetic gene cluster resulting in their inability to produce aflatoxins (Tominaga *et al.* 2006). Particularly, the *aflR* gene is absent or significantly different in some *A. oryzae* strains compared to *A. flavus* (Lee *et al.* 2006). *Aspergillus oryzae* strains can be classified into three groups according to the structure of the aflatoxin biosynthesis gene cluster (Tominaga *et al.* 2006). Group 1 includes strains which has all aflatoxin biosynthesis gene orthologs, group 2 has the region beyond the *ver1* gene deleted, and group 3 has the partial aflatoxin gene cluster up to the *vbs* gene (Chang *et al.* 2009). Isolates assigned to groups 2 and 3 obviously cannot produce aflatoxins due to the loss of part of the gene cluster. Regarding group 1 isolates, the expression level of the *aflR* gene is extremely low, and no expression of several biosynthetic genes (*avnA, verB, omtA, vbs*) was observed. Recent studies clarified that amino-acid substitutions in *AflJ* gene induce inactivation at the protein level (Kiyota *et al.* 2011). Genome sequences of both *A. oryzae* and *A. flavus* are available (Machida *et al*. 2005, Chang & Ehrlich 2010, http://www.aspergillusflavus.org/genomics/).

The genomes of both species are about 37 Mb and consist of 8 chromosomes. A comparative analysis of *A. oryzae* and *A. flavus* genomes revealed striking similarities between them. An

Table 2. Extrolite profiles of species assigned to *Aspergillus* section *Flavi*.

Species	Occurrence	Extrolites produced	Reference
A. arachidicola	Argentina	Aflatoxins B_1,B_2 & G_1,G_2	Pildain *et al.* (2008)
		Aspergillic acid	Pildain *et al.* (2008)
		Chrysogine	Pildain *et al.* (2008)
		Ditryptophenaline	This study
		Kojic acid	Pildain *et al.* (2008)
		Parasiticolides	Pildain *et al.* (2008)
A. avenaceus	UK, USA	Avenaciolide	Brookes *et al.* (1963)
		Aspirochlorine	This study
A. bombycis	Indonesia, Japan	Aflatoxins B_1,B_2 & G_1,G_2	Peterson *et al.* (2001)
		Aspergillic acid	This study
		Kojic acid	This study
A. caelatus	Japan, USA	Aspirochlorin	Pildain *et al.* (2008)
		Kojic acid	Frisvad & Samson (2000)
		Tenuazonic acid	This study
A. coremiiformis	Ivory Coast	Indol alkaloids (not structure elucidated)	This study
A. flavus	Worldwide	Aflatoxins B_1 & B_2	Varga *et al.* (2009)
		Aflatrem	Gallagher & Wilson (1978)
		Aflavarins	TePaske *et al.* (1992)
		Aflavazol	TePaske *et al.* (1990)
		Aspergillic acid	White & Hill (1943)
		Aspergillomarasmines A & B	Haenni *et al.* (1965)
		Cyclopiazonic acid	Luk *et al.* (1977)
		Ditryptophenaline	Springer *et al.* (1977)
		Kojic acid	Birkinshaw *et al.* (1931)
		Miyakamides*	Shiomi *et al.* (2002)
		3-Nitropropionic acid	Bush *et al.* (1951)
		Paspalinine	Cole *et al.* (1981)
A. lanosus	India	Ochratoxins A & B*	Baker *et al.* (2003)
			Palumbo *et al.* (2007)
		Griseofulvin	Frisvad & Samson (2000)
		Kojic acid	Frisvad & Samson (2000)
A. leporis	USA	Antibiotic Y	Frisvad & Samson (2000)
		Kojic acid,	Frisvad & Samson (2000)
		Leporin A	TePaske *et al.* (1991)
		Pseurotin	Frisvad & Samson (2000)
A. minisclerotigenes	Argentina, Australia, Nigeria, USA	Aflatoxins B_1,B_2 & G_1,G_2	Pildain *et al.* (2008)
		Aflavarins	Pildain *et al.* (2008)
		Aflatrems	Pildain *et al.* (2008)
		Aflavinins	Pildain *et al.* (2008)
		Aspergillic acid	Pildain *et al.* (2008)
		Cyclopiazonic acid	Pildain *et al.* (2008)
		Paspalinine	Pildain *et al.* (2008)
A. nomius	Brazil, India, Japan, Thailand, USA	Aflatoxins B_1,B_2 & G_1,G_2	Kurtzmann *et al.* (1987)
		Aspergillic acid	Frisvad & Samson (2000)
		Aspernomine	Staub *et al.* (1992)
		Kojic acid	Frisvad & Samson (2000)
		Nominine	Gloer *et al.* (1989)

Table 2. (Continued).

Species	Occurrence	Extrolites produced	Reference
A. nomius		Paspaline	Staub *et al.* (1993)
		Pseurotin	Frisvad & Samson (2000)
		Tenuazonic acid	Frisvad & Samson (2000)
A. oryzae	China, Japan	Asperfuran	Pfefferle *et al.* (1990)
		Asperopterin A & B*	Matsuura *et al.* (1972)
		Aspirochlorin	Sakata *et al.* (1983)
		Cyclopiazonic acid	Orth (1977)
		Kojic acid	Birkinshaw *et al.* (1931)
		Kojistatin*	Sato *et al.* (1996)
		3-nitropropionic acid	Nakamura & Shimoda (1954)
			Tamogami *et al.* (1996)
		Sporogen AO-1*	Nonoka *et al.* (1997)
		TMC-2A, B, C*	Asai *et al.* (1998)
A. parasiticus	Australia, India, Japan, South America, Uganda USA	Aflatoxins B_1,B_2 & G_1,G_2	Schroeder (1966)
		Aspergillic acid	Assante *et al.* (1981)
		Aspersitin*	Hamasaki *et al.* (1975)
		Kojic acid	Birkinshaw *et al.* (1931)
		Parasperone and ustilaginoidin C*	Brown *et al.* (1993)
		Parasitenone*	Son *et al.* (2002)
		Parasiticolide	Büchi *et al.* (1983)
		Sequoiatones*	Stierle *et al.* (1999, 2001)
		Sequoiamonascins*	Stierle *et al.* (2003)
A. parvisclerotigenus	Nigeria	Aflatoxins B_1,B_2 & G_1,G_2	Frisvad *et al.* (2005)
		Aflatrem	Frisvad *et al.* (2005)
		Aflavarin	Frisvad *et al.* (2005)
		Aspirochlorin	Frisvad *et al.* (2005)
		Cyclopiazonic acid	Frisvad *et al.* (2005)
		Kojic acid	Frisvad *et al.* (2005)
		Paspaline	Frisvad *et al.* (2005)
A. pseudocaelatus	Argentina	Aflatoxins B_1,B_2 & G_1,G_2	This study
		Cyclopiazonic acid	This study
		Kojic acid	This study
A. pseudonomius	USA	Aflatoxin B_1	This study
		Chrysogine	This study
		Kojic acid	This study
A. pseudotamarii	Argentina, Japan	Aflatoxin B_1 ,B_2	Ito *et al.* (2001)
		Cyclopiazonic acid	Ito *et al.* (2001)
		Kojic acid	This study
A. sojae	China, India, Japan	Asperfuran	This study
		Aspergillic acid	Pildain *et al.* (2008)
		Aspirochlorin	This study
		Chrysogine	This study
		Kojic acid	Tanaka *et al.* (2002)
A. tamarii	Worldwide (mostly warmer climates)	Aspirochlorin	Berg *et al.* (1976)
		(-)-canadensolide*	Berg *et al.* (1976)
		Cyclopiazonic acid	Dorner (1983)
		Fumigaclavine A*	Jahardhanan *et al.* (1984)

Table 2. (Continued).

Species	Occurrence	Extrolites produced	Reference
A. tamarii		Kojic acid	Birkinshaw *et al.* (1931)
		Speradine A	Tsuda *et al.* (2003)
A. togoensis	Central Africa	Aflatoxin B_1	Rank *et al.* (2011)
		Sterigmatocystin	Wicklow *et al.* (1989)
A. alliaceus	Worldwide (Argentina, Australia, Canada, Egypt, France, Greece, Hungary, Lybia, Mexico, Netherlands, New Zealand, Russia, Saudi Arabia, Spain, Tunisia, Turkey, UK, USA)	Asperlicins	Liesch *et al.* (1985)
		Isokotanins	Laakso *et al.* (1994)
		Nominine	Laakso *et al.* (1994)
		Ochratoxin A & B	Ciegler (1972)
		Paspaline	Laakso *et al.* (1994)

*We did not detect these compounds in any strains examined in this study.

array based genome comparison found only 43 genes unique to *A. flavus* and 129 genes unique to *A. oryzae* (Georgianna & Payne 2009). *A.oryzae sensu stricto* has been isolated from koji fermentations used for miso, sake and other Japanese, Korean and Japanese fermented products. Sometimes the species has been reported from cereals, soil etc., and it is possible that all these isolates are just floccose variants of *A. flavus*. Therefore the report of aspergillomarasmin, miyakamides, asperopterins etc. from *A. oryzae*, may actually be from *Aspergillus flavus* (see Table 2). The genome sequenced strain of *A. oryzae* (RIB 40) (Machida *et al.* 2005) was isolated from cereals and probably not from industrial settings, so it is possible that this isolate is a brownish to yellowish green spored variant of *A. flavus* too. Figure 16 illustrates the morphology of the ex-type strain of *A. oryzae* (CBS 100925) showing the typical feature of a floccose strain with less abundant sporulation. Conidiophores produce aberrant conidiogenous structures with elongated or inflated phialides and metulae. Conidia are smooth-walled and subglobose to ellipsoidal. Figure 17 shows the strain of *A. oryzae* (RIB 40) with yellow green colonies and a rich sporulation. This strain also produces abundantly sclerotia which are absent in CBS 100925. Conidiophores of RIB 40 are typical bisteriate with regular shaped conidiogenous structures producing globose, smooth to finely roughened conidia. Phenotypically these two strains are distinct and it would be recommendable to genome sequence an *Aspergillus oryzae* strain used for koji fermentation also, for example the ex-type culture.

Regarding the evolutionary origins of *A. oryzae* and *A. flavus*, Chang *et al.* (2009) suggested that, based on the genetic diversity in the region neighbouring the cyclopiazonic acid biosynthesis gene cluster, *A. oryzae* most likely descended from an ancestor that was the ancestor of *A. minisclerotigenes* or *A. parvisclerotigenus* producing both B- and G-type aflatoxins, while *A. flavus* descended from an ancestor of *A. parasiticus*.

Population genetic analyses of restriction site polymorphisms and DNA sequences of several genes indicated that *A. flavus* isolates fell into two reproductively isolated clades (groups I and II). A lack of concordance between gene genealogies among isolates in group I suggested that *A. flavus* has a recombining population structure (Geiser *et al*. 1998, 2000). Regarding the distribution of the mating type genes in *A. flavus* populations, there was no significant difference in the frequency of the two mating types for *A. flavus* (and *A. parasiticus*) in either vegetative compatibility groups (VCG) or haplotype clone-corrected samples. The existence of both mating type genes in equal proportions in these populations together with the observed expression of these genes indicated the possible existence of a sexual state in *A. flavus* (Ramirez-Prado *et al.* 2008). The presence of mating type genes have also been observed in *A. oryzae* isolates (Chang & Ehrlich 2010). Recently the sexual stage of *A. flavus* has been described under the name of *Petromyces flavus* (Horn *et al*. 2009a, 2009b). However, in another study the distribution of mating type genes was uneven within an *A. flavus* population collected from maize fields in Southern Hungary, indicating that the given population reproduces primarily clonally (Tóth B. *et al.* in preparation). Indeed, population genetic analyses of molecular data confirmed that this population is a clonal one (data not shown). Sweany (2010) also observed uneven distribution of mating type genes in *A. flavus* isolates collected from maize with MAT1-2 being dominant (96 %), while the distribution of mating type genes was more balanced in soil isolates (48 % with MAT1-1, and 52 % with MAT1-2 idiomorphs). She also observed that the isolates belonging to different vegetative incompatibility groups of *A. flavus* almost exclusively carried either one or the other mating type gene (Sweany 2010). Differences between the corn and soil populations were suggested to indicate that not all soil isolates are as capable of infecting corn, and that some isolates have become specialised to infect corn.

Multilocus sequence data indicated that several species assigned to section *Flavi* are synonyms of *A. flavus*, including *A. flavus* var. *columnaris, A. kambarensis*, *A. fasciculatus*, *A. thomii* and *A. subolivaceus* (Figs 1–3). Although Peterson (2008) observed that *A. subolivaceus* formed a separate lineage distinct from *A. flavus* based on sequence data of two loci, it could not be distinguished by any other means from *A. flavus* isolates. Some of these species have also been found to be synonyms of *A. flavus* based on sequence analysis of part of their 18 S and 26 S rRNA genes (Nikkuni *et al.* 1998, Peterson 2000). Strains of *A. flavus* var. *columnaris* produce pronounced conidial columns, and most strains accumulate aflatoxin B_2 only. It appears that certain mutations have induced this characteristic phenotype. The *A. kambarensis, A. fasciculatus, A. thomii* and *A. subolivaceus* ex-type strains could not produce aflatoxins, showing that aflatoxin ability can easily be lost in soil strains of *A. flavus*.

