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New ochratoxin A or sclerotium producing species in *Aspergillus* section *Nigri*

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Abstract: *Aspergillus* section *Nigri* includes some of the most important species for biotechnology and its species are of widespread occurrence. During our surveys of various food products and tropical soil we isolated several aspergilli belonging to section *Nigri*. In this paper, four new sclerotium and/or ochratoxin A producing species belonging to this section are proposed. In addition, based on a polyphasic approach using traditional characters, extrolites and β-tubulin sequencing, a provisional revision and synoptic key of section *Nigri* is proposed. *Aspergillus costaricaensis* was isolated from soil in Costa Rica and produces large pink to greyish brown sclerotia. *Aspergillus lacticoffeatus* was found on coffee beans in Venezuela and Indonesia, and is an effective producer of ochratoxin A. *Aspergillus piperis* was isolated from black ground pepper and produces large yellow to pink brown sclerotia. *Aspergillus sclerotioniger* was isolated from a green coffee bean and produces large yellow to red brown sclerotia and abundant ochratoxin A. The species *A. homomorphus* is validated. The ochratoxin A producing black aspergilli are revised. Fifteen species are provisionally accepted in *Aspergillus* section *Nigri*, four of these produce ochratoxin A. Ochratoxin A producing species of section *Nigri* occurring on grapes, raisins and in wine include *A. carbonarius* and to a lesser extent *A. niger*. Four species recovered from coffee, viz. *A. carbonarius*, *A. niger*, *A. lacticoffeatus* and *A. sclerotioniger*, all produce ochratoxin A, but other species of *Nigri* also occur on this substrate, including *A. japonicus* and *A. tubingensis*. The 10 species not producing ochratoxin A are especially interesting for biotechnological exploration, as many other extrolites are produced by these species.

Taxonomic novelties: *Aspergillus costaricaensis* Samson & Frisvad sp. nov., *Aspergillus homomorphus* Frisvad & Samson sp. nov., *Aspergillus lacticoffeatus* Samson & Frisvad sp. nov., *Aspergillus piperis* Samson & Frisvad sp. nov., *Aspergillus sclerotioniger* Steiman, Guiraud, Sage & Seigle-Mur. ex Samson & Frisvad sp. nov.
Key words: *Aspergillus niger,* black aspergilli, ochratoxin A, pyranonigrin, sclerotia.

INTRODUCTION

The black aspergilli are among the most common fungi causing food spoilage and biodeterioration of other materials. They have also been extensively used for various biotechnological purposes, including production of enzymes and organic acids (Schuster *et al.* 2002). The taxonomy of *Aspergillus* section *Nigri* has been studied by many taxonomists and was recently reviewed by Abarca *et al.* (2004). Mosseray (1934) described 35 species black aspergilli, while Raper and Fennell (1965) reduced the number of species accepted within their *A. niger* group to 12. Al-Musallam (1980) revised the taxonomy of the *A. niger* group, primarily based on morphological features. She recognized seven species (*A. japonicus*, *A. carbonarius*, *A. ellipticus*, *A. helicothrix*, *A. heteromorphus*, *A. foetidus*, *A. niger*), and described *A. niger* as an aggregate consisting of seven varieties and two *formae*. Kozakiewicz (1989) distinguished *A. ellipticus*, *A. heteromorphus*, *A. japonicus*, *A. helicothrix*, *A. atroviolaceus* and *A. carbonarius* as species exhibiting echinulate conidial ornamentations distinct from the remaining black *Aspergillus* taxa, which produce verrucose conidia. Within the verrucose category, *A. fonsecaeus*, *A. acidus, A. niger* var. *niger*, *A. niger* var. *phoenicis*, *A. niger* var. *ficuum*, *A. niger* var. *tubingensis*, *A. niger* var. *pulverulentus*, *A. niger* var. *awamori*, *A. citricus* (*A. foetidus*) and *A. citricus* var. *pallidus* were recognized.

Aspergillus niger is the most frequently reported species in this section, and has often been included in biotechnological processes that are Generally Regarded as Safe (GRAS). However, species concepts are uncertain in this complex and occasionally the name *A. niger* has been used for any member of the section. Taxonomic studies using molecular methods have divided the *A. niger* complex into two species, *A. niger* and *A. tubingensis* (for overview see Abarca *et al.* 2004). Some further species have been described but not considered in revisions or reviews. *Aspergillus ellipsoideus* was described as a new species with ellipsoidal greyish black conidia (Rai & Chowdhery 1979). *Aspergillus homomorphus* and *A. pseudohet-*

eromorphus were invalidly described (no designated type, International Code of Botanical Nomenclature Art. 37) (Steiman *et al.* 1994; see Mouchacca 1999). Recently, a new species *A. vadensis,* with a different extrolite profile, colony characters and unusually low citric acid production, was proposed (de Vries *et al.* 2004a, b).

Ueno *et al.* (1991) were the first to report on ochratoxin A (OA) production by a black *Aspergillus* species, *A. foetidus*. This was later confirmed by Téren *et al.* (1996) and Magnoli *et al.* (2003). Abarca *et al.* (1994) reported that two strains of *A. niger* produced OA, which was confirmed in numerous studies (Ono *et al.* 1995, Téren *et al.* 1996, 1997, Nakajima *et al*, 1997, Heenan *et al.* 1998, Accensi *et al.* 2001, Urbano *et al.* 2001, Dalcero *et al.* 2002, Da Rocha *et al.* 2002, Abarca *et al.* 2003, Magnoli *et al.* 2003, Taniwaki *et al.* 2003, Suárez-Quiroz *et al.* 2004). Horie (1995) reported OA in *A. carbonarius*, which was confirmed by Wicklow *et al.* (1996), Téren *et al.* (1996), Heenan *et al.* (1998), Varga *et al.* (2000), Joosten *et al.* (2001), Da Rocha *et al.* (2002), Cabanes *et al.* (2002), Sage *et al.* (2002), Abarca *et al.* (2003), Battilani *et al.* (2003), Taniwaki *et al.* (2003), Bellí *et al.* (2004) and Sage *et al.* (2004). Varga *et al.* (2000) tested about 160 black *Aspergillus* strains from collections and from field isolates for OA production using an immunochemical method and thin layer chromatography. The strains examined included 12 *A. carbonarius* and 45 *A. japonicus* strains from culture collections and field isolates from all over the world, including about 100 strains belonging to the *A. niger* species complex.

Ochratoxin A production was detected in about 6 % of the strains from the *A. niger* species complex (Abarca *et al.* 1994, Téren *et al.* 1996). Of the 13 *A. carbonarius* strains tested, six produced both OA and ochratoxins B (Fig. 8, Téren *et al.* 1996, Wicklow *et al.* 1996). *Aspergillus ellipticus*, *A. heteromorphus*, *A. japonicus* and *A. tubingensis* strains did not produce detectable amounts of ochratoxins. However, *A. japonicus* was later claimed to produce OA (Dalcero *et al.* 2002, Battilani *et al.* 2003).

During our surveys of coffee, black pepper and soil, several isolates of black aspergilli were recovered. The purpose of this paper is to describe four new species from section *Nigri*, distinguished from previously known species by large sclerotia or unusual conidial colours. Furthermore we wanted to suggest a provisional revision of this industrially important section of *Aspergillus* based on a relatively small number of typical strains of each taxon.

MATERIALS AND METHODS

The methods and media for isolation and identification followed the procedures of Samson *et al.* (2004). The names of colours are based on Kornerup & Wanscher (1978). The cultures used for the molecular study were grown in 2 mL malt peptone (MP) broth using 10 % (v/v) of malt extract (Brix 10) and 0.1 % (w/v) bacto peptone (Difco) in 15 mL polystyrene centrifuge tubes. The cultures were incubated at 25 °C without agitation for 7 d in light/darkness. The strains selected included 1 to 8 representatives of the major taxa accepted by Al-Musallam (1980), Kozakiewicz (1989) and Abarca *et al*. (2004) (see Table 1) in addition to the new taxa described here and in de Vries *et al*. (2004b).

