Two novel aflatoxin-producing *Aspergillus* species from Argentinean peanuts

María B. Pildain,[1] Jens C. Frisvad,[2] Graciela Vaamonde,[1] Daniel Cabral,[1] Janos Varga[3,4] and Robert A. Samson[3]

Correspondence
Robert A. Samson
samson@cbs.knaw.nl

[1]Faculty of Ciencias Exactas y Naturales, Pab. II, Lab. 69, University of Buenos Aires, CP EHA1428, Buenos Aires, Argentina

[2]Center for Microbial Biotechnology, BioCentrum-DTU, Building 221, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark

[3]CBS Fungal Biodiversity Centre, PO Box 85167, 3508 AD Utrecht, The Netherlands

[4]Department of Microbiology, Faculty of Sciences, University of Szeged, PO Box 533, H-6701 Szeged, Hungary

Two novel species from *Aspergillus* section *Flavi* from different species of *Arachis* (peanuts) in Argentina are described as *Aspergillus arachidicola* sp. nov. and *Aspergillus minisclerotigenes* sp. nov. Their novel taxonomic status was determined using a polyphasic taxonomic approach with phenotypic (morphology and extrolite profiles) and molecular (β-tubulin and calmodulin gene sequences) characters. *A. minisclerotigenes* resembles *Aspergillus flavus* and *Aspergillus parvisclerotigenus* in producing aflatoxins B_1 and B_2, cyclopiazonic acid, kojic acid and aspergillic acid, but in addition it produces aflatoxins G_1 and G_2, aflavarins, aflatrem, aflavinines, parasiticolides and paspaline. This species also includes several isolates previously assigned to *A. flavus* group II and three Australian soil isolates. *A. arachidicola* produces aflatoxins B_1, B_2, G_1 and G_2, kojic acid, chrysogine and parasiticolide, and some strains produce aspergillic acid. The type strain of *A. arachidicola* is CBS 117610^T =IBT 25020^T and that of *A. minisclerotigenes* is CBS 117635^T =IBT 27196^T. The Mycobank accession numbers for *Aspergillus minisclerotigenes* sp. nov. and *Aspergillus arachidicola* sp. nov. are respectively MB 505188 and MB 505189 (http://www.mycobank.org).

INTRODUCTION

Aflatoxins are the most potent natural carcinogens known (JECFA, 1997), affecting all vertebrate animal species, including humans. Four compounds are commonly produced in foods, aflatoxins B_1, B_2, G_1 and G_2, but other bio-transformed aflatoxins may occur, for example in milk, such as aflatoxins M_1 and M_2 (Cole & Cox, 1981). These mycotoxins have been shown to be produced by *Aspergillus 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.*, 2004) and *A. parvisclerotigenus* (Saito & Tsurota, 1993, Frisvad *et al.*, 2004) in *Aspergillus* section *Flavi*, by *A. ochraceoroseus* (Frisvad *et al.*, 1999, Klich *et al.*, 2000) and *A. rambellii* (Frisvad *et al.*, 2005) in *Aspergillus* section *Ochraceorosei* and in *Aspergillus* section *Nidulantes* or the ascomycete genus *Emericella* by *Emericella astellata* (Frisvad *et al.*, 2004) and *E. venezuelensis* (Frisvad & Samson, 2004a).

The most important aflatoxin producers from a public health point of view are members of *Aspergillus* section *Flavi*, in particular *A. flavus* and *A. parasiticus*. Originally, several isolates of *A. parasiticus* were misidentified as *A. flavus* [for example NRRL 2999, 3000 and 3145 (Hesseltine *et al.*, 1966; Applegate & Chipley, 1973); corrected to *A. parasiticus* by Hesseltine *et al.*, 1970], and therefore G-type aflatoxins were connected with *A. flavus*. Like *A. pseudotamarii* and '*A. flavus*' NRRL 3251, *A. flavus* has later been reported to produce only B-type aflatoxins (Dorner *et al.*, 1984; Klich & Pitt, 1985; Bennett & Papa, 1988; Ito *et al.*, 2001; Ehrlich *et al.*, 2004; Frisvad *et al.*, 2005), while *A. parasiticus*, *A. toxicarius*, *A. nomius* and *A. bombycis* can produce both B- and G-type aflatoxins (Ehrlich *et al.*, 2004; Frisvad *et al.*, 2005). There are many reports to indicate

Abbreviations: CI, consistency index; MP, maximum-parsimony; NJ, neighbour-joining; RI, retention index.

The GenBank/EMBL/DDBJ accession numbers for the β-tubulin and calmodulin gene sequences of the strains examined in this study are shown in Fig. 1 and Supplementary Fig. S1.