Many reports indicate that certain *A. flavus* strains, including micro-sclerotial strains, and strains listed as intermediate between *A. flavus* and *A. parasiticus* can also produce type G aflatoxins (Codner *et al.* 1963, Hesseltine *et al.* 1970, Cotty & Cardwell 1999, Begum & Samajpati 2000). One group of these isolates have been named previously as *A. flavus* var. *parvisclerotigenus* (Saito *et al.* 1986, Saito & Tsuruta 1993), and later raised to species status as *A. parvisclerotigenus* (Frisvad *et al.* 2005). The type strain of *A. parvisclerotigenus* (CBS 121.62 = NRRL A-11612 = IBT 3651 = IBT 3851) was isolated from peanut in Nigeria, and this species has

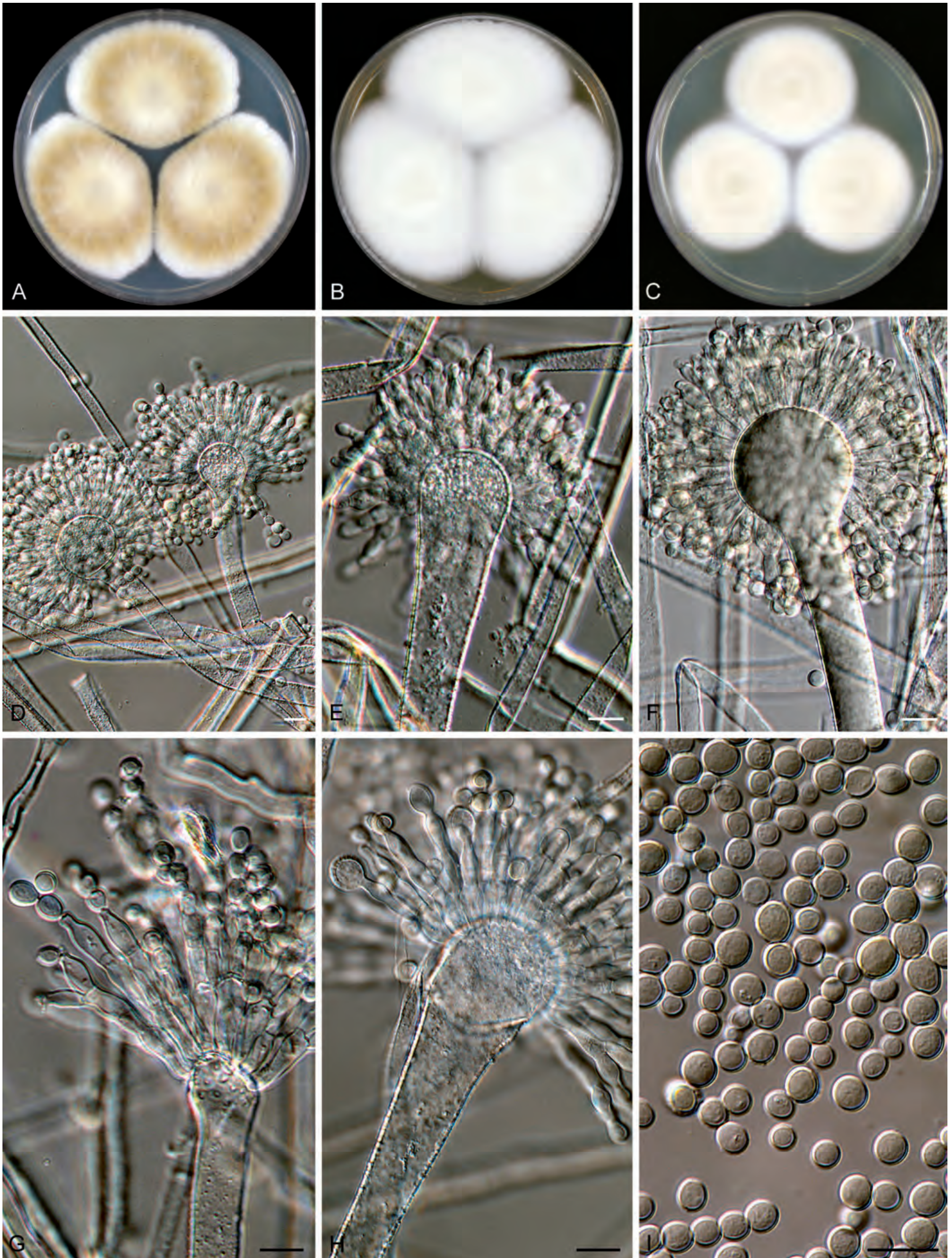

Fig. 16. *Aspergillus oryzae* (ex-type CBS 100925). A–C. Colonies incubated at 25 °C for 7 d, A. CYA, B. MEA, C. YES, D–I. Conidiophores and conidia. Scale bars = 10 µm.

also been identified in grain samples came from Nigeria and Ghana (Perrone *et al.* 2009).

Another group of *A. flavus*-related isolates producing both B- and G-type aflatoxins has also been described as *A. minisclerotigenes*. This species was originally isolated from Argentinean peanuts and had small sclerotia and produced aflatoxins B_1, B_2, G_1, G_2, aspergillic acid, cyclopiazonic acid, kojic acid, parasiticolides and several other extrolites (Pildain *et al.* 2008, Table 2). One of the strains

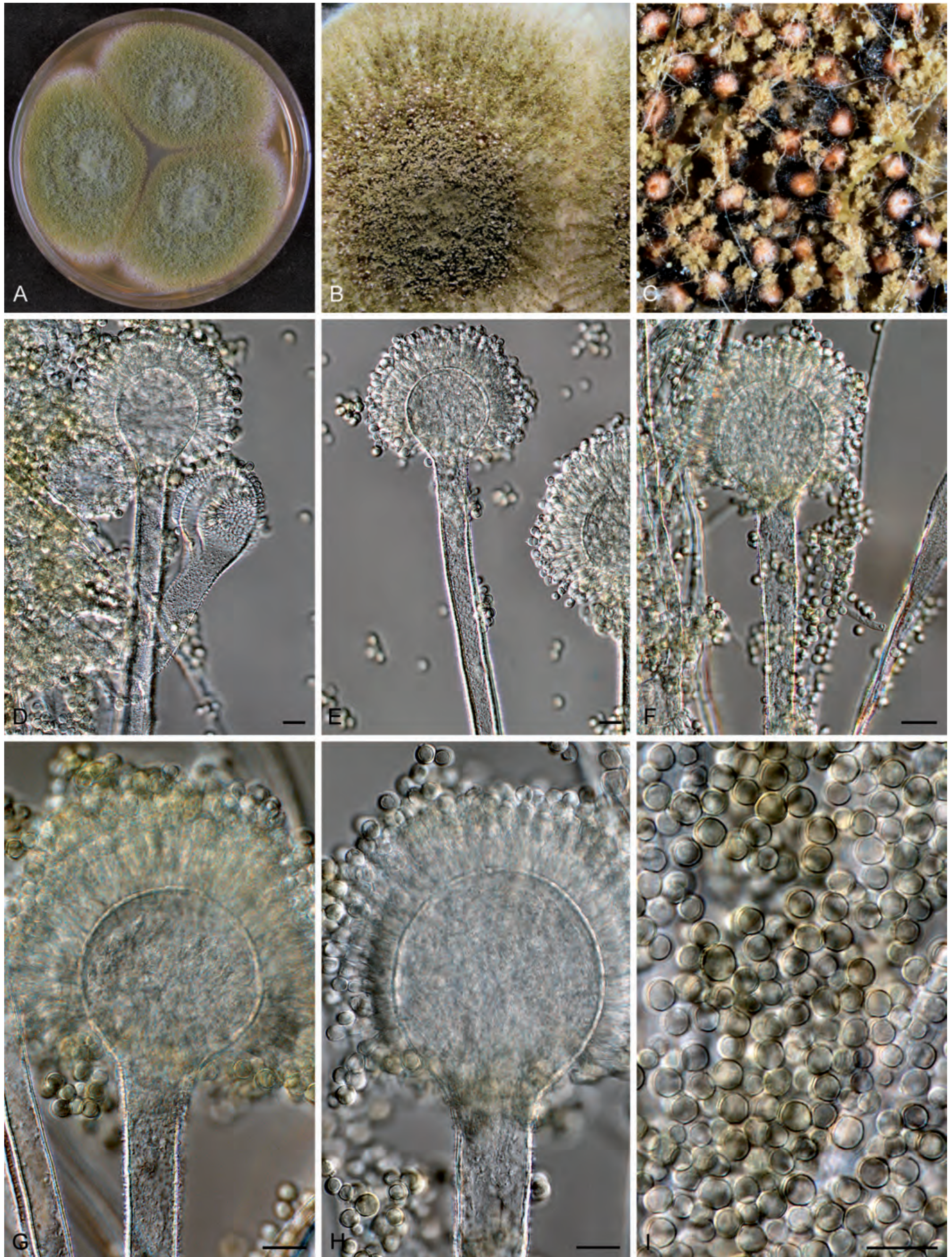

Fig. 17. *Aspergillus oryzae* (RIB 40). A–C. Colonies incubated at 25 °C for 7 d, A. CYA, B. MEA, C. Sclerotia, D–I. Conidiophores and conidia. Scale bars = 10 µm.

listed by Hesseltine *et al.* (1970), NRRL A-11611 = NRRL 6444 also produced aflatoxins B_1, B_2, G_1 and G_2, aflatrem, aflavinines, aspergillic acid, cyclopiazonic acid, parasiticolides, kojic acid, aspergillic acid, paspaline, paspalinine and emindole SB and is an *A. minisclerotigenes*. *Aspergillus parvisclerotigenus* has an extrolite profile very similar to that of *A. minisclerotigenes*, but in contrast

with the Argentinean strains, it also produces parasiticolides, and compound A 30461 (aspirochlorin = oryzachlorin; Table 2). Based on the molecular studies, *A. minisclerotigenes* seems to be quite widespread occurring in Argentina, USA, Nigeria and Australia as well (Pildain *et al.* 2008). Recently, Damann *et al.* (2010) observed sexual recombination between compatible partners of Australian isolates assigned to *A. flavus* groups I and II by Geiser *et al.* (1998). Further studies are needed to clarify the significance of these findings.

A third group of microsclerotial strains, represented by NRRL 3251, actually produces only B-type aflatoxins, but are, except being the S-type, typical *A. flavus*. Even though most strains of *A. flavus* produce large sclerotia, a smaller number of strains can produce small sclerotia. Thus at least three taxa can produce small sclerotia.

Many other isolates producing both aflatoxins B and G and bearing small sclerotia have been reported to date (Bayman & Cotty 1993, Saito & Tsurota 1993, Egel *et al.* 1994, Cotty & Cardwell 1999, Frisvad *et al.* 2005). Isolates came from maize, almond and cocoa beans and assigned to *A. flavus* based on either morphological or ITS sequence data have also been found to belong to different chemotypes based on their abilities to produce aflatoxins B_1, B_2, aflatoxin G_1, G_2 and cyclopiazonic acid (Razzaghi-Abyaneh *et al.* 2006, Giorni *et al.* 2007, Sanchez-Hervas *et al.* 2008, Rodrigues *et al.* 2009). Recently, Donner *et al.* (2009) found that about 8 % of the *Aspergillus* section *Flavi* isolates collected in maize fields in Nigeria produce small sclerotia and both B- and G-type aflatoxins. These isolates which presumably belong to *A. minisclerotigenes* together with *A. parasiticus* were suggested to be the greatest contributors to aflatoxin contamination of maize in regions where they occurred (Donner *et al.* 2009). Further studies are necessary to assign these isolates to species.

Another important aflatoxin producer, *Aspergillus parasiticus* occurs rather commonly in peanuts, and almonds (Rodrigues *et al.* 2009), but is apparently quite rare in other foods (*e.g.* on dried figs; Oktay *et al.* 2009). It is more restricted geographically as compared to *A. flavus*. *Aspergillus parasiticus* produces both B- and G-type aflatoxins (Sargeant *et al.* 1963), and virtually all known isolates are toxigenic. Linkage disequilibrium analyses of variation across 21 intergenic regions also revealed several distinct recombination blocks in *A. parasiticus*, and recombination events have also been observed between different vegetative compatibility groups (Carbone *et al.* 2007). The even distribution of the mating type genes in *A. parasiticus* populations was also indicative of the presence of a cryptic sexual stage (Ramirez-Prado *et al.* 2008). Recently, crosses between strains carrying opposite mating-type genes resulted in the development of ascospore-bearing ascocarps embedded within stromata. Sexually compatible strains belonged to different vegetative compatibility groups (Horn *et al.* 2009b). The sexual state of *A. parasiticus* has been described as *Petromyces parasiticus* (Horn *et al*. 2009c).

Nontoxigenic *A. flavus* and *A. parasiticus* isolates are used to control aflatoxin levels in various agricultural products. Great success in reducing aflatoxin contamination have been achieved by application of nontoxigenic strains of *A. flavus* and *A. parasiticus* in fields of cotton, peanut, maize and pistachio (Brown *et al*. 1991, Pitt & Hocking 2006, Dorner 2008). Significant reductions in aflatoxin contamination in the range of 70 %–90 % have been observed consistently by the use of nontoxigenic *A. flavus* and *A. parasiticus* strains (Pitt & Hocking 2006, Dorner 2008, Yin *et al.* 2008). Actually, two products of nontoxigenic strains have received U.S. Environmental Protection Agency (EPA) registration as biopesticides to control aflatoxin contamination in cotton and peanuts in several states of USA (Dorner 2008). This strategy is based on the application of nontoxigenic strains to competitively exclude naturally toxigenic strains in the same niche and compete for crop substrates. However, the discovery of a sexual cycle in *A. flavus* and in *A. parasiticus* raised concerns about the safety of these products. Indeed, Olarte *et al.* (2010) found that a single generation of sexual reproduction between a nonaflatoxigenic *A. flavus* isolate containing a single mutation in the aflatoxin biosynthesis gene cluster and an aflatoxigenic parent can restore aflatoxin production due to a crossing over within the aflatoxin biosynthesis gene cluster. In other crosses involving strains with either a partial aflatoxin gene cluster or strains missing the entire cluster and an aflatoxigenic *A. flavus* strain also regained toxicity via independent assortment of chromosomes, questioning the safety of using non-aflatoxigenic *A. flavus* or *A. parasiticus* strains for lowering aflatoxin levels in agricultural products. *Aspergillus toxicarius*, which also produces B- and G-type aflatoxins (Murakami *et al.* 1966, Murakami 1971), was suggested to be conspecific with *A. parasiticus* by Kozakiewicz (1989), which view is supported by the sequence data. *Aspergillus terricola* var. *americanus* (which does not produce aflatoxins!) and *A. parasiticus* var. *globosus* (which produces all the known aflatoxins) could also not be distinguished from *A. parasiticus* by neither phylogenetic analysis of multilocus sequence data nor by extrolite profiles indicating that these are also synonyms of *A. parasiticus* (Figs 1–3). *Aspergillus sojae* is the domesticated variety of *A. parasiticus*, which can hardly be distinguished from it apart from its inability to produce aflatoxins (Rigó *et al.* 2002, Chang *et al.* 2007). The lack of aflatoxin-producing ability of some *A. sojae* isolates results primarily from an early termination point mutation in the pathway-specific *AflR* regulatory gene, which causes the truncation of the transcriptional activation domain of *AflR* and the abolishment of interaction between *AflR* and the *AflJ* co-activator. In addition, a defect in the polyketide synthase gene also contributes to its nonaflatoxigenicity (Chang *et al.* 2007). Recently, Garber *et al.* (2010) identified *A. parasiticus* lineages associated with maize and peanut cultivation in USA, Asia and Africa, and a presumably new species with an ancient, global and almost exclusive association with sugarcane (*Saccharum* sp.). Again a soil-borne form of *A. parasiticus, A. terricola* var. *americanus*, and the domesticated forms (*A. sojae*) cannot produce aflatoxins similar to the examples in *A. flavus*.