Extrolite analysis

Extrolites (includes secondary metabolites; for definition see Samson & Frisvad 2004) were 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). Standards of ochratoxin A and B, aflavinine, asperazine, austdiol, kotanin and other extrolites from the collection at Biocentrum-DTU were used to compare with the extrolites from the species under study. Pyranonigrin A was identified by comparison with literature UV and MS data (Hiort 2003, Hiort *et al*. 2004)

DNA Extraction, sequencing and analysis

The total fungal genomic DNA was isolated using UltracleanTM Microbial DNA Isolation Kit (MoBio, Solana Beach, U.S.A.) according to the manufacturer's instructions. Amplification of β-tubulin gene was mostly performed using the primers Bt2a and Bt2b. Some strains in this study Bt-T10 (5'ACG ATA GGT TCA CCT CCA GAC 3') an Bt2b (Glass 1995). PCR was performed in a 25 µL reaction mixture containing 1 µL of genomic DNA (10 ng/µL), 0.75 µL of $MgCl_2$ (50mM provided with BioTaq), 2.5 µL Buffer with 10× NH_4 (provided with BioTaq), 17.8 µL of ultra pure sterile water, 1.85 µL dNTP (1 mM), 0.50 µL of each primer (10 pmol/µL) and 0.1 µL BioTaq polymerase (5 U/µL, BiotaqTM DNA Polymerase, Bioline Randolph, U.S.A.). Amplification was performed in a GeneAmp PCR system 9700 (AB Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands); programmed for 5 min 94 °C followed by 35 cycles of 1 min denaturation at 94 °C followed by primer annealing 1 at 55 °C and primer extension 1 min. at 72 °C and a final 7 min elongation step at 72 °C. After amplification of the β-tubulin gene, excess primers and dNTP's were removed from the reaction mixture using a commercial GFX column, PCR DNA Purification kit (Amersham Bioscience, Roosendaal, The Netherlands). The purified PCR fragments were resuspended in 50 µL of TE buffer. The PCR fragments were directly sequenced in both directions with the primers Bt2a or BtT10 and Bt2b using a

Table 1. Cultures examined.

Taxon name	Strain number(s)	Substratum and origin	GenBank accession no.
A. "aculeatus"	CBS 620.78 = NRRL 2053	Unknown	AY 585538
A. "aculeatus"	CBS 114.80	Soil, India	AY 585539
A. "*brasiliensis*"	CBS 101740 = IMI 381727	Soil, Brazil	AY 820006
A. aculeatus	CBS 119.49	Unknown substratum, Indonesia	AY 585541
	CBS 172.66 = ATCC 16872 = IMI 211388 = WB 5094 **T**	Tropical soil, unknown origin	AY 585540
A. carbonarius	CBS 116.49	Unknown	AY 819997
	CBS 101697 = IBT 21854	External finga-coffee bean, Kenya	AY 819994
	CBS 126.49 = ATCC 10698 = IFO 6648 = NRRL 363 (received as *A. phoenicis*)	Unknown	AY 819995
	CBS 111.26 = ATCC 1025; = ATHUM 2854 = CBS 556.65 = IMI 016136 = IMI 016136ii = LSHB Ac11 = MUCL 13583 = NCTC 1325 = NRRL 369 = NRRL 1987 = QM 331 = WB 369 **NT**	Paper, unknown origin	AY 585532
A. costaricaensis	CBS 115574 = CBS 23401 **T**	Soil in Gaugin Garden on Taboga Island, Costa Rica	AY 820014
A. ellipticus	CBS 677.79 = IMI 278383 (Type of *A. helicothrix*)	Sector in colony of *Aspergillus ellipticus*, CBS 482.65, Costa Rica	AY 819993
A. ellipticus	CBS 707.79 = IMI 278384 **T**	Soil, Costa Rica	AY 585530
A. flavus (outgroup)	CBS 100927 = ATCC 16883 = CBS 569.65 = IMI 124930 = LCP 89.2565 = WB 1957	Cellophane, South Pacific Islands	AY 819992
A. foetidus	CBS 564.65 =ATCC 16874 = IFO 4121 = IMI 104688 = IMI 104688ii = WB 4796 (Type of *A. foetidus* var. *acidus*)	Unknown substratum, Japan	AY 585533
	CBS 565.65 = ATCC 16884 = IFO 4123 = IMI 175963 = WB 4797 (Type of *A. foetidus* var. *pallidus*)	Unknown substratum, Japan	AY 585534
A. heteromorphus	CBS 117.55 = ATCC 12064 = IMI 172288 = QM 6954 = WB 4747 **T**	Culture contaminant of *Trichophyton* culture, Brazil	AY 585529
A. homomorphus	CBS 101889 **T**	Soil of death sea area, Israel	AY 820015
A. japonicus	CBS 115.80 = IFO 5330 (Type of *A. yezoensis*)	Unknown	AY 820017
	CBS 611.78 = NRRL 5118	Tropical soil, unknown origin	AY 585544
	CBS 113.48 = IMI 312983 = IMI 016135ii = LSHB Ac44 = MUCL 13578 = NCTC 3792 = NRRL 4839 = WB 4839 (Type of *A. atro-violaceus*)	Unknown	AY 585531
	CBS 568.65 = ATCC 16873 = IMI 211387 = NRRL 1782 = WB 1782	Soil, Panama	AY 820018
	CBS 101.14 = IFO 4030 (received as *A. atropurpureus)*	Unknown	AY 585543
	CBS 522.89	Air, the Netherlands	AY 820019
	CBS 114.51 **T**	Unknown	AY 585542
A. lacticoffeatus	CBS 101884	Beans of *Coffea arabica*, Venezuela, Rubio district	AY 819999
	CBS 101886 = IBT 22032	Soil under *Coffea robusta*, Indonesia, Sumatra	AY 820003
	CBS 101883 **T**	Surface sterilized beans *Coffea robusta*, Indonesia, South Sumatra	AY 819998
A. niger	CBS 101699 = IBT 6461	Foodstuff, unknown origin	AY 585537
	CBS 618.78 = IFO 739 = IMI 041871 = LSHB Ac72 = MUCL 28130 = NCTC 1692 = VTT D-71001 = NRRL 337 (believed to be Wehmer's isolate of *A. niger-citricus nom. nud*., as *A. foetidus*)	Unknown	AY 820004

	CBS 420.64 = ATCC 8740 = DSM 872 = IMI 041875 = MUCL 30479 = NRRL 67 = NRRL 605 = NRRL 1737 = QM 330 = WB 67 (Isotype of *A. fonsecaeus*)	Unknown	AY 820002
	CBS 101705 = IBT 18741	Carpet dust from school, Canada	AY 820005
	CBS 101698 = IBT 21853	Mesocarp finga - coffee bean, Kenya	AY 820000
	CBS 120.49 = ATCC 9029 = CECT 2088 = DSM 2466 = IMI 041876 = MUCL 30480 = NRRL 3 = NRRL 566 = VKM F-3747 = VTT D-85240 = WB 3 = WB 566 (='*A. usamii*')	Unknown substratum, U.S.A.	AY 585535
	CBS 557.65 = ATCC 16877 = IMI 211394 = IOC 230 = WB 4948 (type of *A. awamori*)	Unknown	AY 820001
	CBS 554.65 = ATCC 16888 = IFO 33023 = IHEM 3415 = IMI 050566 = IMI 050566ii = JCM 10254 = NRRL 326 = WB 326 **T**	Unknown	AY 585536
A. piperis	CBS 112811 = IBT 26239 **T**	Grounded black pepper of tropical origin, Denmark	AY 820013
A. pseudoheteromorphus	CBS 101888 **T**	Soil of death sea area, Israel	AY 820016
A. sclerotioniger	CBS 115572 = IBT 22905 **T**	Green *Arabica* coffee, India, Karnataka	AY 819996
A. tubingensis	CBS 117.32 (received as *A. ficuum*)	Unknown	AY 820012
	CBS 136.52 = ATCC 11362 = CBS 552.65 = IMI 211395 = WB 4757 (Type strain of *A. saitoi*; as *A. phoenicis*)	Kuro-koji, Japan	AY 820008
	CBS 425.65 = IAM 2170 (received as *A. pulverulentus*)	Unknown substratum, Japan	AY 820009
	CBS 126.52 = WB 4860 = IFO 4115 (received as *A. miyakoensis*, identified as *A. awamori*)	Unknown	AY 585528
	CBS 115657 = IBT 23434	Desert sand, Namibia	AY 820011
	CBS 161.79 = NRRL 4700	Unknown substratum, India	AY 585527
	CBS 134.48 = Biourge 726 = WB 4875 **T**	Unknown	AY 820007
A. vadensis **T**	CBS 113365 = IMI 313493 **T**	Dead plant tissue, unknown origin	AY 585531

T = ex-type culture.

DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Bioscience, Roosendaal, The Netherlands). The sequence PCR reaction mixture, total reaction mix is 10 µL, contained 1 µL of template DNA (10–15 ng/µL), 4 µL Dye terminator RR mix, 4 µL ultra pure sterile water and 1 µL primer Bt2a or Bt2b (4 pmol/µL). The reaction was performed in a GeneAmp PCR system 9700 run in 9600 mode (AB Applied Biosystems, Nieuwerkerk a/d Yssel, The Netherlands); programmed for 25 cycles of 10 s denaturation at 96 °C followed by primer annealing 5 s at 50 °C and primer extension 4 min at 60 °C. Sequencing products were purified according to the manufacturer's recommendations with Sephadex G-50 superfine column (Amersham Bioscience, Roosendaal, The Netherlands) in a multiscreen HV plate (Millipore, Amsterdam, The Netherlands) and with MicroAmp Optical 96-well reaction plate (AB Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). The samples were analyzed on an ABI PRISM 3700 Genetic Analyzer (AB Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). A concensus was computed from the forward and reverse sequences with software package Seqman and Editseq from the lasergene package (DNAStar Inc., Madison, WI). The alignments of the partial β-tubulin gene sequence data were performed using the software package BioNumerics from Applied Maths and manual adjustments for improvement were made by eye where necessary. The phylogenetic analyses of sequence data were done using PAUP (Phylogenetic Analysis Using Parsimony) v. 4.0b10 (Swofford 2000). Alignment gaps were treated as fifth character state, missing data were identified by '?', 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. The robustness of the most parsimonious trees was evaluated by 1000 bootstrap replications (Hillis & Bull 1993). Other measures

including tree length, consistency index, retention index and rescaled consistency index (CI, RI and RC) were also calculated.

RESULTS

All strains of the black aspergilli produced a large number of known and as yet unknown extrolites. Some of the most important extrolites are listed in Table 1. Two strains of *Aspergillus aculeatus* (CBS 172.66 and CBS 119.49) produced secalonic acid D as earlier reported for this taxon (Andersen *et al*. 1977) and in addition they both produced neoxaline. The latter metabolite was first reported from *A. japonicus* (Hirano *et al*. 1979, Konda *et al*. 1980), but we only found neoxaline in *A. aculeatus* in this study. Two other strains identified as *A. aculeatus* were quite dissimilar to the two typical strains above: CBS 620.78 produced secalonic acid D and some indole compounds, while CBS 114.80 produced the same indole compounds and okaramin H and I. Those okaramins have earlier been reported from *A. aculeatus* (Hayashi *et al*. 1999). *A. brasiliensis* CBS 101740 produced some naphtho-γ-pyrones including aurasperone B (Tanaka *et al*. 1972) and a series of compounds that have not been structure elucidated yet.

All four strains of *A. carbonarius* produced pyranonigrin A (earlier reported from *A. niger*, Hiort *et al*. 2004), ochratoxin A and naphtho-γ-pyrones. *Aspergillus costaricaensis* produced trace amounts of aurasperone B, pyranonigrin A, 14-epi-14-hydroxy-10,23-dihydro-24,25-dehydroaflavinine, 10,23-dihydro-24,25-dehydroflavinine (those aflavinines were found earlier in *A. tubingensis* CBS 161.79 = NRRL 4700, TePaske *et al*. 1989a), a funalenone-like compound (see Inokoshi *et al*. 1999) or a corymbiferan lactone-like compound (see Overy & Blunt 2004). *A. ellipticus* CBS 677.79 and CBS 707.79 both produced austdiol earlier reported from *Aspergillus ustus* (Vleggaar *et al*. 1974). *Aspergillus foetidus* CBS 564.65 and CBS 565.65 both produced asperazine, earlier erroneously reported from *A. niger* (Varoglou *et al*. 1997). The strain examined by Varoglou *et al*. was actually an *A. tubingensis* (unpublished results, J.C. Frisvad). Furthermore CBS 564.65 and CBS 565.65 produced antafumicin (Fujimoto *et al*. 1993). Both strains also produced naphtho-γ-pyrones and pyranonigrin A. *Aspergillus heteromorphus* CBS 117.55 produced several as yet unknown extrolites, including some indol-alkaloids. *Aspergillus homomorphus* CBS 101889 and *A. pseudoheteromorphus* had identical profiles of extrolites, including secalonic acid D. *Aspergillus japonicus* CBS 101.14, CBS 114.51 and CBS 522.89 did not produce any known extrolites. *Aspergillus lacticoffeatus* CBS 101886, CBS 101883, CBS 101884 and CBS 101885 all produced ochratoxin A, pyranonigrin A, orlandin (see Cutler *et al*. 1979), kotanin and desmethylkotanin. All eight strains of *A. niger* investigated produced pyranonigrin A and naphtho-γ-pyrones. CBS 101705, CBS 101698 and CBS 554.65 produced orlandin, kotanin and desmethylkotanin and CBS 618.78, CBS 420.64, CBS 101705, and CBS 101698 produced ochratoxin A and B. *A. piperis* CBS 112811 produced aurasperone B, 14-epi-14-hydroxy-10,23-dihydro-24,25-dehydroaflavinine, and 10,23-dihydro-24,25-dehydroaflavinine. *Aspergillus sclerotioniger* CBS 115572 produced pyranonigrin A, naphtho-γ-pyrones, ochratoxin A and B, and compounds related to funalenone or corymbiferan-lactones. All eight strains of *A. tubingensis* produced asperazine, except CBS 161.79. The latter strain produced tubingensin A and B (TePaske *et al*. 1989b, c), dihydrotubingensin A and B (Sings *et al*. 2001) and 14-epi-14-hydroxy-10,23-dihydro-24,25-dehydroaflavinine, 10,23-dihydro-24,25-dehydroaflavinine and 10,23-dihydro-24,25-dehydro-21-oxo-aflavinine (TePaske *et al*. 1989a) indicating a difference between CBS 161.79 and other strains of *A. tubingensis*. All eight strains of *A. tubingensis* (Table 1) also produced pyranonigrin A and naphtho-γ-pyrones. *Aspergillus vadensis* CBS 113365 produced nigragillin, asperazine, aurasperone B (a naphtho-γ-pyrone) and a polar orlandin-like compound.

Among the isolates listed in Table 1, four species were able to produce OA. Ochratoxin A was consistently produced by *A. carbonarius* strains, in agreement with most other studies on this species (Abarca *et al.* 2004). Ochratoxin A was only produced by some strains of *A. niger sensu stricto*, also in agreement with numerous studies (Abarca *et al.* 2004). The other producers of OA were the new species that are described below, namely *A. lacticoffeatus* and *A. sclerotioniger*. Both of these new species were isolated from coffee. On the other hand OA production by *A. japonicus* (Dalcero *et al.* 2002, Battilani *et al.* 2003) was not confirmed. Similarly, no strains of *A. foetidus sensu stricto* produced OA. The strain CBS 618.78 has been identified by different authors as *A. foetidus*, *A. foetidus* var. *citricus* or *A. citricus* and produced OA (Téren *et al.* 1996). It was listed among isolates of *A. foetidus* by Raper & Fennell (1965). Ochratoxin A production by this strain was confirmed here, but this strain has been shown to be *A. niger* and not *A. foetidus* (Kusters-van Someren *et al*. 1991, Parenicová *et al*. 1997, Accensi *et al*. 1999).

Sclerotium production was not necessarily correlated with OA production. It was suggested by Wicklow *et al*. (1996), that ochratoxin A was associated with sclerotium production of *A. carbonarius*. *Aspergillus carbonarius* occasionally produced sclerotia and OA, but non-sclerotial strains of *A. carbonarius* could also produce ochratoxin A. *A. tubingensis* occasionally produces sclerotia but never produces ochratoxin

A. No strains of *A. niger* have been found to produce sclerotia yet, and other sclerotium producers, such as *A. ellipticus*, *A. aculeatus*, *A. costaricaensis*, and *A. piperis* also did not produce OA. *Aspergillus sclerotioniger*, however, produced abundant sclerotia and OA.