A neighbour-joining phylogenetic tree based on calmodulin gene sequences and colour versions of Figs 2 and 3 are available as supplementary material with the online version of this paper.

that certain *A. flavus* strains, microsclerotial strains, and strains listed as intermediate between *A. flavus* and *A. parasiticus* can also produce G-type aflatoxins (Codner *et al.*, 1963; Hesseltine *et al.*, 1970; Cotty & Cardwell, 1999). Many aflatoxin B- and G-producing strains have been reported to produce small sclerotia, but they do not obviously belong to *A. parvisclerotigenus* (Saito & Tsurota, 1993; Cotty & Cardwell, 1999; Bayman & Cotty, 1993; Egel *et al.*, 1994; Frisvad *et al.*, 2005). Isolates in section *Flavi* producing small sclerotia apparently produce the same mixture of indoloditerpene alkaloids, whether they produce only B-type aflatoxins (NRRL 3251) or both B- and G-type aflatoxins [CBS 121.62 (=NRRL A-11612) and Nigerian and Indonesian strains] (Tanaka *et al.*, 1989). There is phylogenetic evidence that *Aspergillus flavus sensu lato* may consist of several species (Geiser *et al.*, 1998, 2000; Chang *et al.*, 2006). However, this is difficult to evaluate, as most strains examined in those studies were generally not deposited in major culture collections. In order to find out whether such B+G-type aflatoxin producers belonged to one or more species, we surveyed various *Arachis* species in Argentina because such wild specimens of *Arachis* could be expected to harbour a more diverse mycobiota than domesticated peanuts (*Arachis hypogaea*), which have been examined in depth by many authors (e.g. Austwick & Ayerst, 1963). Isolates representing *A. flavus* group II as defined by Geiser *et al.* (1998, 2000) and soil isolates from Australia kindly provided by J. I. Pitt (CSIRO, North Ryde, Australia) were also included in the analyses.

We have used a polyphasic taxonomic approach in order to determine the taxon delimitation (Frisvad & Samson, 2004b; Varga *et al.*, 2007; Houbraken *et al.*, 2007, Samson *et al.*, 2007a, b). For the phenotypic analyses, macro- and micromorphology, extrolite profiles and growth temperatures were studied. For the phylogenetic analyses, β-tubulin and calmodulin gene sequences were used.

METHODS

Isolates. The strains examined listed in Table 1 were cultures from the CBS (CBS Fungal Biodiversity Centre, Utrecht, Netherlands), NRRL (NCAUR Culture Collection, Peoria, IL, USA) or IBT (at BioCentrum-DTU, Kgs. Lyngby, Denmark) collections or they were freshly isolated from seeds and leaves of cultivated peanut (*Arachis hypogaea* L.) and leaves of autochthonous peanut species [*Arachis villosa* Benth., *Arachis correntina* (Burkart) Krapov. & W. Gregory, *Arachis glabrata* Benth. and *Arachis burkartii* Handro] from Argentina. The Argentinean strains were chosen based on being representatives of known vegetative compatibility groups (VCGs) of *A. flavus* and from different hosts and agroecological zones within Argentina (Vaamonde *et al.*, 1995; Novas & Cabral, 2002; Pildain *et al.*, 2003, 2004).

Morphology and extrolite profiles. For macromorphological observations, isolates were grown on Czapek yeast autolysate (CYA), malt extract agar (MEA), Czapek agar (CZA), yeast extract sucrose (YES) agar, oatmeal agar (OA) and creatine sucrose agar (CREA) (Samson *et al.*, 2004). Aspergillus flavus and parasiticus agar (AFPA; Pitt *et al.*, 1983) was used to determine the production of aflatoxins on agar medium. The strains were inoculated at three points and incubated at 25 °C in the dark for 7 days and/or at 37 and 42 °C on CYA. For micromorphological observations, microscope mounts were made in lactic acid from MEA colonies and a drop of alcohol was added to remove air bubbles and excess conidia. Extrolites were analysed by HPLC using alkylphenone retention indices and diode array UV-VIS detection according to Frisvad & Thrane (1993), as modified by Smedsgaard (1997).