Aspergillus arachidicola was isolated from leaves of *Arachis glabrata* in Argentina, and produce aflatoxins B_1, B_2, G_1 and G_2, aspergillic acid, chrysogine, aspirochlorin, parasiticolide, ditryptophenaline and the extrolite NO2. All strains had a floccose colony texture, a conidium colour similar to *A. flavus* but, except for the production of chrysogine by most isolates, they exhibited extrolite profiles similar to those of *A. parasiticus* isolates (Pildain *et al.* 2008, Table 2).

Aflatoxins have been shown to be produced by *A. flavus*, *A. parasiticus* (Codner *et al.* 1963, Schroeder 1966), *A. nomius* (Kurtzman *et al.* 1987), *A. pseudotamarii* (Ito *et al.* 2001), *A. bombycis* (Peterson *et al.* 2001), *A. toxicarius* (Murakami 1971, Murakami *et al.* 1982, Frisvad *et al.* 2005), *A. parvisclerotigenus* (Saito & Tsurota 1993, Frisvad *et al.* 2005), *A. minisclerotigenes, A. arachidicola* (Pildain *et al.* 2007) and *A. pseudonomius* and *A. pseudocaelatus* in *Aspergillus* section *Flavi.* Aflatoxin-producing species are scattered throughout the phylogenetic trees indicating that aflatoxin-producing ability was lost (or gained) several times during evolution.

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New taxa in *Aspergillus* section *Usti*

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Abstract: Based on phylogenetic analysis of sequence data, *Aspergillus* section *Usti* includes 21 species, inclucing two teleomorphic species *Aspergillus heterothallicus* (= *Emericella heterothallica*) and *Fennellia monodii*. *Aspergillus germanicus* sp. nov. was isolated from indoor air in Germany. This species has identical ITS sequences with *A. insuetus* CBS 119.27, but is clearly distinct from that species based on β-tubulin and calmodulin sequence data. This species is unable to grow at 37 °C, similarly to *A. keveii* and *A. insuetus*. *Aspergillus carlsbadensis* sp. nov. was isolated from the Carlsbad Caverns National Park in New Mexico. This taxon is related to, but distinct from a clade including *A. calidoustus, A. pseudodeflectus, A. insuetus* and *A. keveii* on all trees. This species is also unable to grow at 37 °C, and acid production was not observed on CREA. *Aspergillus californicus* sp. nov. is proposed for an isolate from chamise chaparral (*Adenostoma fasciculatum)* in California. It is related to a clade including *A. subsessilis* and *A. kassunensis* on all trees. This species grew well at 37 °C, and acid production was not observed on CREA. The strain CBS 504.65 from soil in Turkey showed to be clearly distinct from the *A. deflectus* ex-type strain, indicating that this isolate represents a distinct species in this section. We propose the name *A. turkensis* sp. nov. for this taxon. This species grew, although rather restrictedly at 37 °C, and acid production was not observed on CREA. Isolates from stored maize, South Africa, as a culture contaminant of *Bipolaris sorokiniana* from indoor air in Finland proved to be related to, but different from *A. ustus* and *A. puniceus.* The taxon is proposed as the new species *A. pseudoustus.* Although supported only by low bootstrap values, *F. monodii* was found to belong to section *Usti* based on phylogenetic analysis of either loci BLAST searches to the GenBank database also resulted in closest hits from section *Usti*. This species obviously does not belong to the *Fennellia* genus, instead it is a member of the *Emericella* genus. However, in accordance with the guidelines of the Amsterdam Declaration on fungal nomenclature (Hawksworth *et al.* 2011), and based on phylogenetic and physiological evidence, we propose the new combination *Aspergillus monodii* comb. nov. for this taxon. Species assigned to section *Usti* can be assigned to three chemical groups based on the extrolites. *Aspergillus ustus, A. granulosus* and *A. puniceus* produced ustic acid, while *A. ustus* and *A. puniceus* also produced austocystins and versicolorins. In the second chemical group, *A. pseudodeflectus* produced drimans in common with the other species in this group, and also several unique unknown compounds. *Aspergillus calidoustus* isolates produced drimans and ophiobolins in common with *A. insuetus* and *A. keveii*, but also produced austins. *Aspergillus insuetus* isolates also produced pergillin while *A. keveii* isolates produced nidulol. In the third chemical group, *E. heterothallica* has been reported to produce emethallicins, 5'-hydroxyaveranthin, emeheterone, emesterones, 5'-hydroxyaveranthin.

Key words: Ascomycetes, *Aspergillus* section *Usti,* ITS, calmodulin, extrolites, β-tubulin, polyphasic taxonomy.
Taxonomic novelties: *Aspergillus carlsbadensis* Frisvad, Varga & Samson sp. nov., *Aspergillus californicus* Frisvad, Varga & Samson sp. nov., *Aspergillus germanicus* Varga, Frisvad & Samson sp. nov. , *Aspergillus monodii* (Locquin-Linard) Varga, Frisvad & Samson comb. nov., *Aspergillus pseudoustus* Frisvad, Varga & Samson sp. nov., *Aspergillus turkensis* Varga, Frisvad & Samson sp. nov.

INTRODUCTION

Aspergillus ustus is a common filamentous fungus found in foods, soil and indoor air environments (Samson *et al.* 2004). This species was considered as a relatively rare human pathogen that can cause invasive infection in immunocompromised hosts (Weiss & Thiemke 1983, Stiller *et al.* 1994, Verweij *et al.* 1999, Nakai *et al.* 2002, Pavie *et al.* 2005, Panackal *et al.* 2006, Yildiran *et al.* 2006, Krishnan-Natesan *et al.* 2008, Florescu *et al.* 2008, Vagefi *et al.* 2008). However, recent studies clarified that infections attributed to *A. ustus* are caused in most cases by another species, *A. calidoustus* (Houbraken *et al.* 2007, Varga *et al.* 2008, Balajee *et al.* 2009, Peláez *et al.* 2010). This species is also common in indoor air (Houbraken *et al.* 2007, Slack *et al.* 2009) and is able to colonise water distribution systems (Hageskal *et al.* 2011). Other species related to *A. ustus* can also cause human or animal infections; *A. granulosus* was found to cause disseminated infection in a cardiac transplant patient (Fakih *et al.* 1995), while *A. deflectus* has been reported to cause disseminated mycosis in dogs (Jang *et al.* 1986, Kahler *et al.* 1990, Robinson *et al.* 2000, Schultz *et al.* 2008, Krockenberger *et al.* 2011).

Raper & Fennell (1965) classified *A. ustus* to the *Aspergillus ustus* species group (*Aspergillus* section *Usti* according to Gams *et al.* 1985) together with four other species: *A. panamensis, A. puniceus, A. conjunctus* and *A. deflectus*. Later, Kozakiewicz (1989) revised the taxonomy of the group, and included *A. ustus*, *A. pseudodeflectus, A. conjunctus, A. puniceus, A. panamensis* and *A. granulosus* in the *A. ustus* species group, and established the *A. deflectus* species group including *A. deflectus, A. pulvinus* and *A. silvaticus* based on morphological studies. Klich (1993) treated *A. granulosus* as member of section *Versicolores,* and found that *A. pseudodeflectus* is only weakly related to this section based on morphological treatment of section *Versicolores*. Peterson (2000) transferred *A. conjunctus, A. funiculosus, A. silvaticus, A. panamensis* and *A. anthodesmis* to section *Sparsi*. More recently, Peterson (2008) examined the relationships of the *Aspergillus* genus using phylogenetic analysis of sequences of four loci, and assigned 15 species to this section (see below).

We examined the evolutionary relationships among species assigned to section *Usti*. We have used a polyphasic taxonomic approach in order to determine the delimitation and variability of known and new species. For phenotypic analyses, macro- and micromorphology of the isolates was examined, and secondary

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Table 1. Isolates in *Aspergillus* section *Usti* and related species examined in this study.

Species	Strain No.	Source
A. amylovorus	CBS 600.67^T = NRRL 5813 = IMI 129961 = VKM F-906 = IBT 23158	Wheat starch, Ukraine
A. calidoustus	CBS 112452	Indoor air, Germany
	CBS 113228	ATCC 38849; IBT 13091
	CBS 114380	Wooden construction material, Finland
	CBS 121601; 677	Bronchoalveolar lavage fluid, proven invasive aspergillosis, Nijmegen, the Netherlands†
	CBS 121610; 91	Post-cataract surgery endophthalmitis, Turkey
A. californicus	CBS 123895^T = IBT 16748	Ex chamise chaparral (*Adenostoma fasciculatum*), in the foothills of the San Gabriel Mountains on Baldy Mountain Road near Shinn Road Intersection, North of Claremont and near San Antonio Dam, California, USA, Jeff S. La Favre, 1978. A wildfire occurred here 31/8 1975.
A. carlsbadensis	CBS 123893 = IBT 16753	Soil, Galapagos Islands, Ecuador
	CBS 123894^T = IBT 14493	Lechuguilla Cave, Carlsbad Caverns National Park, New Mexico, USA, D.E. Northup, 1992
	CBS 123903 =IBT 18616	Soil, Carthage, Tunesia
A. cavernicola	CBS 117.76^T = NRRL 6327	Soil, cave wall, Romania
A. deflectus	CBS 109.55^T = NRRL 2206 = IBT 24665	Soil, Rio de Janeiro, Brazil
	NRRL 4235 = IBT 25291	Potting soil
	NRRL 13131 = IBT 25254	Unknown
A. egyptiacus	CBS 123892 = IBT 16345 = RMF 9515	Soil, Iraq
	CBS 656.73^T = NRRL 5920	Sandy soil, under *Olea europaea*, Ras-El-Hikma, Egypt
	CBS 991.72C	Bare ferruginous soil, Dahkla Oasis, Western desert, Egypt
	CBS 991.72A	Bare ferruginous soil, Dahkla Oasis, Western desert, Egypt
	CBS 991.72B	Bare ferruginous soil, Dahkla Oasis, Western desert, Egypt
	CBS 991.72F	Bare ferruginous soil, Dahkla Oasis, Western desert, Egypt
	CBS 991.72E	Bare ferruginous soil, Dahkla Oasis, Western desert, Egypt
A. elongatus	CBS 387.75^T = NRRL 5176	Alkaline Usar soil, Lucknow, India
A. germanicus	CBS 123887^T = DTO 27-D9 = IBT 29365	Indoor air, Stuttgart, Germany
A. granulosus	CBS 588.65^T	Soil, Fayetteville, Arkansas, USA
	CBS 119.58	Soil, Texas, USA
A. heterothallicus	CBS 489.65^T	Soil, Costa Rica
	CBS 488.65	Soil, Costa Rica
A. insuetus	CBS 107.25^T = NRRL 279	South Africa
	CBS 119.27 = NRRL 4876	Soil, Iowa, USA
	CBS 102278	Subcutaneous infection, Spain
A. kassunensis	CBS 419.69^T = NRRL 3752 = IMI 334938 = IBT 23479	Soil, Damascus, Syria
A. keveii	CBS 209.92	Soil, La Palma, Spain
	CBS 561.65 = NRRL 1974	Soil, Panama
	IBT 10524 = CBS 113227 = NRRL 1254	Soil, Panama
	IBT 16751	Soil at trail from Pelican Bay to inland, Isla Santa Cruz, Galapagos Islands, Ecuador, Tjitte de Vries and D.P. Mahoney, 1968
A. lucknowensis	CBS 449.75^T = NRRL 3491	Alkaline Usar soil, Lucknow, India
A. monodii	CBS 434.93	Dung of *Procavia* sp. (daman), Darfur, Sudan
	CBS 435.93^T	Dung of sheep, Ennedi, Chad
A. pseudodeflectus	CBS 596.65	Sugar, USA, Louisiana
	CBS 756.74^T	Desert soil, Egypt, Western Desert
	NRRL 4846 = IBT 25256	Unknown
A. pseudoustus	ATCC 36063 = NRRL 5856 = CSIR 1128 = CBS 123904^T = IBT 28161	Stored maize, South Africa
	MRC 096 = IBT 31044	Contaminant in a *Bipolaris sorokiniana* strain (MRC 093), South Africa

Table 1. (Continued).