Maximum parsimony analysis of the sequence data was restricted to 5000 equally most parsimonious trees (TL = 719 steps, CI= 0.701 RI = 0.898, RC = 0.630), one of which is shown in Fig. 1. The tree was rooted using *A. flavus*. This species was chosen after examining the results of Peterson (2000). The bootstrap support, based on fast stepwise addition, from 1000 replicates is shown at the nodes. The cladogram indicates that there are five major clades in section *Nigri*. The first clade contains *A. heteromorphus* and *A. ellipticus*, but these two species are clearly very distantly related. The next clade contains *A. carbonarius* and *A. sclerotioniger* and this clade is a sister clade to a major clade containing species usually included in the *A. niger* complex (Al-Musallam 1979), but it also includes the two new species *A. piperis* and *A. costaricaensis*. This major clade includes two subclades, one with *A. niger* and *A. lacticoffeatus*, and one with *A. vadensis*, *A. tubingensis*, *A. foetidus*, *A. piperis* and *A. costaricaensis*. The next clade includes *A. homomorphus* and the closely related *A. pseudoheteromorphus* and two strains identified as *A. aculeatus*. This is the only clade containing both uniseriate and biseriate aspergilli. The last clade includes the two common uniseriate species *A. aculeatus sensu stricto* and *A. japonicus*. This analysis, along with the phenotypic data, supports our recognition of the newly described species. We also conclude that CBS 101740 ("*A. brasiliensis"*) represents a new species distinct from all the other species of *Aspergillus,* and this is supported by 70 % majority-rule consensus analyses but with a low bootstrap value (51 %). This species is represented by several isolates (Varga *et al.* 2000) and will be described elsewhere. *Aspergillus piperis* has similar sequences to *A. foetidus* and differs only by six base pair changes; however it is supported by consensus and bootstrap (85 %). *Aspergillus costaricaensis* is a new species supported by consensus but with a poor bootstrap value (53 %). *Aspergillus sclerotioniger* is a new species supported by consensus and bootstrap (100 %). All the three strains of *A. lacticoffeatus* have identical sequences to the eight strains of *A. niger* studied. The strains of *A. lacticoffeatus* have strikingly different in colony colour and morphology and also have a different extrolite pattern. Multilocus DNA sequences might reveal genetic differences between *A. lacticoffeatus* and *A. niger*. CBS 101888 and 101889, the ex-type strains of *Aspergillus homomorphus* and *A. heteromorphus*, had identical sequences.

DISCUSSION

Approximately 108 taxa (species, subspecies and varieties) have been described in *Aspergillus* section *Nigri* (Mosseray 1934, Raper & Fennell 1965, Samson 1979, 1992, Al-Musallam 1980, Kozakiewicz 1989). Of these, we provisionally accept 15 taxonomic entities, including the four new species described here. The reason for the multiplicity of proposed names may be that isolates of section *Nigri* are readily isolated world-wide but are difficult to distinguish.

Often very small differences in texture or conidial colour have been used as the basis for distinguishing new taxa. Phenotypic comparisons of a broad collection of black aspergilli showed that 15 taxa can be distinguished. Most of these species could be distinguished by combinations of colony and micromorphological characters and extrolite profiles, including the new species described below in addition to *A. ellipticus*, *A. carbonarius*, *A. japonicus*, *A. vadensis*, and *A. heteromorphus*. However, *A. niger, A. tubingensis* and *A. foetidus* remain difficult to differentiate using phenotypic methods. These taxa can be differentiated by DNA sequences of the cytochrome b gene (Yokoyama *et al.* 2001), ITS (Parenicová *et al*. 2001) and β-tubulin (De Vries *et al*. 2004b) and by RFLP and other fingerprinting methods (Abarca *et al.* 2004). No phenotypic methods have yet been found that can distinguish between *A. niger*, *A. foetidus* and *A. tubingensis*, except that they can be differentiated based on production of the extrolites asperazine, antafumicins and ochratoxin A and/or orlandins (Table 2). However old deteriorated strains sometimes do not produce any of these compounds and then molecular methods would be necessary to distinguish them.

The β-tubulin nucleotide sequence cladogram (Fig. 1) is divided into four clades with no obvious sister group relationships, thus it is not possible to infer any deeper phylogenetic relationships between these groups. Within the four clades the phylogenetic structure is more resolved. In the first clade, two very unique species, *A. heteromorphus* and *A. ellipticus*, appear to be distantly related. The discovery of more taxa in section *Nigri* or the use of more than one gene for constructing the cladogram may help resolve this relationship. *Aspergillus heteromorphus* and *A. ellipticus* are also phenotypically very different. The next major clade consists of *A. carbonarius*, the *A. niger* complex and the four new species. Most of these species produce pyranonigrin A and naphtha-γ-pyrones.

Table 2. Production of sclerotia, ochratoxin A and other extrolites by species in *Aspergillus* section *Nigri.*

Species	Ochratoxin A	Sclerotia	Pyranonigrin	N-γ-P[1]	Asp[2]	SeD[3]	Ant[4]	Afl[5]	Cor[6]	Kot[7]
A. aculeatus	–	+/–	–	–	–	+	–	–	–	–
A. brasiliensis	–	–	–	+	–	–	–	–	–	–
A. carbonarius	+	+/–	+	+	–	–	–	–	–	–
A. costaricaensis	–	+	–	+	–	–	–	+	+	–
A. ellipticus	–	+	–	–	–	–	–	–	–	–
A. foetidus	–	–	+	+	+	–	+	–	–	–
A. heteromorphus	–	–	–	–	–	–	–	–	–	–
A. homomorphus[8]	–	–	–	–	–	+	–	–	–	–
A. japonicus	–	–	–	–	–	–	–	–	–	–
A. lacticoffeatus	+	–	+	–	–	–	–	–	–	+
A. niger	+/–	–	+	+	–	–	–	–	–	+/–
A. piperis	–	+	+	+	–	–	–	+	–	–
A. sclerotioniger	+	+	+	+	–	–	–	–	+	–
A. tubingensis	–	+/–	+	+	+	–	–	–	–	–
A. vadensis	–	-	–	+	+	–	–	–	–	–

[1]N-γ-P: Naphtho-γ-pyrones; [2]Asp = asperazine; [3]SeD = secalonic acid D; [4]Ant = antafumicin; [5]Afl = aflavinines; [6]Cor = Corymbiferan lactones; [7]Kot = Kotanins (kotanin, desmethylkotanin, orlandin); [8]*A. pseudoheteromorphus* was not different from *A. homomorphus*, but none of the species have been validly described.