Genotypic analysis. The cultures used for molecular studies were grown in 2 ml malt peptone (MP) broth, containing 10 % (v/v) malt extract (Brix 10) and 0.1 % (w/v) bacto peptone (Difco), in 15 ml tubes. The cultures were incubated at 25 °C for 7 days in light/darkness. DNA was extracted from the cells using the Masterpure yeast DNA purification kit (Epicentre Biotechnologies) according to the instructions of the manufacturer. A fragment of the 5′ portion of the β-tubulin gene was amplified using primers bt2a and bt2b (Glass & Donaldson, 1995), while a segment of the calmodulin gene was amplified using primers cmd5 and cmd6 as described by Hong *et al.* (2006). The amplified DNA fragments were purified using a QIAquick PCR purification kit (Qiagene). DNA sequences were determined using a BigDye Terminator v3.1 cycle sequencing kit (ABI) and an ABI 3100 DNA sequencer. Both strands of each fragment were sequenced.

DNA sequences were edited with the DNASTAR computer package and an alignment of the sequences was performed using the CLUSTAL W program (Thompson *et al.*, 1994). The neighbour-joining (NJ) method was used for the phylogenetic analysis. For NJ analysis, the data were first analysed using the Tamura–Nei parameter distance calculation model with gamma-distributed substitution rates, which were then used to construct the NJ tree with MEGA version 3.1 (Kumar *et al.*, 2004). To determine the support for each clade, a bootstrap analysis was performed with 1000 replications.

Phylogenetic analysis of sequence data was also performed using PAUP* 4.0b10 (Swofford, 2000). Alignment gaps were treated as a fifth character state, uninformative characters were excluded and all characters were unordered and weighted equally. Maximum-parsimony (MP) analysis was performed for all datasets 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 and retention index (CI and RI, respectively) were also calculated. Sequences were deposited at GenBank under accession numbers listed in Fig. 1 and Supplementary Fig. S1 (available in IJSEM Online).

RESULTS

Morphological analysis

In our survey, six species from *Aspergillus* section *Flavi* were isolated from Argentinean wild peanut species: *A. caelatus*, *A. flavus*, *A. tamarii* and *A. parasiticus* and two taxa related to *A. parvisclerotigenus* and *A. parasiticus*. All *Aspergillus* isolates analysed by microscope examination exhibited conidial heads in shades from yellow–green to brown and had similar colony characteristics and growth rates on all media analysed in this study, and they all grew very fast at 37 °C. These are typical morphological features associated with *Aspergillus* section *Flavi* (Raper & Fennell, 1965). The strains from Argentinean peanuts were compared to ex type and authentic strains of species in *Aspergillus* section *Flavi* (see Table 1) and could be divided