Species	Strain No.	Source
A. pseudoustus	IBT 22361	Indoor air, Finland
A. puniceus	CBS 495.65[T]	Soil, Zarcero, Costa Rica
	CBS 128.62	Soil, Louisiana, USA
A. subsessilis	CBS 502.65[T] = NRRL 4905 = IMI 135820 = IBT 23160	Desert soil, Mojave desert, CA, USA
	CBS 988.72 = NRRL 4907 = IMI 335782 = IBT 23165	Desert soil, USA
A. turkensis	CBS 504.65[T] = NRRL 4993 = WB 4993 = IBT 22553	Soil, Turkey
A. ustus	CBS 116057	Antique tapestries, Krakow, Poland
	CBS 114901	Carpet, The Netherlands
	CBS 261.67[T]	Culture contaminant, USA
	CBS 133.55	Textile buried in soil, Netherlands
	CBS 239.90	Man, biopsy of brain tumor, Netherlands
	CBS 113233 = IBT 14495	Cave wall, Lechuguilla Cave, Carlsbad, New Mexico
	CBS 113232 = IBT 14932	Indoor air, Denmark

metabolite profiles were studied. For genotypic studies, partial sequences of the β-tubulin and calmodulin genes and the ITS region of the rRNA gene cluster were analysed.

MATERIALS AND METHODS

Isolates

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

Morphological analysis

For macromorphological observations, Czapek Yeast Autolysate (CYA), Malt Extract Autolysate (MEA) agar, Yeast Extract Sucrose Agar (YES), Creatine Agar (CREA), and Oatmeal Agar (OA) were used (Samson *et al.* 2004). 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, microscopic mounts were made in lactic acid with cotton blue 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), with minor modifications as described by Smedsgaard (1997).

Genotypic analysis

The cultures used for the molecular studies were grown on malt peptone (MP) broth using 1 % (w/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 (Houbraken *et al.* 2007, Varga *et al.* 2007, 2008).

Data analysis

DNA sequences were edited with the DNASTAR computer package. Alignments of the sequences were performed using MEGA v. 4 (Tamura *et al.* 2007). Phylogenetic analysis of sequence data was 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 (CI and RI, respectively) were also calculated. *Aspergillus versicolor* CBS 583.65[T] was used as outgroup in these analyses. Sequences were deposited at GenBank under accession numbers FJ531124–FJ531191.

RESULTS AND DISCUSSION

Phylogenetic analysis

For the molecular analysis of the isolates, three genomic regions, the ITS region, and parts of the calmodulin and β-tubulin genes were amplified and sequenced. Phylogenetic analysis of the data was carried out using parsimony analysis. For the analysis of part of the β-tubulin gene, 589 characters were analysed, 197 of which were found to be parsimony informative. One of the 78 MP trees based on partial β-tubulin genes sequences is shown in Fig. 1 (tree length: 661 steps, consistency index: 0.6445, retention index: 0.8922). The calmodulin data set included 475 characters, with 266 parsimony informative characters. One of the 119 MP trees based on partial calmodulin gene sequences is shown in Fig. 2 (tree length:

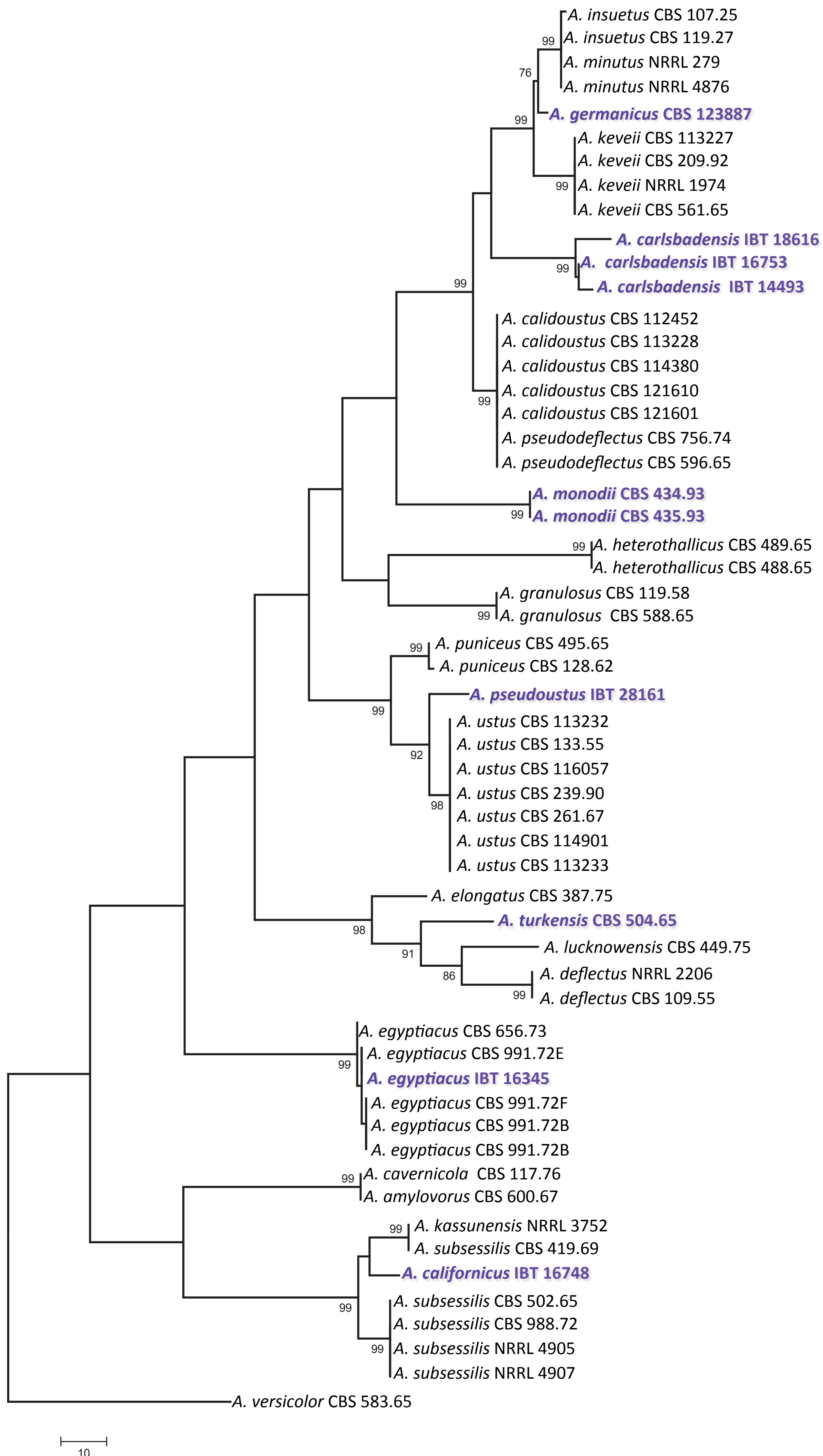

Fig. 1. The single MP tree obtained based on phylogenetic analysis of β-tubulin sequence data of *Aspergillus* section *Usti.* Numbers above branches are bootstrap values. Only values above 70 % are indicated.

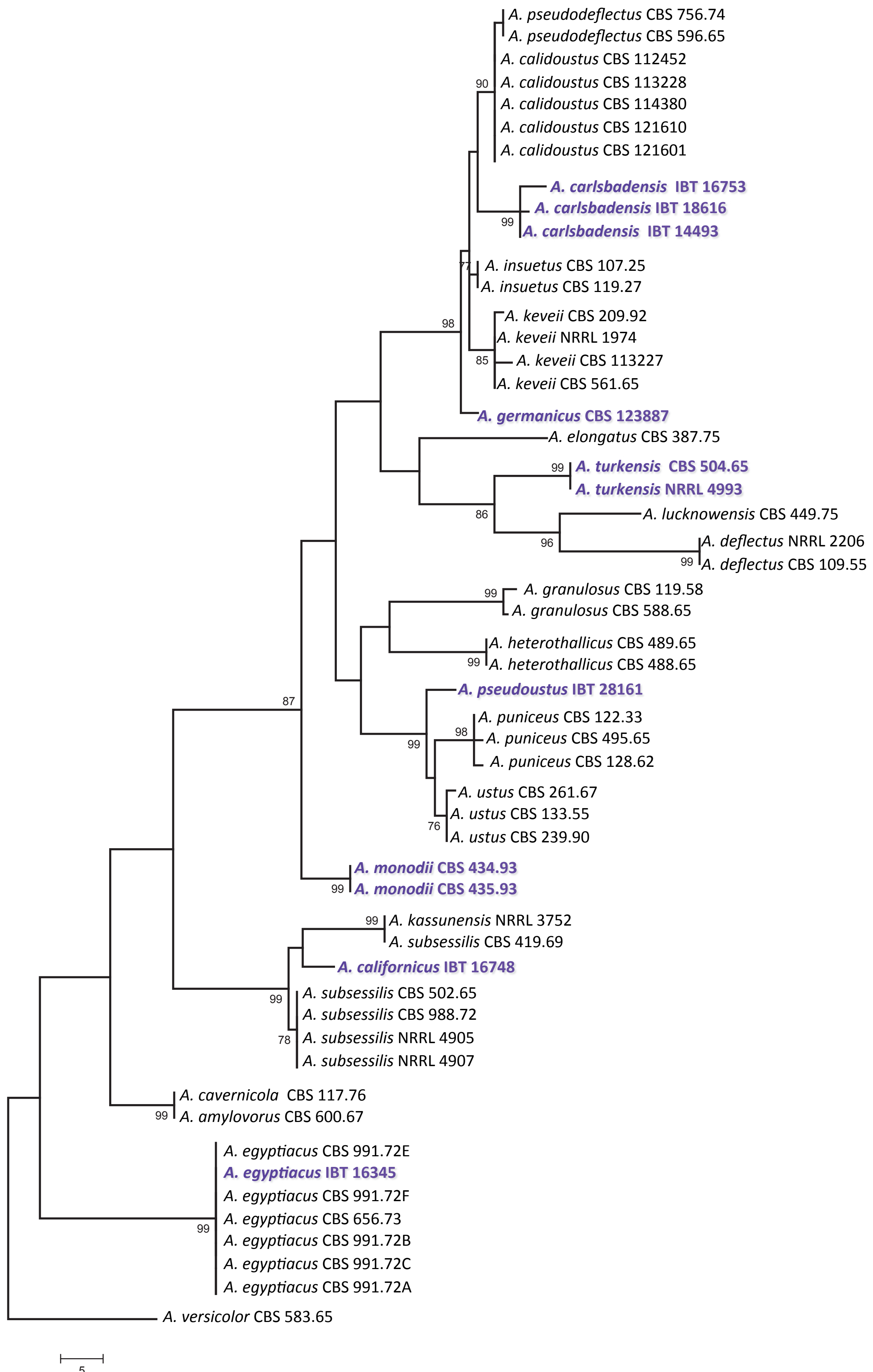

Fig. 2. One of the MP trees obtained based on phylogenetic analysis of calmodulin sequence data of *Aspergillus* section *Usti*. Numbers above branches are bootstrap values. Only values above 70 % are indicated.

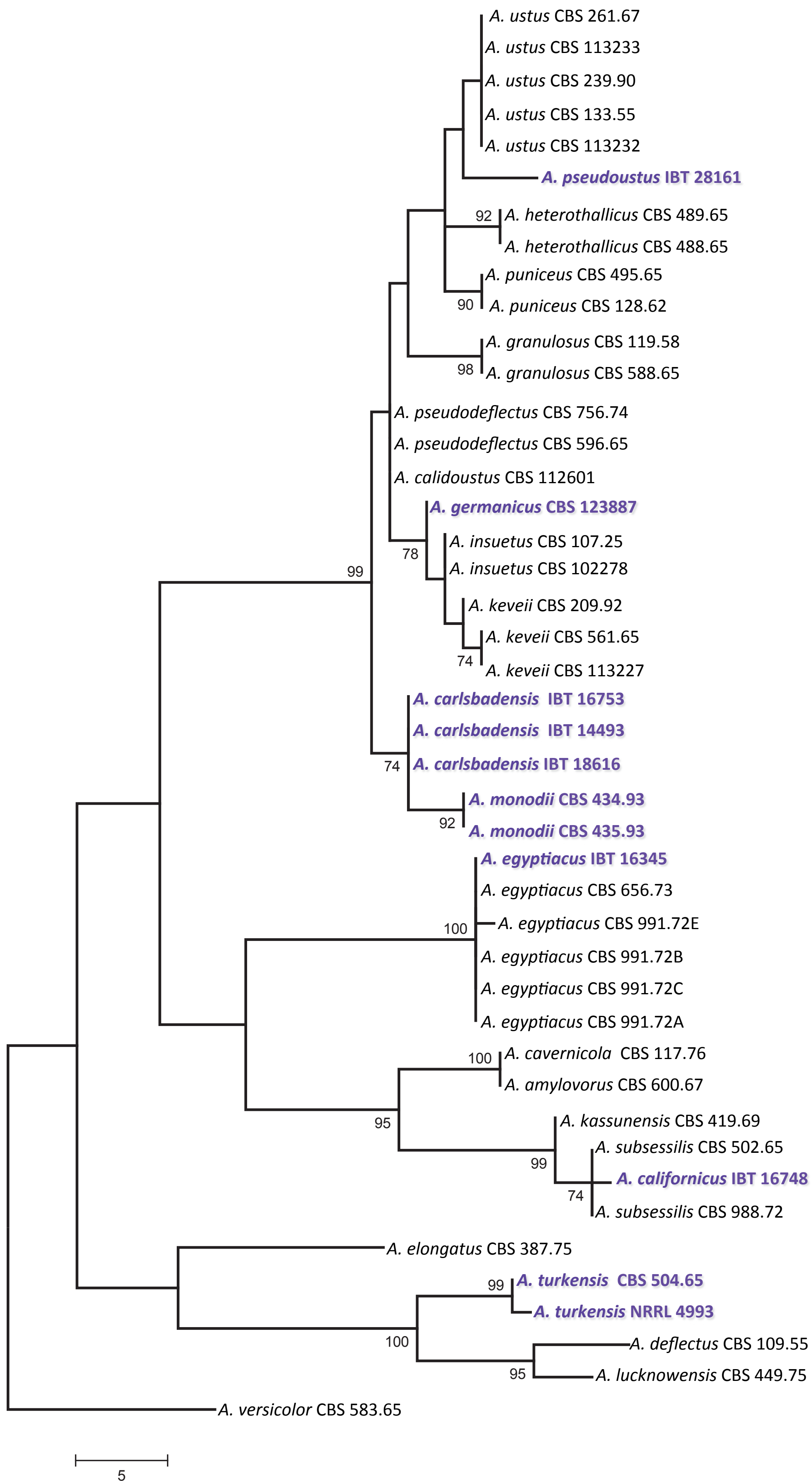

Fig. 3. One of the MP trees obtained based on phylogenetic analysis of ITS sequence data of *Aspergillus* section *Usti*. Numbers above branches are bootstrap values. Only values above 70 % are indicated.