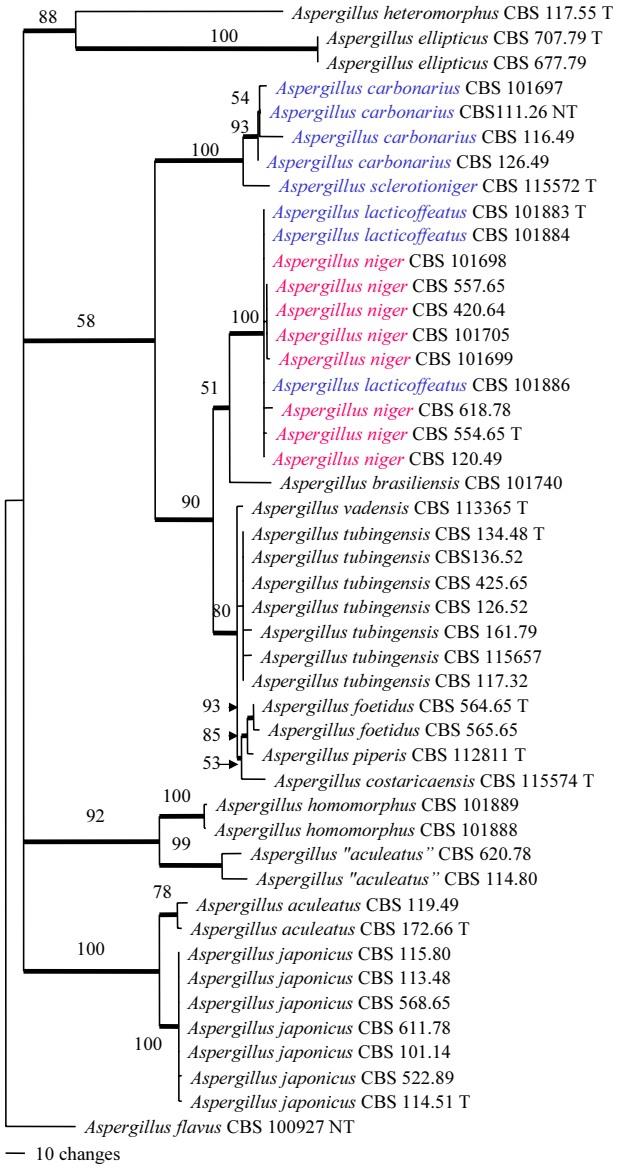

Fig. 1. One of the 5000 equally MPT of 719 steps based on heuristic search partial β-tubulin sequences with *A. flavus* as an outgroup. The branches in bold are 100 % in the 70 % majority-rule consensus of equally parsimonious trees. The numbers represent bootstrap percentages > 50 % (CI = 0.701, RI = 0.898 RC = 0.630, HI = 0.299). Names in blue are ochratoxin producing taxa. Taxa in red contain isolates which can produce ochratoxin.

The first subclade, sister group to the *A. niger* complex, consists of species with large conidia and ability to produce sclerotia and ochratoxin A: *A. carbonarius* and *A. sclerotioniger*. The two latter species also share the slow groth at 37 ºC. These species share the ochratoxin A production with *A. niger* and *A. lacticoffeatus* in the next subclade and the ability to produce sclerotia with *A. tubingensis*, *A. foetidus*, *A. piperis* and *A. costaricaensis* in the last subclade. Sclerotium production in *A. tubingensis* and *A. foetidus* is rare, however. In the next subclade *A. niger sensu stricto* and *A. lacticoffeatus* cannot be separated based on their β-tubulin sequences. In agreement with this, they share the ability to produce OA, pyranonigrin, kotanins and in not having the ability to produce sclerotia. Again, there are several differences, including lack of naphtha-γ-pyrones in *A. lacticoffeatus*, the sulphur yellow mycelium on YES agar and the smooth to finely roughened light brown to dark blonde conidia of *A. lacticoffeatus*. The third subclade consists of *A. vadensis*, *A. tubingensis, A. foetidus, A. piperis* and *A. costaricaensis*. The three first species are united by production of naphtho-γ-pyrones and asperazine, while the latter two species produce naphtho-γ-pyrones and aflavinins. The large central clade consisting of *A. carbonarius, A. sclerotioniger, A. niger, A. lacticoffeatus, A. brasiliensis, A. vadensis, A. tubingensis, A. foetidus, A. piperis* and *A. costaricaensis* appears to be monophyletic and all species share the ability to produce naphtho-γ-pyrones and pyranonigrin A, except that the naphtho-γ-pyrones has been lost in *A. lacticoffeatus* and pyranonigrin A has been lost in *A. vadensis*. The last two clades include uniseriate species and the biseriate *A. homomorphus*. On the other hand all isolates of *A. aculeatus* and *A. homomorphus* share the production of secalonic acid D, not found in any other black *Aspergillus* species. Unexpectedly the distinction between uniseriate and biseriate species is only partly supported by nucleotide sequence data. Originally the two types of black aspergilli were distinctly separated (Peterson 2000, Varga *et al.* 2000, Parenicová *et al.* 2001). The separation of *A. aculeatus sensu lato* into two separate clades indicate that more than one species may exist. The group of uniseriate black aspergilli should be further examined before taxonomic conclusions for that group are drawn. However, our examination of the type isolates of *Aspergillus homomorphus* and *A. pseudoheteromorphus* show that these species are identical. Both species were invalidly described and below we validate the species and name it *A. homomorphus*.

In combination with the phenotypc and extrolite characters the β-tubulin sequences revealed the distinction of the 15 taxa incluing four new species. However a multigen sequence approach will be necessary to get a better insight in the species complexes of *A. niger/tubingensis* and *A. aculeatus/A. japonicus*.

Taxonomy

Aspergillus costaricaensis Samson & Frisvad **sp. nov.** MycoBank MB500007.

Aspergillo nigro similis, capitulis biseriatis, sed sclerotiis roseis vel grisello-luteis et vesiculis metulisque majoribus differens. Typus CBS H-13437

Type: CBS 115574 = IBT 23401, ex soil in Gaugin Garden on Taboga Island, **Costa Rica**, Martha Christensen, Nov. 2000.

Colony diameters at 7 d 25 °C, in mm: CYA: 63–78 mm, MEA 26–62 mm, YES: 77–80 mm, OAT: 41–56 mm, CREA: 38–50 mm, thin colonies with poor sporulation, strong acid production, CYA at 37 ºC: 58–62 mm. *Colony colours and texture*. On CYA25 and MEA only a few conidiophores are produced, conidial areas are black; mycelium white, inconspicuous; sclerotia abundantly present, large (1.2–1.8 mm), subglobose to ellipsoidal, pink to grayish yellow. Reverse on CYA pale yellow, on MEA medium–yellow. Conidial heads radiate, splitting into 5–8 defined columns, stipes (800–)1000–1700(–1900) × (12–)13–20(–22) µm, walls thick, smooth, hyaline; vesicles large (40–)45–70(–90) µm wide, globose; biseriate; metulae covering entire vesicle, measuring 30–60 × 3–4 (at base) to 8–11 µm (at top); phialides 7–9.5 × 3–5 µm; conidia globose to subglobose, (3.1–)3.5–4.3(–4.5), smooth when young, becoming distinct rough walled, dark brown.

Extrolites: Aurasperone B and pyranonigrin A, 14-epi-14-hydroxy-10,23-dihydro-24,25-dehydro-aflavinine, and 10,23-dihydro-24,25-dehydro-aflavi-nine, funalenone-like compound similar to the corymbiferan lactones (see *A. sclerotioniger*).

Distinguishing features: This species is characterised by its pink to greyish yellow sclerotia and large vesicles and metulae.

Aspergillus lacticoffeatus Frisvad & Samson **sp. nov.** MycoBank MB500008.

Aspergillo nigro similis, capitulis biseriatis, sed coloniis dilute brunneis et vesiculis metulisque majoribus et conidiis asperellis differens. Typus CBS H-13436

Type: CBS 101883 = IBT 22031 ex surface disinfected green robusta coffee bean in coffee farm, Labu Kompong of Ngarip Village, Ulu Belu territory, Lampung highlands of southern Sumatra, **Indonesia**, J.M. Frank.