890, consistency index: 0.5753, retention index: 0.8788). The ITS data set included 541 characters with 100 parsimony informative characters. One of the 8 MP trees is shown in Fig. 3 (tree length: 224, consistency index: 0.7366, retention index: 0.9230).

Based on phylogenetic analysis of sequence data, *Aspergillus* section *Usti* includes now 21 species, at least two of which are able to reproduce sexually: *Aspergillus heterothallicus* (=*Emericella heterothallica*) and *Fennellia monodii*. Although supported only by low bootstrap values, *F. monodii* was found to belong to section *Usti* based on phylogenetic analysis of either loci (Figs 1–3). BLAST searches to the GenBank database also resulted in closest hits from section *Usti* (*A. pseudodeflectus* and *A. calidoustus* for the ITS and calmodulin sequence data, and *A. ustus* and *A. insuetus* for the β-tubulin sequences). *Fennellia monodii* was described in 1990 by Locquin-Linard from dung of herbivores in Tchad and Sudan. This species is characterised by two-valved ascospores with low, wrinkled equatorial crests. The anamorph of this species has not yet been observed in spite of repeated attempts using various media (data not shown). This species obviously does not belong to the *Fennellia* genus, instead it is a member of the *Emericella* genus. However, in accordance with the guidelines of the Amsterdam Declaration on fungal nomenclature (Hawsksworth *et al.* 2011), and based on phylogenetic and physiological evidence, we propose the new combination *Aspergillus monodii* comb. nov. for this interesting species.

Another new species in this section was isolated from indoor air in Germany. This species has identical ITS sequences with *A. insuetus* CBS 119.27, but is clearly distinct from that species based on β-tubulin and calmodulin sequence data. This species is unable to grow at 37 °C, similarly to *A. keveii* and *A. insuetus*. We propose the name *A. germanicus* sp. nov. for this taxon.

Isolate IBT 16753 from Galapagos Islands, Ecuador, and IBT 14493 isolated from Lechuguilla Cave, Carlsbad Caverns National Park in New Mexico, USA were found to be related to, but clearly distinct from a clade including *A. calidoustus, A. pseudodeflectus, A. insuetus* and *A. keveii* on all trees. This species is also unable to grow at 37 °C, and acid production was not observed on CREA. We propose the name *A. carlsbadensis* sp. nov. for this taxon.

Isolate IBT 16748 was isolated from chamise chaparral (*Adenostoma fasciculatum)* in California, USA in 1978. It was found to be related to a clade including *A. subsessilis* and *A. kassunensis* on all trees. This species grew well at 37 °C, and acid production was not observed on CREA. We propose the name *A. californicus* sp. nov. for this taxon.

The "*A. deflectus*" isolate CBS 504.65 came from soil in Turkey is clearly distinct from the *A. deflectus* type strain on all trees, indicating that this isolate represents a distinct species in this section. This species grew, although rather restrictedly at 37 °C, and acid production was not observed on CREA. We propose the name *A. turkensis* sp. nov. for this taxon.

Another new species in this section, tentatively called *A. pseudoustus* sp. nov., is represented by NRRL 5856 = IBT 28161, which was found to be related to, but clearly different from *A. ustus* and *A. puniceus* on all trees (Figs 1–3). This isolate came from stored maize, South Africa. Other isolates belonging to this species include a culture contaminant of *Bipolaris sorokiniana* from South Africa (IBT 31044), and one isolate came from indoor air in Finland (IBT 22361).

Isolate IBT 16345 from soil, Iraq is a new isolate of *A. egyptiacus* based on all sequence data. The isolate grew well at 37 °C, and acid production was not observed on CREA. This is the first isolate of this species which was isolated outside Egypt.

In agreement with the data of Peterson (2008), *A. kassunensis*, which was treated as a synonym of *A. subsessilis* (Samson 1979, Samson & Moucchaca 2004), is also a valid species, related to *A. subsessilis* and *A. californicus* (Figs 1–3). *Aspergillus cavernicola* was treated as a synonym of *A. varians* by Samson (1979); however, based on sequence data, it is conspecific with *A. amylovorus* and belongs to section *Usti*, while the *A. varians* type strain belongs to *Aspergillus* section *Nidulantes* (data not shown). *Aspergillus amylovorus* was invalidly described (nom. inval., Art. 37) from wheat starch (Panasenko 1964), and subsequently validated by Samson (1979), while *A. cavernicola* was described in 1969 from cave wall from Romania. This species was validly described and hence is the correct name for *A. cavernicola* (= *A. amylovorus*).

Extrolites

The mycotoxins and other secondary metabolites found to be produced by the examined species in this study are listed in Table 2. Species assigned to section *Usti* could clearly be assigned to three chemical groups based on the extrolites produced by them. *Aspergillus ustus, A. granulosus* and *A. puniceus* produced ustic acids in common. *Aspergillus ustus* and *A. puniceus* also produced austocystins and versicolorins. In the second chemical group, *A. pseudodeflectus* produced drimans (Hayes *et al.* 1996) in common with the other species in this group, and also several unique unknown compounds. *Aspergillus calidoustus* isolates produced drimans and ophiobolins (Cutler *et al.* 1984) in common with *A. insuetus* and *A. keveii*, but also produced austins (Chexal *et al.* 1976) not identified in other species of section *Usti*. *Aspergillus insuetus* isolates also produced pergillin (Cutler *et al.* 1980), while *A. keveii* isolates produced nidulol. In the third chemical group, *E. heterothallica* has been reported to produce emethallicins A–F (Kawahara *et al.* 1989, 1990a, b), 5'-hydroxyaveranthin (Yabe *et al.* 1991), emeheterone (Kawahara *et al.* 1988), emesterones A & B (Hosoe *et al.* 1998), 5'-hydroxyaveranthin (Yabe *et al.* 1991), Mer-NF8054X (Mizuno *et al.* 1995). This latter compound, an 18,22-cyclosterol derivative, is closely related to the emesterones, and was also identified in an isolate identified as *A. ustus* (Mizuno *et al.* 1995). *Aspergillus deflectus* produces several antibiotics, including desferritriacetylfusigen, which inhibits the growth of bacteria (Anke 1977), and deflectins, angular azaphilons, which have antibiotic properties, and exhibit lytic activities against bacteria and erythrocytes (Anke *et al.* 1981). *Aspergillus egyptiacus* has been suggested to be more closely related to *E. nidulans* than to *A. versicolor* based on its biochemical behavior (Zohri & Ismail 1994). *Aspergillus egyptiacus* produces fumitremorgins and verruculogen, thus resembling *A. caespitosus* in that aspect. However *A. caespitosus* is placed within *Aspergillus* section *Nidulantes* (Peterson 2008, J. Varga, unpubl. data). *Aspergillus elongatus* CBS 387.75 produced fumitremorgin C, but other fumitremorgins and verruculogen could not be detected in that strain. The same strain also produced a member of the norgeamide / notoamide / aspergamide / stephacidin family of secondary metabolites (notoamide E). This type of compound has also been found in a strain of *A. versicolor* (Greshock *et al.* 2008).

Of particular interest is *A. pseudoustus* NRRL 5856 = CSIR 1128, which was originally identified as *A. ustus* and the first strain from which austamides, austdiols and austocystins (Table 2) were isolated (Steyn 1971, 1973, Steyn & Vleggaar 1974, 1976a, b, Vleggaar *et al.* 1974). This very toxic species has, however, only been isolated from maize in South Africa twice, and once in indoor

Table 2. Extrolites produced by species assigned to *Aspergillus* section *Usti*.

Species	Extrolites produced
A. amylovorus	An asperugin, monascorubramin-like extrolites, (CANO, SCYT, SENSTER, STARM)
A. calidoustus	Austins, drimans, ophiobolins G and H, TMC-120B, (ALTIN, FAAL, KNOF)
A. californicus	An arugosin, (CANDU, SAERLO, SCAM, SEND, XANXU)
A. carlsbadensis	Brevianamide A (only in IBT 14493), [An arugosin, DRI, TRITRA, TIDL (not in IBT 16753), GNI (only in IBT 18616), EMO (only in IBT 14493)]
A. deflectus	Desferritriacetylfusigen, deflectins A & B, emerin, a shamixanthone, (FUMU, RED2)
A. egyptiacus	Fumitremorgin A, fumitremorgin B, verruculogen, (FYEN, UTSCABI, TOPLA, FUMU, PRUD, HØJV)
A. elongatus	Fumitremorgin C, notoamide E, (DYK, SENT, TERRET)
A. germanicus	Drimans, (DRUL, KNAT, SLOT, SNOF)
A. granulosus	Asperugins, ustic acids, nidulol, drimans, (KMET, PUBO, SENSTER, SFOM)
A. heterothallicus	Emethallicins A, B, C, D, E & F, emeheterone, emesterones A & B and Mer-NF8054X, 5'-hydroxyaveranthin, stellatin, sterigmatocystin, (DRI, NIDU)
A. insuetus	Asperugins, drimans, ophiobolins G and H, pergillin-like compound, (AU, HETSCYT, INSU)
A. kassunensis	Asperugins, Mer-NF8054X, (FYRT, SAERLO, SENSCAB, SENSTER)
A. keveii	Asperugins, drimans, ophiobolins G and H, nidulol, (DRI, HETSCYT, INSU, PUBO, SENSTER, UP)
A. lucknowensis	An arugosin, (GULT, PULK, RED1)
A. monodii	Terrein, (DYVB, METK)
A. pseudodeflectus	Drimans, (DRI, DRUL, HUT, SLOT), asperugins in NRRL 4846
A. pseudoustus	Asperugins, austamide, prolyl-2-(1',1'-dimethylallyl) tryptophyldiketopiperazine, 12,13-dihydroaustamide, 12,13-dehydroprolyl-2-(1',1'-dimetylallyl)-tryptophyldiketopiperazine, 10,20-dehydro[12,13-dehydropropyl-2-1',1'-dimethylallyl)tryptophyldiketopiperazine], 12,13-dihydro-12-hydroxyaustamide, austdiol, dihydrodeoxy-8-epi-austdiol, austocystin A, B, C, D, E, F, G, H, I, norsolorinic acid, versicolorin C, averufin, (DRI, HETSCYT, SENSTER, UZ)
A. puniceus	Ustic acids, austocystins (and versicolorins), phenylahistin, nidulol, (SENSTER)
A. subsessilis	Mer-NF8054X, (SENSCAB, VIRO)
A. turkensis	An austocystin, deflectins, emerin, a shamixanthone, (RED2)
A. ustus	Ustic acids, austocystins (and versicolorins), austalides, nidulol, (SENSTER)

All designations in parenthesis with capital letters are secondary metabolites with characteristic chromophores (UV spectra) and retention-times, but their chemical structure is not yet known.

air in Finland. All three strains examined produced austamides, austdiol and austocystins. The austocystins have been found in *A. ustus, A. puniceus* and *A. pseudoustus* and one austocystin has also been found in *A. turkensis*. The austocystins seem to be another biosynthetic family of secondary metabolites that are derived from the versicolorins. In other species in sections *Aenei, Versicolores* and *Nidulantes*, versicolorins are precursors of sterigmatocystin and in few species, the aflatoxins (Frisvad *et al.* 2005, Varga *et al.* 2009). Sterigmatocystin has not yet been found in any species in section *Usti*, but a related metabolite, listed as SENSTER in Table 2 is common in this section, and may be related to sterigmatocystin, as it has a similar UV spectrum.

Comparing the secondary metabolite profiles of section *Usti* with other sections within subgenus *Nidulantes,* nidulol, and versicolorins are also produced by members of sections *Versicolores* and *Nidulantes* (Cole & Schweikert 2003). Interestingly, versicolorin, sterigmatocystin and 5'-hydroxyaveranthin are intermediates of the aflatoxin biosynthetic pathway and also produced by species assigned to *Aspergillus* sections *Flavi* and *Ochraceorosei* (Yabe *et al.* 1991, Frisvad *et al.* 2005). Other extrolites found in species in section *Usti* are also found in other sections in subgenus *Nidulantes*: arugosins, asperugins, austins and the metabolite DRI are present in species of the different sections. On the other hand, several metabolites have only been found in section *Usti*, including austamide, austdiol, austocystins, deflectins, drimans, emethallicins, emeheterones and ustic acids (Table 2). Two species produce red pigments, *A. amylovorus* produce a large number of monascorubramin like red pigments, while *A. turkensis* produce few monascorubramin-like extrolites.

Species descriptions

Aspergillus carlsbadensis Frisvad, Varga & Samson, **sp. nov.** MycoBank MB560399 Fig. 4.

Coloniis flavo-brunneis, cum caespitulis ex conglomerationibus cellularum obtegentium ("Hülle"). Cellulis obtegentibus ("Hülle") hyalinis, crassitunicatis, globosis vel late ellipsoideis, 15–30 µm. Conidiophoris biseriatis, stipitibus plerumque levibus, brunneis, 4–5 µm latis. Vesiculis globosis, 10–14 µm diam. Conidiis conspicue ornamentatis, echinulatis vel verrucosis, ellipsoideis, 2.5–3.0 × 3.0–3.5 µm.

Typus: **USA,** from soil, Lechuguilla Cave, Carlsbad Caverns National Park, New Mexico, isolated by D.E. Northup, 1992, (CBS H-30634 -- holotypus, culture ex-type CBS 123894).

CYA, 1 wk, 25 °C: 30–32 mm (poor to medium sporulation, cream yellow to dark brown reverse, Hülle cells), MEA, 1 wk, 25 °C: 7–29 mm (rather poor sporulation, light yellow to cream reverse), YES, 1 wk, 25 °C: 35–45 mm (no sporulation, yellow to curry yellow), OA, 1 wk, 25 °C: 25–32 mm (Hülle cells), CYA, 1 wk, 37 °C: no growth, CREA: good growth (18–22 mm) and no acid production.

Colonies yellow brown with white tufts of conglomerates of Hülle cells. Hülle cells hyaline, thick-walled, globose to broadly ellipsoidal, 15–30 µm. Conidiophores biseriate with typical smooth-walled, brown, 4–5 µm wide stipes. Vesicles globose, 10–14 µm in diam. Conidia, distinctly ornamented with spines or warts, ellipsoidal 2.5–3.0 × 3.0–3.5 µm.

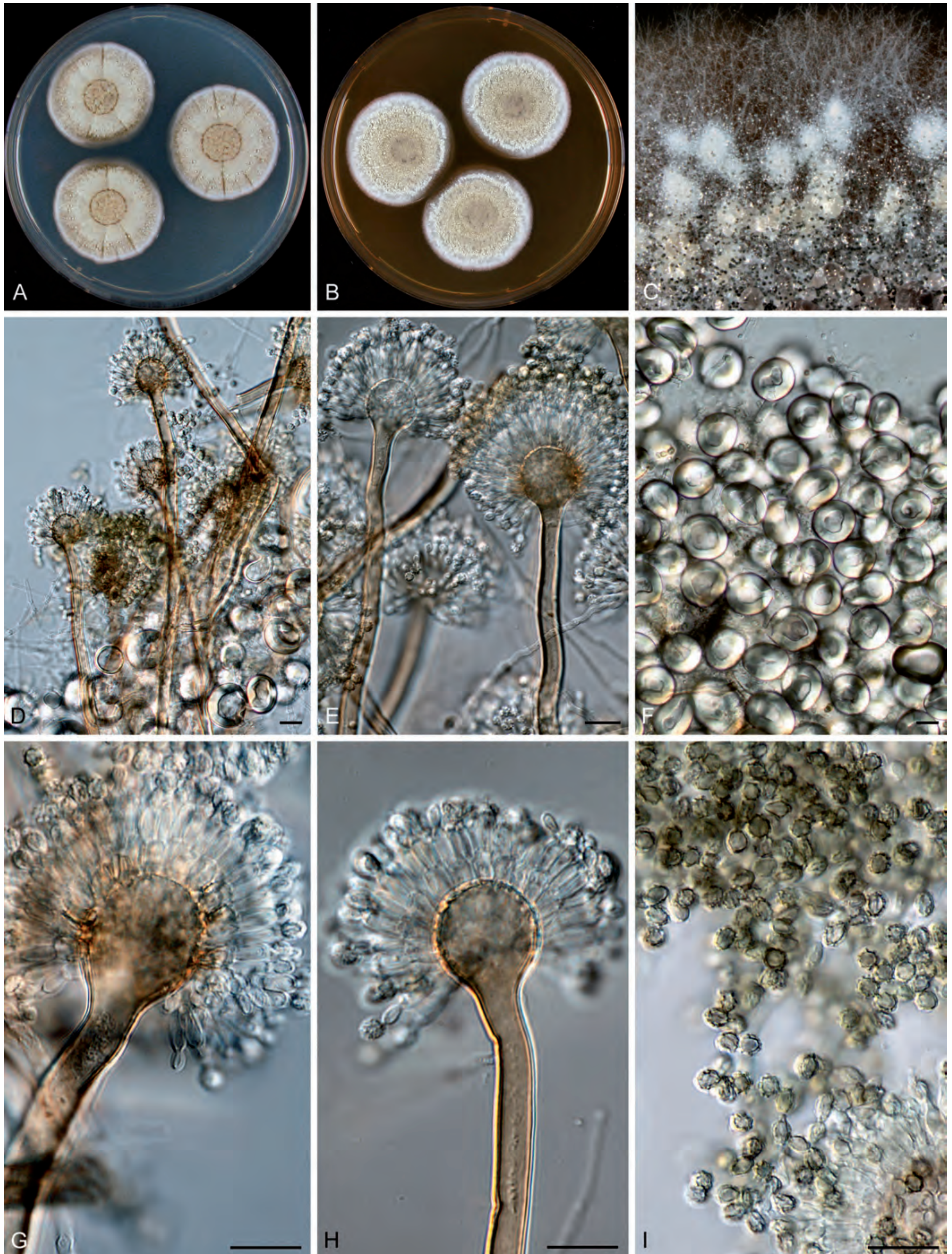

Fig. 4. *Aspergillus carlsbadensis* Frisvad, Varga & Samson sp. nov. A–C. Colonies incubated at 25 °C for 7 d, A. CYA, B. MEA, C. Tufts of Hülle cells. D–E, G–I. Conidiophores and conidia. F. Hülle cells. Scale bars = 10 µm.

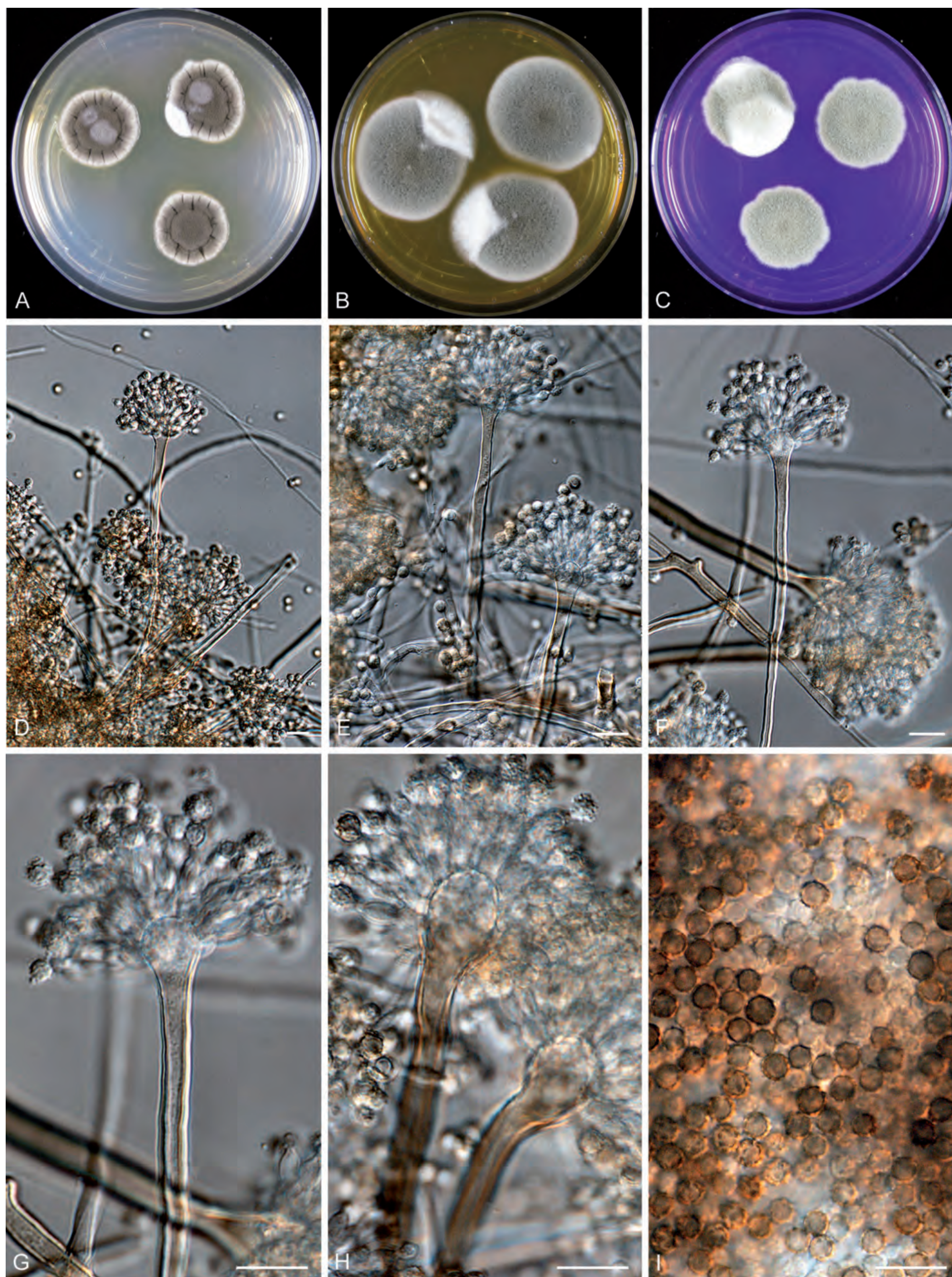

Fig. 5. *Aspergillus californicus* Frisvad, Varga & Samson sp. nov. A–C. Colonies incubated at 25 °C for 7 d, A. CYA, B. MEA, C. CREA, D–I. Conidiophores and conidia. Scale bars = 10 µm.

The taxon is related to, but clearly distinct from a clade including *A. calidoustus, A. pseudodeflectus, A. insuetus* and *A. keveii* on all trees. This species is also unable to grow at 37 °C, and acid production was not observed on CREA.

Aspergillus californicus Frisvad, Varga & Samson, **sp. nov.** MycoBank MB560400. Fig. 5.

Coloniis clare flavis, cum caespitulis albidis ex conglomerationibus cellularum obtegentium ("Hülle"). Cellulis obtegentibus ("Hülle") hyalinis, crassitunicatis, globosis vel late ellipsoideis. Conidiophoris biseriatis, stipitibus levibus, clare brunneis, 3.5–5 µm latis. Vesiculis globosis,. 11–16 µm in diam. Conidiis levibus vel subtiliter exasperates, subglobosis vel globosis, hyalinis vel viridibus, 2.5–3.0 µm.

Typus: **USA**, foothills of San Gabriel Mountains, California, ex chamise chaparral (*Adeonostoma fasciculatum*), Jeff S. La Favre, 1978 (CBS H-20635 -- holotypus, culture ex-type CBS 123895).

CYA, 1 wk, 25 °C: 18–20 mm (poor sporulation, yellow brown reverse, Hülle cells), MEA, 1 wk, 25 °C: 6–9 mm (rather poor sporulation, yellow brown reverse), YES, 1 wk, 25 °C: 23–26 mm (no sporulation, cream yellow reverse), OA, 1 wk, 25 °C: 18–21 mm (Hülle cells), CYA, 1 wk, 37 °C: no growth, CREA: good growth and no acid production.

Colonies light yellow with white tufts of conglomerates of Hülle cells. Hülle cells hyaline, thick-walled, globose to broadly ellipsoidal, 25–50 µm. Conidiophores biseriate with smooth-walled, light brown, 3.5–5 µm wide stipes. Vesicles globose, 11–16 µm in diam. Conidia, smooth to finely roughened, subglobose to globose, hyaline to greenish, 2.5–3.0 µm.

This species grew well at 37 °C, and acid production was not observed on CREA. It was found to be related to species in a clade including *A. subsessilis* and *A. kassunensis*.

Aspergillus germanicus Varga, Frisvad & Samson, **sp. nov.** MycoBank MB560401. Fig. 6.

Coloniis in agaro CYA brunneis et in agaro MEA griseo-brunneis, cellulis tectegentibus ("Hülle") nullis. Conidiophoris biseriatis, stipitibus plerumque levibus, brunneis, 6–9 µm latis. Vesiculis spathuliformibus, 14–22 µm diam. Conidiis conspicue echinulatis, globosis, brunneis, 3.5–5.0 µm diam.

Typus: **Germany**, ex indoor air, Stuttgart. Isolated by U. Weidner (CBS H-20636 -- holotypus, culture ex-type CBS 123887).

CYA, 1 wk, 25 °C: 22–26 mm (poor to medium sporulation, yellow brown to orange reverse, pigment diffusing, Hülle cells), MEA, 1 wk, 25 °C: 12–16 mm (good sporulation, light yellow to cream reverse), YES, 1 wk, 25 °C: 32–37 mm (some sporulation, yellow brown reverse), OA, 1 wk, 25 °C: 28–32 mm, CYA, 1 wk, 37 °C: 7–9 mm, CREA: good growth and no acid production.

Colonies on CYA brown, on MEA greyish brown. Hülle cells not observed. Conidiophores biseriate with typical smooth-walled, brown, 6–9 µm wide stipes. Vesicles spathulate, 14–22 µm diam. Conidia, distinctly echinulate, globose, brown, 3.5–5.0 µm.

This species has identical ITS sequences with *A. insuetus* CBS 119.27, but is clearly distinct from that species based on β-tubulin and calmodulin sequence data.

Aspergillus monodii (Locquin-Linard) Varga, Frisvad & Samson, **comb. nov.** MycoBank MB560402. Fig. 7.

Basionym: *Fennellia monodii* Locquin-Linard, *Mycotaxon* **39**: 10, 1990.

CYA, 1 wk, 25 °C: 2–21 mm (no sporulation, white to cream reverse), MEA, 1 wk, 25 °C: 6–8 mm (ascomata, light yellow reverse), YES, 1 wk, 25 °C: 8–23 mm (no sporulation, yellow to red brown reverse, yellow obverse), OA, 1 wk, 25 °C: 9–19 mm (ascomata), CYA, 1 wk, 37 °C: 0–2 mm, CREA: poor growth and no acid production.