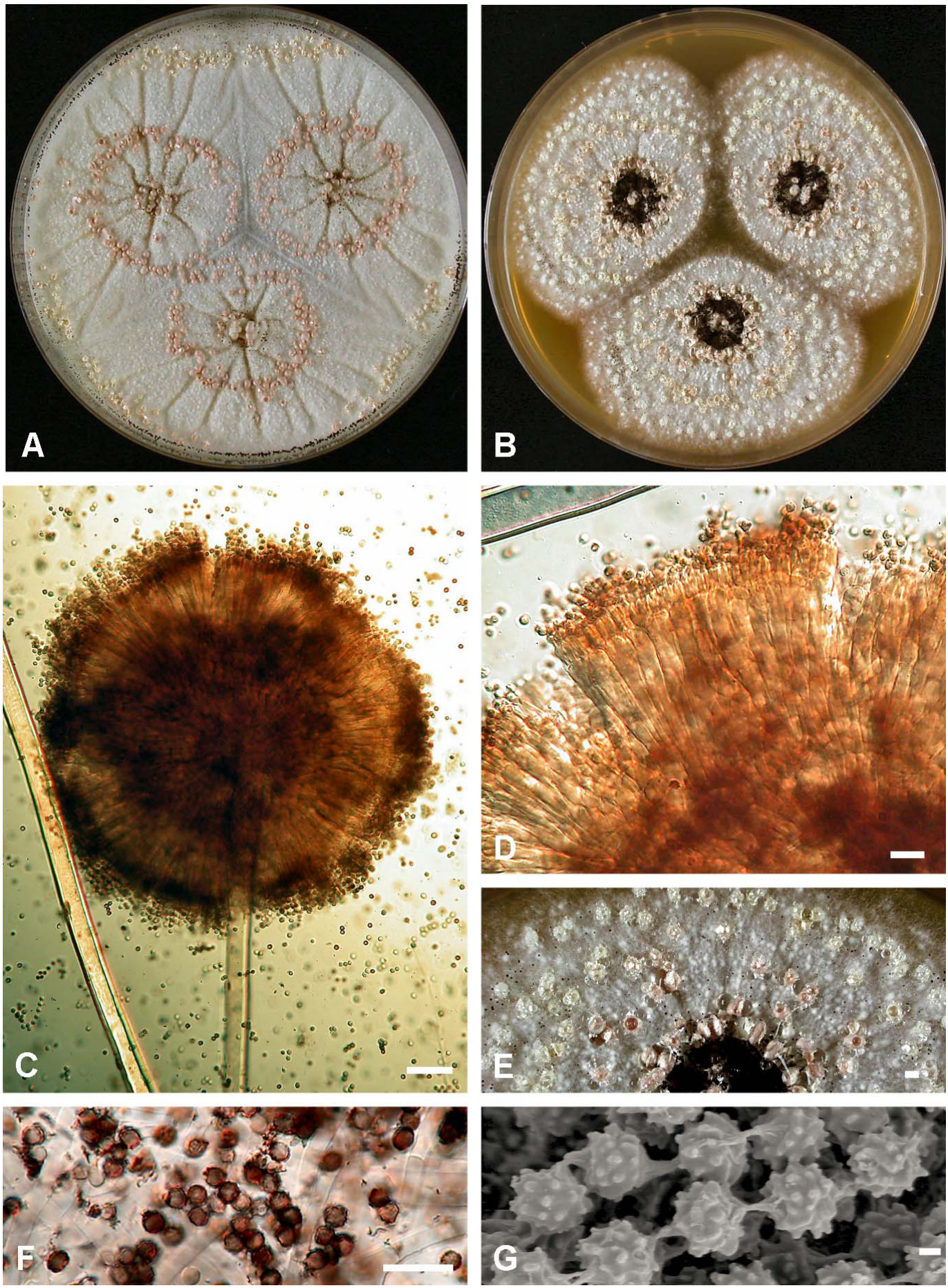

Fig. 2. *Aspergillus costaricaensis*. Seven-day-old cultures on A. CYA and B. MEA. C. Conidiophore. D. Detail of a conidiophore showing large metulae. E. Detail of a 7-day-old colony showing sclerotia. F. Conidia. G. Scanning electron micrograph photo of conidia. Scale bars: C, D, F = 10 µm, E = 1 mm, G = 1 µm.

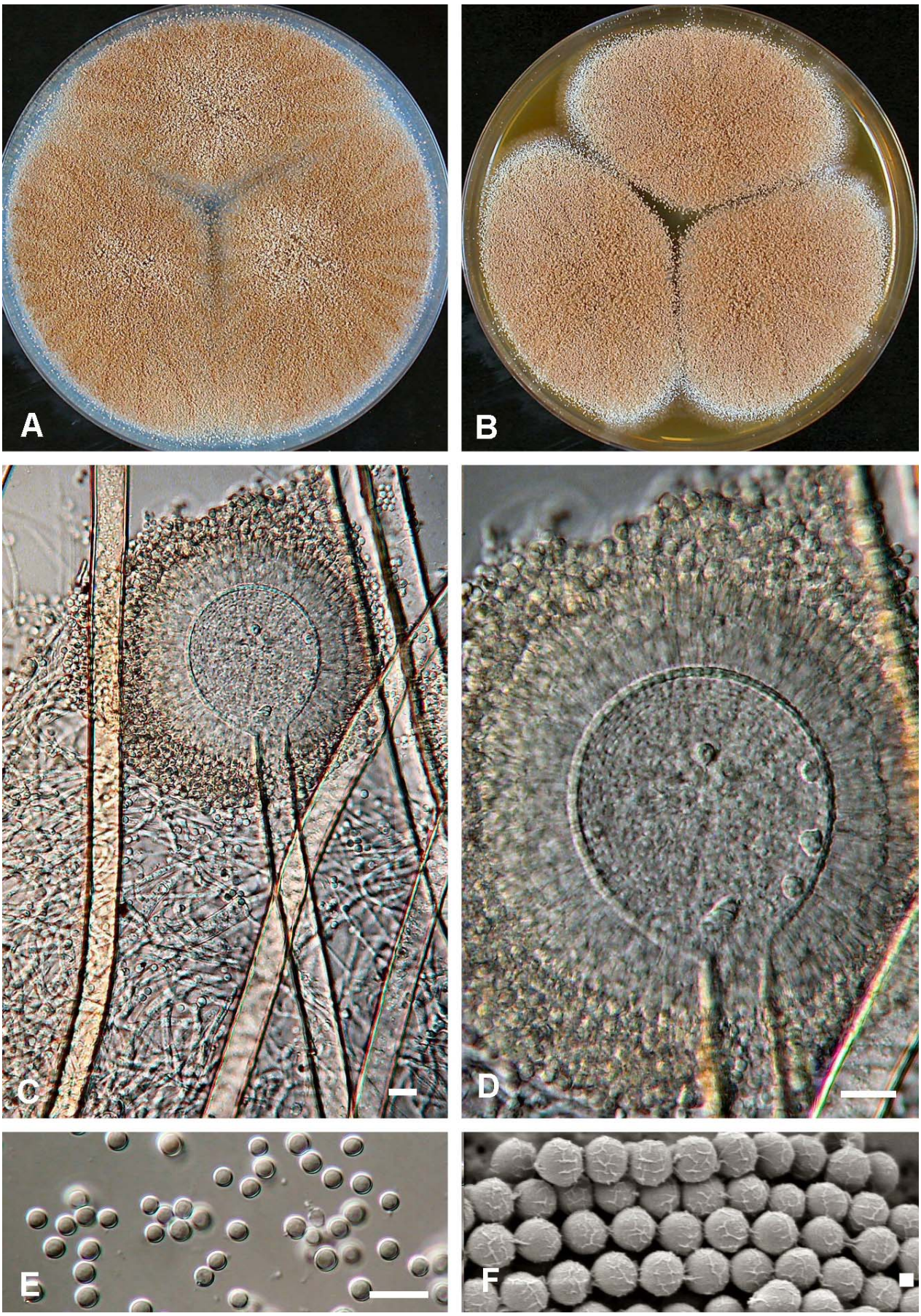

Fig. 3. *Aspergillus lacticoffeatus*. Seven-day-old cultures on A. CYA and B. MEA. C, D. Conidiophores. E. Conidia. F. Scanning electron micrograph photos of conidia. Scale bars:: C–E = 10 µm, F = 1 µm.