Colonies producing an orange brown crusts of stromata with ascomata 200–350 µm in diam. Hülle cells forming the structure of the stromata, globose to ellipsoidal, 8–40 µm diam. Asci 8–10 × 10–13 µm. Ascospores 3.0–3.5 × 4.5–5.0 µm, hyaline, smooth-walled with two equatorial rings. *Aspergillus* anamorph not observed on various media and after cultivation at different temperatures.

This species occurs on dung and found on sheep dung in Chad and daman dung in Soudan.

Aspergillus pseudoustus Frisvad, Varga & Samson, **sp. nov.** MycoBank MB560403. Fig. 8.

Coloniis in agaro CYN cinnamomeo-brunneis et in agaro MEA flavo-brunneis, cellulis obtegentibus ("Hülle") nullis. Conidiophoris biseriatis, stipitibus plerumque levibus, brunneis, 3.5–5 µm latis. Vesiculis globosis, 10–14 µm diam. Conidiis levibus vel distinct echinulatis, globosis, brunneis vel viridibus, 2.5–3.0 µm.

Typus: **South Africa**, ex stored maize (CBS H-20637 -- holotypus, culture ex-type CBS 123904).

CYA, 1 wk, 25 °C: 30–32 mm (medium sporulation, yellow brown reverse), MEA, 1 wk, 25 °C: 15–25 mm (rather poor sporulation, light yellow reverse), YES, 1 wk, 25 °C: 35–45 mm (no sporulation, curry yellow to brown reverse), OA, 1 wk, 25 °C: 30–36 mm, CYA, 1 wk, 37 °C: no growth, CREA: 28–34 mm, no acid production.

Colonies on CYA cinnamon brown, on MEA yellow brown. Hülle cells not observed. Conidiophores biseriate with typical smooth-walled, brown, 3.5–5 µm wide stipes. Vesicles globose, 10–14 µm in diam. Conidia, smooth to distinctly echinulate, globose, brown to greenish, 2.5–3.0 µm.

Other strains: MRC 096 = IBT 31044, contaminant in *Bipolaris sorokiniana*, isolated from maize, South Africa; IBT 22361, indoor air, Finland

Aspergillus pseudoustus sp. nov., is related to, but clearly different from *A. ustus* and *A. puniceus* on all trees. This isolate came from stored maize, South Africa. Other isolates belonging to this species include a culture contaminant of *Bipolaris sorokiniana* from South Africa (IBT 31044), and one isolate came from indoor air in Finland (IBT 22361).

Aspergillus turkensis Varga, Frisvad & Samson **sp. nov.** MycoBank MB560404. Fig. 9.

Coloniis in agaro CYN clare brunneis et in agaro MEA flavo-brunneis, cellulis obtegentibus ("Hülle") nullis. Conidiophoris minute biseriatis, stipitibus plerumque levibus, clare brunneis, 2.5–3 µm latis. Vesiculis spathuliformibus, 5–8 µm diam. Conidiis levibus, globosis, hyalinis, 2.5–3.0 µm diam.

Typus: **Turkey**, ex soil isolated by K.B. Raper in 1950 (CBS H-20638 -- holotypus, culture ex-type CBS 504.65).

CYA, 1 wk, 25 °C: 13–18 mm (poor sporulation, red orange reverse), MEA, 1 wk, 25 °C: 4–10 mm (rather poor sporulation, cream yellow reverse), YES, 1 wk, 25 °C: 35–45 mm (no sporulation, orange

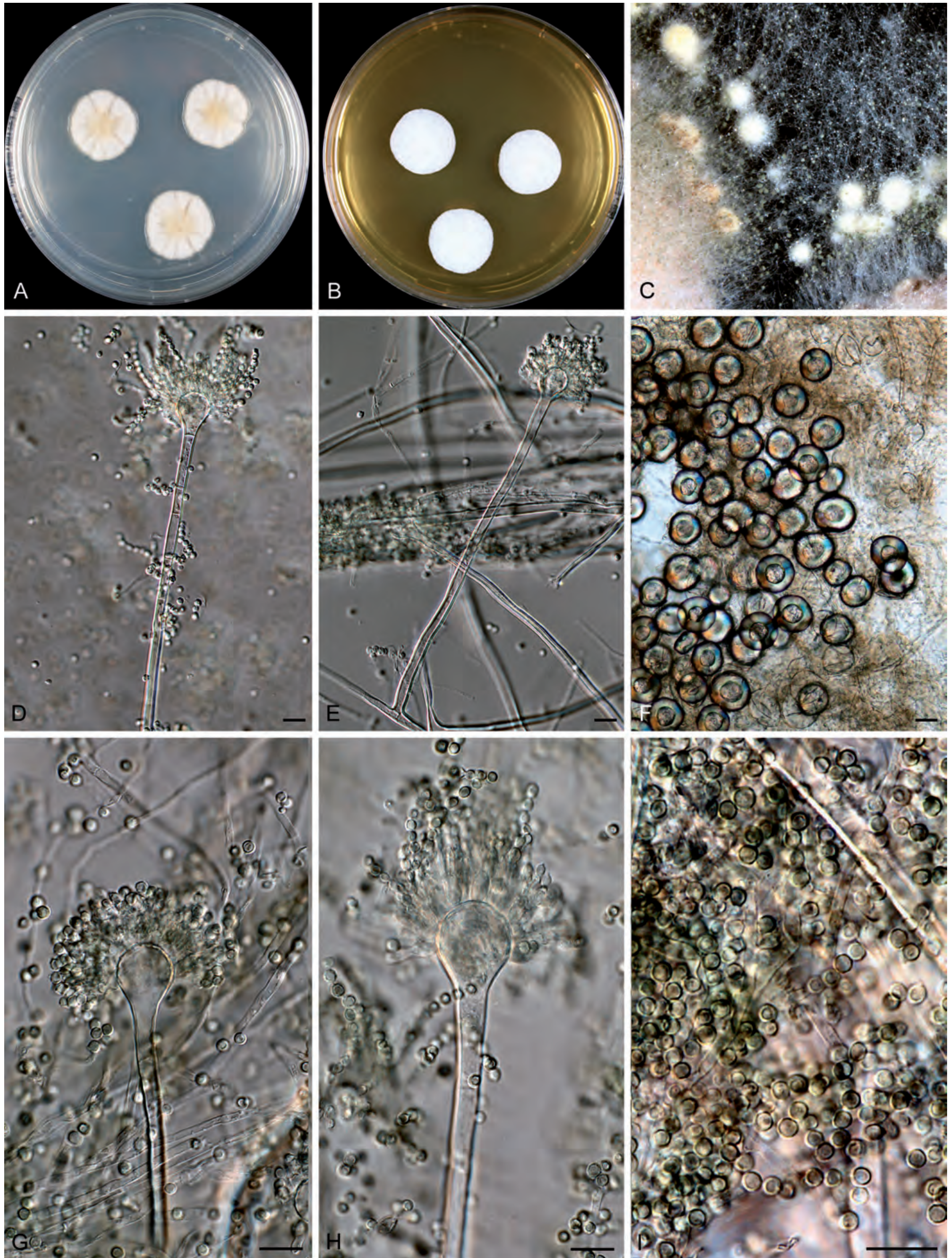

Fig. 6. *Aspergillus germanicus* Varga, Frisvad & Samson sp. nov. A–C. Colonies incubated at 25 °C for 7 d, A. CYA, B. MEA, C. Tufts of Hülle cells. D–E, G–I. Conidiophores and conidia. F. Hülle cells. Scale bars = 10 µm.

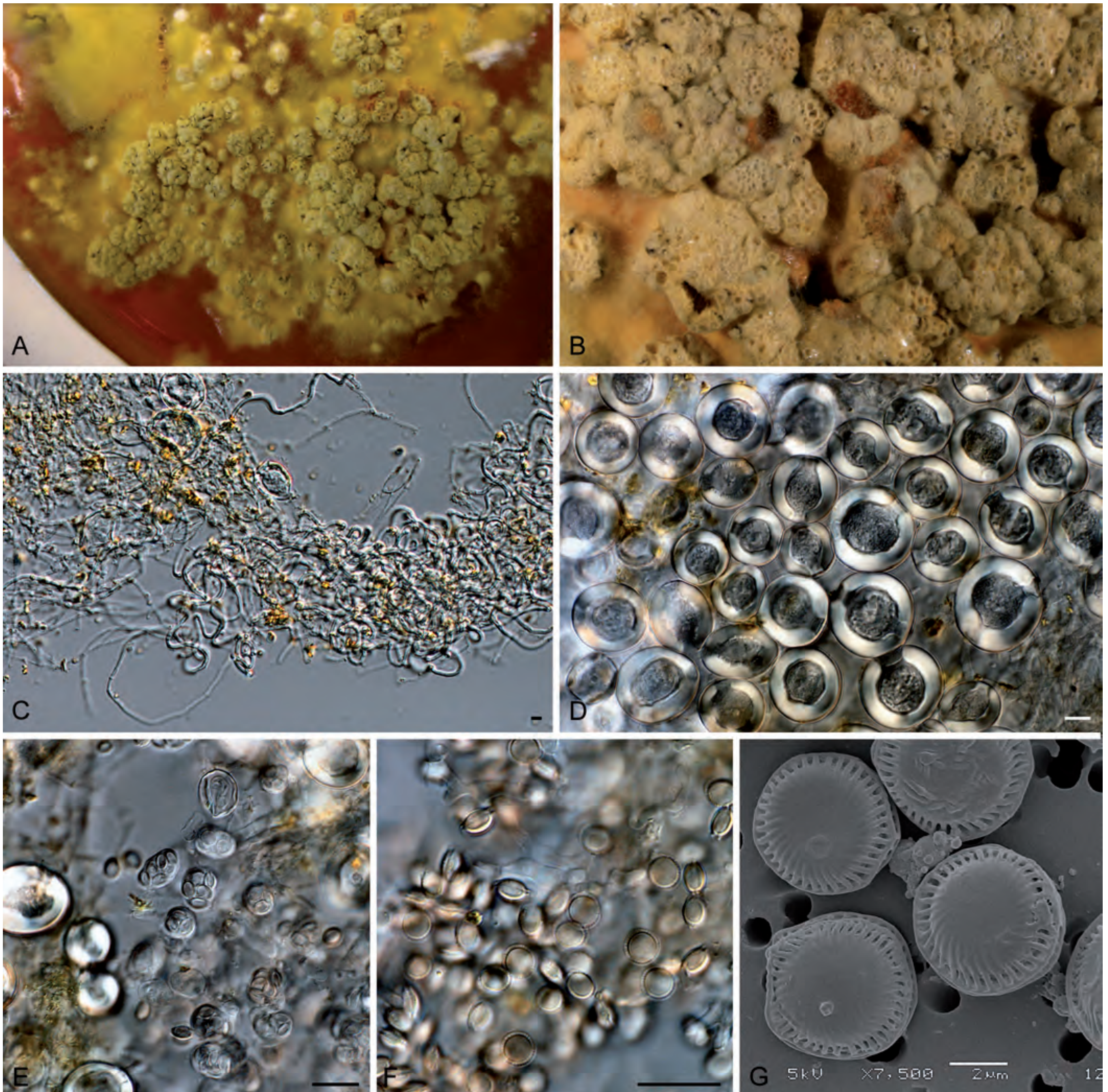

Fig. 7. *Aspergillus monodii* (Locquin-Linard) Varga, Frisvad & Samson comb. nov. A–B. Stromata containing ascomata, grown at 25 °C for 7 d, C. Mycelium with ascoma initials. D. Hülle cells, E–G. Asci and ascospores. Scale bars = 10 µm.

yellow reverse, yellow obverse), OA, 1 wk, 25 °C: 14–17 mm (yellow reverse and obverse), CYA, 1 wk, 37 °C: 6–14 mm, CREA: weak growth and no acid production.

Colonies on CYA light brown, on MEA pale yellow brown. Hülle cells not observed. Conidiophores small biseriate with typical smooth-walled, light brown, 2.5–3 µm wide stipes. Vesicles spathulate, 5–8 µm diam. Conidia, smooth-walled, globose, hyaline, 2.5–3.0 µm.

Isolate CBS 504.65 is distinct from the *A. deflectus* ex-type strain on all trees, indicating that this isolate represents a distinct species in this section. This species grew, although rather restrictedly at 37 °C, and acid production was not observed on CREA.

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Fig. 8. *Aspergillus pseudoustus* Frisvad, Varga & Samson 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.