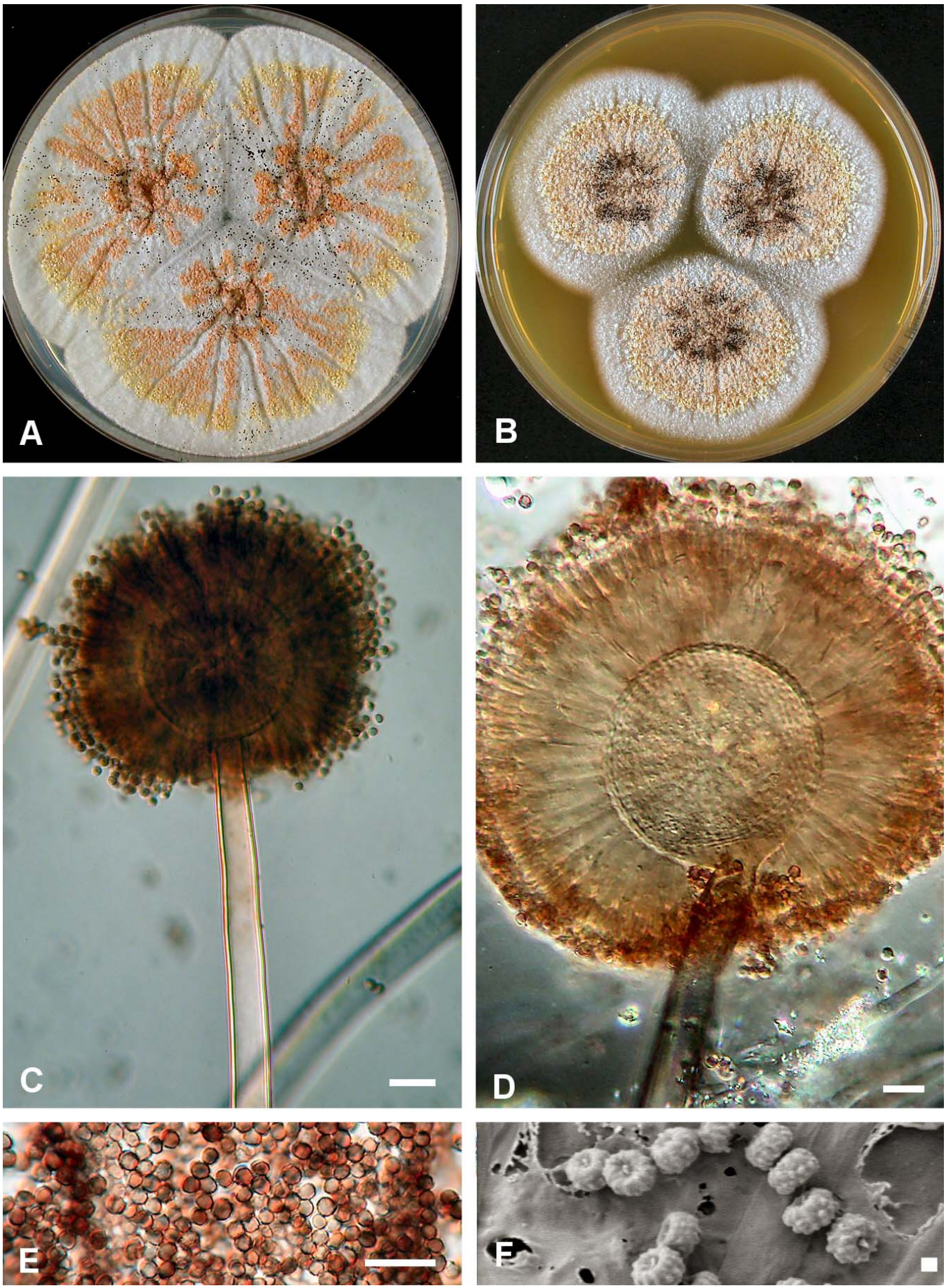

Fig 4. *Aspergillus piperis*. Seven-day-old cultures on A. CYA and B. MEA. C, D. Conidiophores. E. Conidia. F. Scanning electron micrograph photo of conidia. Scale bars: C–E = 10 µm, F = 1 µm.

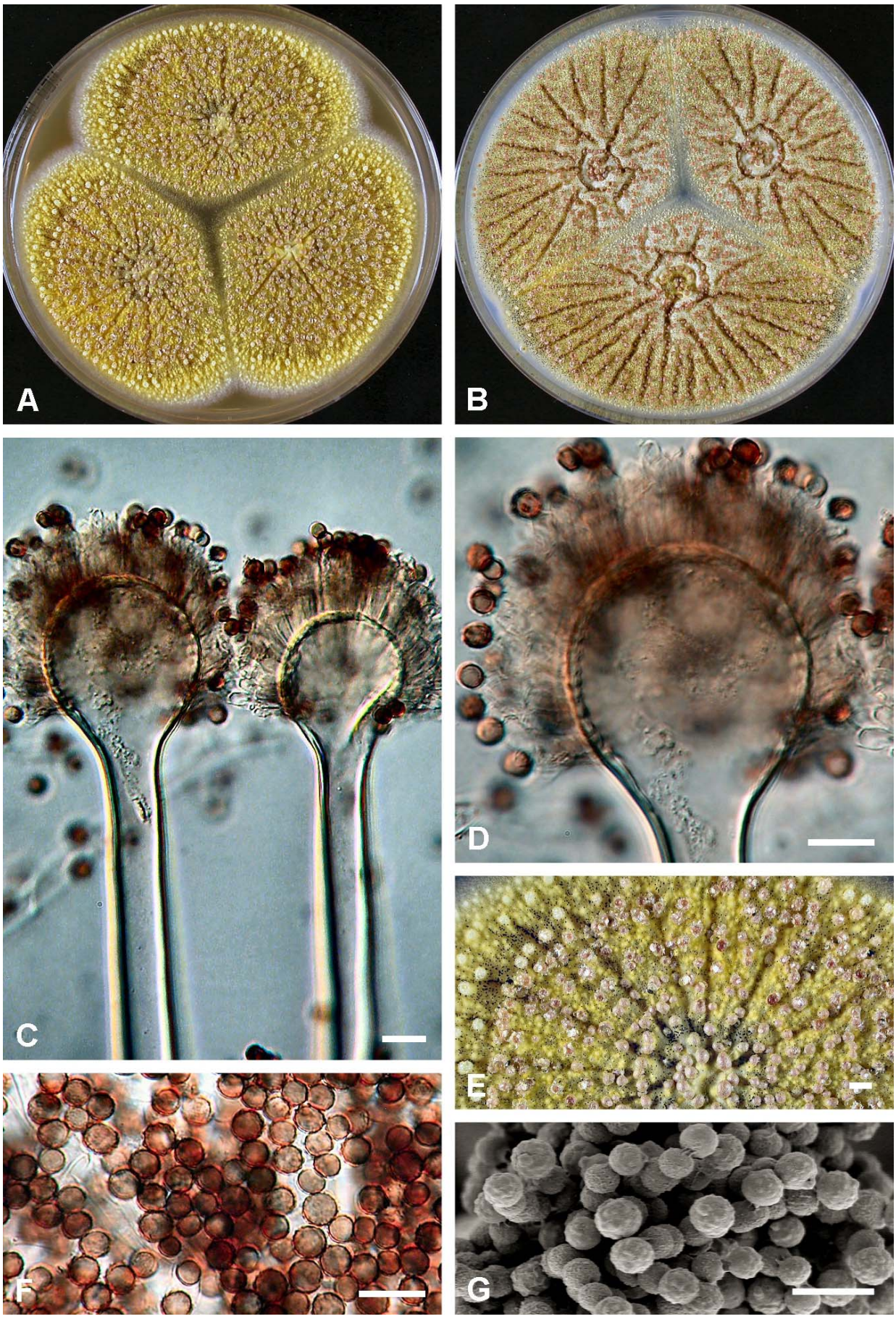

Fig. 5. *Aspergillus sclerotioniger*. Seven-day-old cultures on A. CYA and B. MEA. C. Conidiophore. D. Conidial head. E. detail of a 7-day-old colony showing sclerotia. F. Conidia. G. Scanning electron micrograph photo of conidia. Scale bars: C–D, F–G = 10 µm, E = 2 mm.

Other strains: CBS 101885 = IBT 22029, ex surface disinfected ripe green arabica coffee bean, farm Agua Blanco, Rubio district, **Venezuela**, J.M. Frank; CBS 101884 = IBT 22030, ex surface disinfected ripe green arabica coffee bean, farm Agua Blanco, Rubio district, Venezuela, J.M. Frank; CBS 101886 = IBT 22032, ex soil under robusta cherry coffee of a compacted soil drying yard, Karangsari, Pulo Pannggung subdistrict, Sumatra, **Indonesia**, J.M. Frank

Colony diameters at 7 d 25 °C, in mm: CYA: 71–76 mm, MEA 52–70 mm, YES: 75–80 mm, OAT: 32–36 mm, CREA: 32–44 mm, thin colonies with poor sporulation, strong acid production, CYA at 37 ºC: 59–75 mm. *Colony colours and texture.* Conidial areas first white then becoming hair brown (5E4) to dark blonde (5D4) and densely packed on CYA25, hyphae usually inconspicuous, no sclerotia on any medium, no exudates present, reverse cream to light brown on CYA, colony granular, sometimes sulcate. The conidial heads are globose at first and later occasionally developing into several conidial columns on each head. Colonies on CZ similar as on CYA, only reverse is uncoloured on CZ. Growth on YES is characterized by sulfur yellow mycelium formation. Conidial heads radiate; stipes short (200–)300–1200 × (7–)10–15(–18) µm, walls thick, smooth, orange–brown; vesicles (40–)45–60(–65) µm wide, nearly spherical; biseriate; metulae covering virtually the entire surface of the vesicle, measuring 12–25 × 3–6 µm; phialides 7–10 × 3–4 µm; conidia subglobose, 3.5–4.1 × 3.4–3.9 µm, usually smooth to very finely roughened. No sclerotia observed

Extrolites: Ochratoxin A, ochratoxin B, pyranonigrin A, orlandin, kotanin.