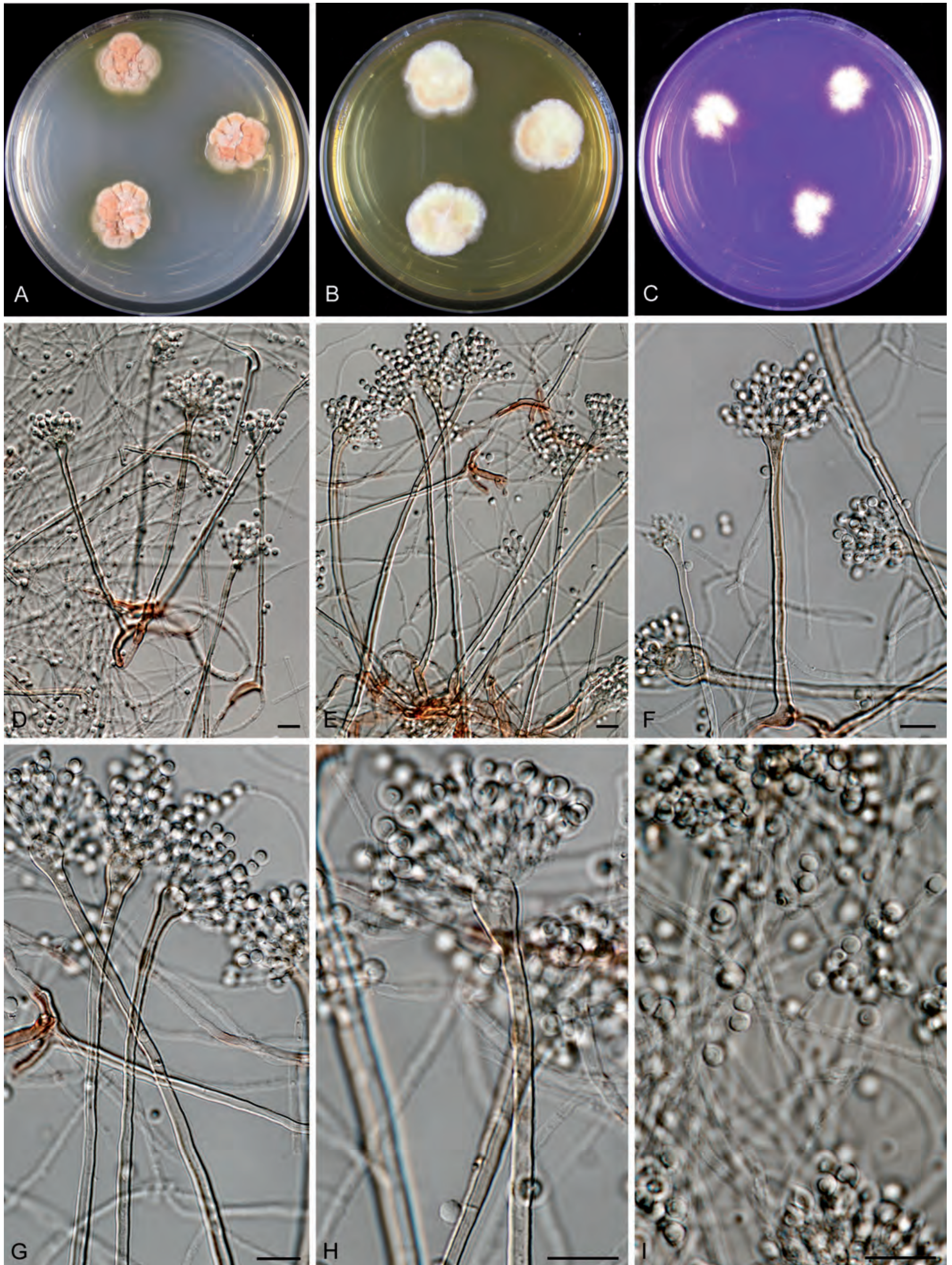

Fig. 9. *Aspergillus turkensis* Varga, Frisvad & Samson sp. nov. A–C. Colonies incubated at 25 °C for 7 d, A. CYA, B. MEA, C. CREA, D–I. Conidiophores and conidia. Scale bars = 10 µm.

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SUPPLEMENTARY INFORMATION

Table 1. Primers used in this study.

	Primer 5' to 3'	
gene	**dw**	**up**
***araR* 5' flank**	GGTACCCTTTGATGTTAGTTG	GGATCCATCGCGGGGAAAC
***araR* 3' flank**	GCATGCTTAAATTATCTTCCGCC	AAGCTTTCAATTTTTGTGTCTGGAG
xlnR	CTTGGTTGGTCTCCGTCTG	GGGAAGTGCGGAGGGAGTG
abfA	AGGGTGGCAACTCATCCAG	GCCAGCACCGTCAACTTG
abfB	ACCCGCGCCCTATACAGC	CTGCTTCGTGCCATCGTTG
ladA	AGATCTCTACCGCAACTGTTCTCG	CTGCAGTTTAAATCTTCTGACCAG
xdhA	AGATCTGCACCCAGAACACCAACG	CTGCAGAATTCTATGAATCGACACC
xyrA	AACAGCGGCTACGACATGC	TCTGCTTCAACCGCTGAGG
xkiA	CATCGGCTTCGACCTCTC	CAGTGCTTCCCTTCCTGG

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Table 2. Fungal genomes used for the phylogenetic analysis.

Species anamorph	Species teleomorph	website	(sub)phylum	order
Aspergillus clavatus		http://www.broad.mit.edu/annotation/genome/aspergillus_group/Blast.html	Ascomycota	*Eurotiales*
Aspergillus fischerianus	*Neosartorya fischeri*	http://www.broad.mit.edu/annotation/genome/aspergillus_group/Blast.html	Ascomycota	*Eurotiales*
Aspergillus flavus		http://www.broad.mit.edu/annotation/genome/aspergillus_group/Blast.html	Ascomycota	*Eurotiales*
Aspergillus fumigatus		http://www.broad.mit.edu/annotation/genome/aspergillus_group/Blast.html	Ascomycota	*Eurotiales*
Aspergillus nidulans	*Emericella nidulans*	http://www.broad.mit.edu/annotation/genome/aspergillus_group/Blast.html	Ascomycota	*Eurotiales*
Aspergillus niger JGI		http://www.broad.mit.edu/annotation/genome/aspergillus_group/Blast.html	Ascomycota	*Eurotiales*
Aspergillus oryzae		http://www.broad.mit.edu/annotation/genome/aspergillus_group/Blast.html	Ascomycota	*Eurotiales*
Aspergillus terreus		http://www.broad.mit.edu/annotation/genome/aspergillus_group/Blast.html	Ascomycota	*Eurotiales*
Botrytis cinerea	*Botryotinia fuckeliana*	http://www.broad.mit.edu/annotation/genome/botrytis_cinerea/Home.html	Ascomycota	*Helotiales*
Candida albicans		http://www.broad.mit.edu/annotation/genome/candida_group/MultiHome.html	Ascomycota	*Saccharomycetales*
Candida robusta	*Saccharomyces cerevisiae*	http://www.broad.mit.edu/annotation/genome/saccharomyces_cerevisiae/Home.html	Ascomycota	*Saccharomycetales*
Coccidioides immitis		http://www.broad.mit.edu/annotation/genome/coccidioides_group/MultiHome.html	Ascomycota	*Onygenales*
Coprinus cinereus	*Coprinopsis cinerea*	http://www.broad.mit.edu/annotation/genome/coprinus_cinereus/Home.html	Basidiomycota	*Agaricales*
Cryptococcus neoformans	*Filobasidiella neoformans*	http://www.broad.mit.edu/annotation/genome/cryptococcus_neoformans/Home.html	Basidiomycota	*Tremellales*
Fusarium graminearum	*Gibberella zeae*	http://www.broad.mit.edu/annotation/genome/fusarium_group/MultiHome.html	Ascomycota	*Hypocreales*
Fusarium oxysporum		http://www.broad.mit.edu/annotation/genome/fusarium_group/MultiHome.html	Ascomycota	*Hypocreales*
Fusarium verticillioides	*Gibberella moniliformis*	http://www.broad.mit.edu/annotation/genome/fusarium_group/MultiHome.html	Ascomycota	*Hypocreales*
Histoplasma capsulatum	*Ajellomyces capsulatus*	http://www.broad.mit.edu/annotation/genome/histoplasma_capsulatum/Home.html	Ascomycota	*Onygenales*
Laccaria bicolor		http://genome.jgi-psf.org/Lacbi1/Lacbi1.home.html	Basidiomycota	*Agaricales*
Mucor circinelloides		http://genome.jgi-psf.org/Mucci1/Mucci1.home.html	Mucormycotina	*Mucorales*
Nectria haematococca		http://genome.jgi-psf.org/Necha1/Necha1.home.html	Ascomycota	*Hypocreales*
Neurospora crassa		http://www.broad.mit.edu/annotation/genome/neurospora/Home.html	Ascomycota	*Sordariales*
Paracercospora fijiensis var. *difformis*	*Mycosphaerella fijiensis*	http://genome.jgi-psf.org/Mycfi1/Mycfi1.home.html	Ascomycota	*Capnodiales*
Penicillium chrysogenum		http://www.ncbi.nlm.nih.gov/genomeprj	Ascomycota	*Eurotiales*
Penicillium marneffei		http://www.ncbi.nlm.nih.gov/genomeprj	Ascomycota	*Eurotiales*
Talaromyces stipitatus		http://www.ncbi.nlm.nih.gov/genomeprj	Ascomycota	*Eurotiales*
Phycomyces blakesleeanus		http://genome.jgi-psf.org/Phybl1/Phybl1.home.html	Mucormycotina	*Mucorales*
Podospora anserina		http://podospora.igmors.u-psud.fr/	Ascomycota	*Sordariales*
Postia placenta		http://genome.jgi-psf.org/Pospl1/Pospl1.home.html	Basidiomycota	*Polyporales*
Pyricularia oryzae	*Magnaporthe oryzae*	http://www.broad.mit.edu/annotation/genome/magnaporthe_grisea/Home.html	Ascomycota	*Magnaporthales*
Rhizopus oryzae		http://www.broad.mit.edu/annotation/genome/rhizopus_oryzae/Home.html	Mucormycotina	*Mucorales*
Schizosaccharomyces japonicus		http://www.broad.mit.edu/annotation/genome/schizosaccharomyces_japonicus	Ascomycota	*Schizosaccharomycetales*

SUPPLEMENTARY INFORMATION

Table 2. (Continued).

Species anamorph	Species teleomorph	website	(sub)phylum	order
Septoria tritici	*Mycosphaerella graminicola*	http://genome.jgi-psf.org/Mycgr1/Mycgr1.home.html	Ascomycota	*Capnodiales*
Sporotrichum pruinosum	*Phanerochaete chrysosporium*	http://genome.jgi-psf.org/Phchr1/Phchr1.home.html	Basidiomycota	*Corticiales*
Trichoderma reesei	*Hypocrea jecorina*	http://genome.jgi-psf.org/Trire2/Trire2.home.html	Ascomycota	*Hypocreales*
Trichoderma virens	*Hypocrea virens*	http://genome.jgi-psf.org/Trive1/Trive1.home.html	Ascomycota	*Hypocreales*
Uncinocarpus reesii		http://www.broad.mit.edu/annotation/genome/uncinocarpus_reesii/Home.html	Ascomycota	*Onygenales*
Ustilago maydis		http://www.broad.mit.edu/annotation/genome/ustilago_maydis/Home.html	Basidiomycota	*Ustilaginales*
Verticillium albo-atrum		http://www.broadinstitute.org/annotation/genome/verticillium_dahliae/MultiHome.html	Ascomycota	*Hypocreales*

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Fig. 1. Maximum Parsimony bootstrap tree (1000 bootstraps) of pentose catabolic pathway genes. XDH = xylitol dehydrogenase, LAD = L-arabitol dehydrogenase, XKI = D-xylulose kinase, XYR = D-xylose reductase.

SUPPLEMENTARY INFORMATION

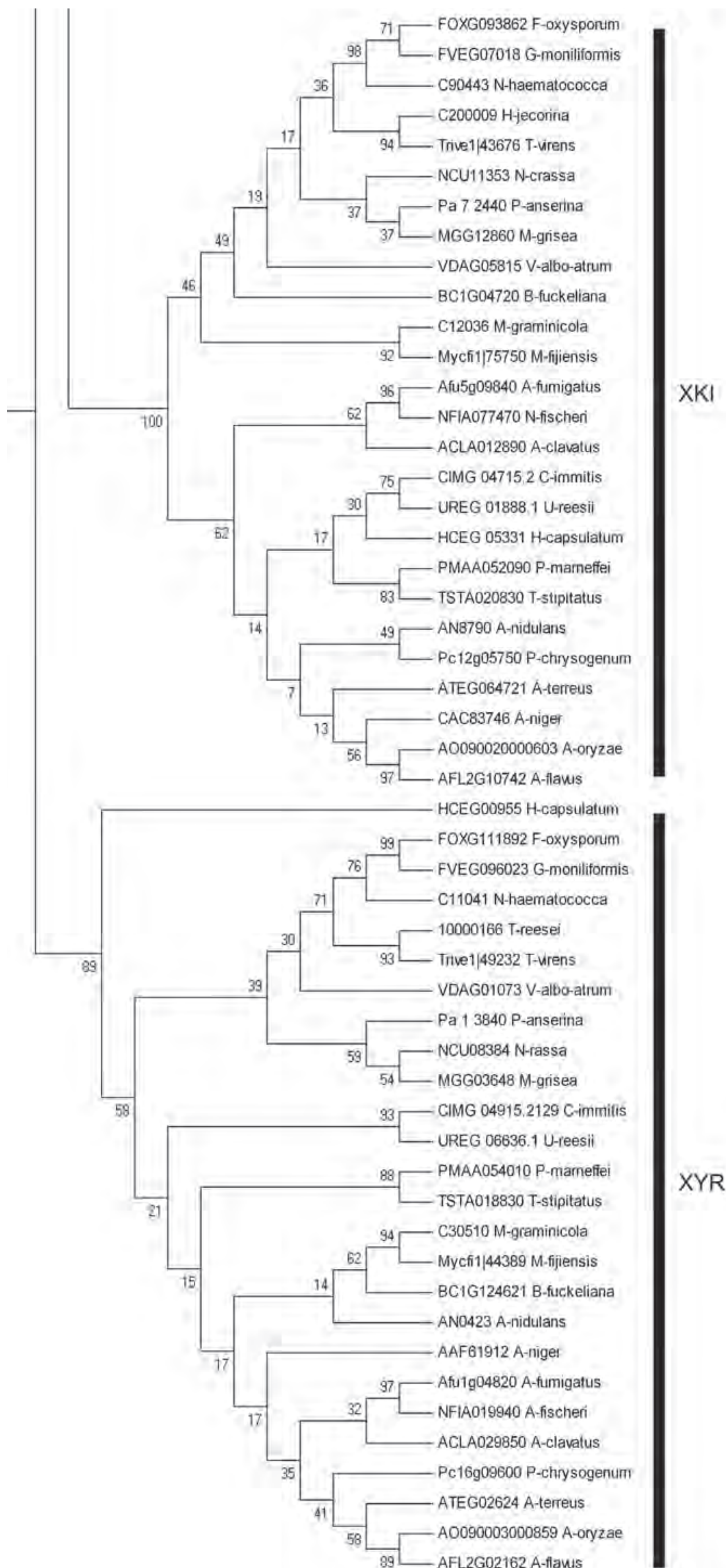

Fig. 1. (Continued).