Distinguishing features: This species is characterized by its hair brown to dark blonde colonies, biseriate conidial heads with large vesicles and smooth to very finely roughened conidia.

Aspergillus piperis Samson & Frisvad **sp. nov.** MycoBank MB500009.

Aspergillo nigro similis, capitulis biseriatis, sed sclerotiis luteis vel roseo-brunneis et conidiis subglobosis vel late ellipsoideis distincte asperatis differens. Typus CBS H-13434.

Type: CBS 112811 = IBT 26239, ex grounded black pepper of tropical origin, Kgs. Lyngby, **Denmark**, K.F. Nielsen.

Colony diameters at 7 d 25 °C, in mm: CYA: 60–75 mm, MEA 59–78 mm, YES: 79–83 mm, OAT: 45–54 mm, CREA: 43–48 mm, thin colonies with poor sporulation, strong acid production, CYA at 37 ºC: 64–82 mm. *Colony colours and texture*. Conidial areas black and sparsely produced, after sub-culturing many aspergilla are produced on all media; hyphae inconspicuous, white; large sclerotia (1-17 mm) abundantly produced on all media, white when young becoming yellow to pink brown at age; exudate present like small hyaline droplets; reverse uncoloured, pale to creamy. Conidial heads radiate; stipes (300–)400–3000 × (7–)12–15(–20) µm, walls thick, smooth, hyaline; vesicles (40–)45–50(–55) µm wide, nearly spherical; biseriate; metulae covering virtually the entire surface of the vesicle, measuring (20–)25–30(–35) × 3–6 µm; phialides (5.5–)6–7.5(–8) × 3–4 µm; conidia subglobose to broadly ellipsoidal, 2.8–3.6 × 2.8–3.4 µm, smooth when young to very rough with irregular bars/striations.

Extrolites: Aurasperone B, 14-epi-14-hydroxy-10,23-dihydro-24,25-dehydroaflavinine, and 10,23-dihydro-24,25-dehydroaflavinine.

Distinguishing features: This species is characterized by its yellow to pink brown sclerotia, subglobose to broadly ellipsoidal and distinctly roughened conidia.

Aspergillus sclerotioniger Samson & Frisvad **sp. nov.** MycoBank MB500010.

Aspergillo carbonario similis, capitulis biseriatis, sed mycelio luteo, sclerotiis luteis vel aurantiacis vel rubro-brunneis, hyphis spicularibus luteis in agaro YES formatis et conidiis majoribus differens. Typus CBS H-13433.

Type: CBS 115572 = IBT 22905 ex surface disinfected green *Arabica* coffee bean, Karnataka, **India**, J.M. Frank.

Colony diameters at 7 d 25 °C, in mm: CYA: 71–78 mm, MEA 60–72 mm, YES: 72–80 mm, OAT: 42–56 mm, CREA: 19–25 mm, thin colonies with poor sporulation, strong acid production, CYA at 37 ºC: 7–16 mm. *Colony colours and texture*. On CYA25 and MEA only a few conidiophores are produced, conidial areas are black; mycelium yellow, conspicuous; sclerotia abundantly present, large (1–1.6 mm), (sub)globose, yellow to orange to red brown covered by yellow mycelium. Reverse on CYA pale, on MEA medium–yellow. Conidial heads radiate; stipes short (400–)500–800(–1200) × (12–)14–16(–18) µm, walls thick, smooth, hyaline; vesicles (30–)35–45(–50) µm wide, pyriform; biseriate; metulae covering three quarters of the vesicle, measuring 8–14 × 4–6 µm; phialides 6.5–9.5 × 3–5 µm; conidia subglobose, (4.7–)5–6(–6.4) × (4.5–)4.9–5.6(–6.1) µm, smooth when young, becoming verruculose, dark brown.

Extrolites: Ochratoxin A, ochratoxin B, traces of aurasperone B, and pyranonigrin A. The isolates produce a compound with a chromophore like that of the corymbiferans produced by *Penicillium hordei*

Stolk (Overy & Blunt 2004). A compound with a chromophore close to these compounds is funalenone, isolated from a fungus identified as *A. niger* (Inokoshi *et al.* 1999). This funalenone-like extrolite is also produced by *A. costaricaensis*, but has not been found in any strain of *A. niger* or *A. tubingensis*.

Distinguishing features: This species is characterized yellow mycelium, yellow to orange to red brown sclerotia, yellow spicular hyphae on YES agar and large conidia. This species is related to *Aspergillus carbonarius*.

Aspergillus homomorphus Steiman, Guiraud, Sage & Seigle-Mur. ex Samson & Frisvad, **sp. nov.** MycoBank MB500011.

Latin description: Systematic and Applied Microbiology 17(4): 621. 1995.

= *Aspergillus homomorphus* Steiman, Guiraud, Sage & Seigle-Mur., Systematic and Applied Microbiology 17(4): 621. 1995. [Nom.inval., Art. 37.4.]
= *Aspergillus pseudo-heteromorphus* Steiman, Guiraud, Sage & Seigle-Mur., Systematic and Applied Microbiology 17(4): 622. 1995. [Nom.inval., Art. 37.4.]

Type: CBS 101889, soil of death sea area, **Israel**.

Both species were described without designating a holotype specimen. Both taxa are identical and we are validating the name by depositing herb. CBS 101889 as holotype.

Extrolites: secalonic acid.

Distinguishing features: Short metulae, echinate conidia (spines up to 1.5 µm), secalonic acid D

Provisional synoptic key to species in *Aspergillus* section *Nigri*

Species list:
1. *A. aculeatus*
2. *A. brasiliensis* ined
3. *A. carbonarius*
4. *A. costaricaensis*
5. *A. ellipticus*
6. *A. japonicus*
7. *A. foetidus*
8. *A. heteromorphus*
9. *A. homomorphus*
10. *A. lacticoffeatus*
11. *A. niger*
12. *A. piperis*
13. *A. sclerotioniger*
14. *A. tubingensis*
15. *A. vadensis*

Conidia more than 6 µm diam: 3, (5)
Conidia spinulose: (3), 5, 6, 8, 9
Conidia strongly ellipsoidal: (1), 5, (6)
Metulae not produced: 1, 6
Metulae less than 15 µm in length: (7), (8), 9, (10), (11), 13, (14), (15)
Production of sclerotia: (1), (3), 4, (5), (6), 12, 13, (14)
Sclerotia yellow to orange: 13
Sclerotia yellow to pinkish brown: 12
Sclerotia pint to grayish yellow: 3
Colony diameter at 25 ºC on CYA, 7 d, less than 30 mm: 15
Colony diameter at 37 ºC on CYA, 7 d, larger than 70 mm: 2, 7, 10, 11, 12, 14
Colony diameter at 37 ºC on CYA 7.d, between 55 and 65 mm: 4, 15
Colony diameter at 37 ºC on CYA 7 d, less than 40 mm: 1, 3, 5, 6, 8, 9, 13
Colony diameter at 37 ºC on CYA, 7 d, 0 mm: (5), 8
Acid production on CREA agar weak or not present: (1), (7), 8, 9
Conidium colour *en masse* light brown to dark blonde: 10, 15
Conidium colour *en masse* greenish–olive: 8, (15)
Production of ochratoxin A: 3, 10, (11), 13
Production of pyranonigrin A: 3, 7, 10, 11, 12, 13, 14
Production of one or more naphtha-γ-pyrones: 2, 3, 4, 7, 11, 12, 13, 14, 15
Production of asperazine: 7, 14, 15
Production of secalonic acid D: 1, 9
Production of aflavinines: 4, 12, (14)
Production of antafumicins: 7
Production of corymbiferan lactone/funalenone-like compounds: 4, 13
Production of kotanin, desmethylkotanin and/or orlandin: 10, (11)
Production of austdiol: 5
Production of neoxaline: (1)

(Numbers in parentheses: feature not always present)

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