Table 1. *Aspergillus* isolates examined

Isolate number	Source*	Species name
CBS 117610^T =IBT 25020^T	*Arachis glabrata* leaf; CO, Argentina	*A. arachidicola*
CBS 117611 =IBT 27185	*Arachis glabrata* leaf; CO, Argentina	*A. arachidicola*
CBS 117612 =IBT 27190	*Arachis glabrata* leaf; CO, Argentina	*A. arachidicola*
CBS 117615 =IBT 27178	*Arachis glabrata* leaf; CO, Argentina	*A. arachidicola*
CBS 117614 =IBT 27183	*Arachis glabrata* leaf; CO, Argentina	*A. arachidicola*
IBT 27215	*Arachis glabrata* leaf; CO, Argentina	*A. arachidicola*
NRRL 3353 =IBT 14897	Diseased alkali bee, USA	*A. arachidicola*
CBS 117187^T =NRRL 26010^T	Frass in a silkworm rearing house; Japan	*A. bombycis*
CBS 117616	*Arachis burkartii* leaf; CO, Argentina	*A. caelatus*
CBS 763.97^T =NRRL 25528^T	Soil; USA	*A. caelatus*
CBS 764.97 =NRRL 25404	Soil; USA	*A. caelatus*
CBS 110.55^T	Air contaminant; Brazil	*A. fasciculatus*
CBS 484.65^T	Air contaminant; Brazil	*A. flavofurcatus*
CBS 117623	*Arachis hypogaea* seed; CD, Argentina	*A. flavus*
CBS 117622	*Arachis hypogaea* seed; CD, Argentina	*A. flavus*
CBS 117621	*Arachis hypogaea* seed; CD, Argentina	*A. flavus*
CBS 117631	*Arachis hypogaea* seed; SA, Argentina	*A. flavus*
CBS 117632	*Arachis hypogaea* seed; FO, Argentina	*A. flavus*
CBS 117624	*Arachis glabrata* leaf; CO, Argentina	*A. flavus*
CBS 100927^T	Cellophane; South Pacific Islands	*A. flavus*
CBS 117733	Hospital strain; Netherlands	*A. flavus*
CBS 116.48	Unknown source; Netherlands	*A. flavus*
CBS 616.94	Man, orbital tumour; Germany	*A. flavus*
CBS 485.65^T	Butter; Japan	*A. flavus* var. *columnaris*
CBS 117731	*Dipodomys spectabilis* cheek pouch; New Mexico, USA	*A. flavus* var. *columnaris*
CBS 542.69^T	Stratigraphic core sample; Japan	*A. kambarensis*
CBS 151.66^T	Dung of *Lepus townsendii*; USA	*A. leporis*
CBS 117620 =IBT 27198	*Arachis hypogaea* seed; SA, Argentina	*A. minisclerotigenes*
CBS 117633	*Arachis hypogaea* seed; FO, Argentina	*A. minisclerotigenes*
CBS 117634 =IBT 27197	*Arachis hypogaea* seed; SA, Argentina	*A. minisclerotigenes*
CBS 117635^T =IBT 27196^T	*Arachis hypogaea* seed; CD, Argentina	*A. minisclerotigenes*
CBS 117636	*Arachis hypogaea* seed; FO, Argentina	*A. minisclerotigenes*
CBS 117637 =IBT 27177	*Arachis hypogaea* seed; SA, Argentina	*A. minisclerotigenes*
CBS 117639 =IBT 27195	*Arachis hypogaea* seed; SA, Argentina	*A. minisclerotigenes*
IBT 27213, IBT 27214, IBT 27217, IBT 27199, IBT 27200, IBT 27179	*Arachis hypogaea* seed; SA, Argentina	*A. minisclerotigenes*
NRRL A-11611 =NRRL 6444	Soil, peanut field, Nigeria	*A. minisclerotigenes*
FRR 4086	Freshly pulled peanuts, Kingaroy, Queensland, Australia	*A. minisclerotigenes*
FRR 4937	Soil, Australia	*A. minisclerotigenes*
FRR 5309	Soil, Australia	*A. minisclerotigenes*
1-22	Soil, peanut field, Australia	*A. minisclerotigenes*
4-2	Soil, peanut field, Australia	*A. minisclerotigenes*
NPL TX12-10-2s	Soil, peanut field, Texas, USA	*A. minisclerotigenes*
CBS 260.88^T =NRRL 13137^T	Wheat; USA	*A. nomius*
CBS 100925^T	Unknown source; Japan	*A. oryzae*
CBS 117638	*Arachis hypogaea* seed; CO, Argentina	*A. parasiticus*
CBS 117617	*Arachis villosa* leaf; CO, Argentina	*A. parasiticus*
CBS 117618	*Arachis correntina* leaf; CO, Argentina	*A. parasiticus*
CBS 100926^T	*Pseudococcus calceolariae*, sugar cane mealy bug; Hawaii, USA	*A. parasiticus*
CBS 260.67^T	Unknown source; Japan	*A. parasiticus* var. *globosus*
CBS 121.62^T	*Arachis hypogaea*; Nigeria	*A. parvisclerotigenus*
CBS 766.97^T =NRRL 25517^T	Soil; USA	*A. pseudotamarii*
CBS 766.97	Soil; USA	*A. pseudotamarii*
CBS 100928^T	Soy sauce; Japan	*A. sojae*
CBS 100929	Soy sauce; Japan	*A. sojae*
CBS 501.65^T	Cotton lintafelt, UK	*A. subolivaceus*
CBS 104.13^T	Activated carbon	*A. tamarii*

Table 1. cont.

Isolate number	Source*	Species name
CBS 117626	*Arachis hypogaea* seed; SA, Argentina	*A. tamarii*
CBS 580.65^T	Soil; USA	*A. terricola* var. *americanus*
CBS 119.51	Japan	*A. terricola* var. *americanus*
CBS 120.51^T	Culture contaminant	*A. thomii*
CBS 822.72^T	*Arachis hypogaea*; Uganda	*A. toxicarius*

*Peanut-producing provinces in Argentina: CD, Córdoba; CO, Corrientes; FO, Formosa; SA, Salta.

into two groups. One group of isolates (represented by CBS 117626) was similar to *A. tamarii* and was characterized by dark-brown conidia with conspicuously roughened to tuberculate thick walls and colonies with a dark-brown reverse on AFPA. The only species with such characteristics included *A. tamarii*, *A. pseudotamarii* and *A. caelatus*. The remaining strains had light to dark yellow–green conidia and less conspicuously roughened conidia. They also had a cadmium orange- or cream-coloured reverse on AFPA.

Isolates of *A. flavus* have been reported to produce two types of sclerotia, small (S) and large (L) (Cotty, 1989). In our study, we found 16 strains (represented by CBS 117620, CBS 117633–117635 and CBS 117639) with small sclerotia, which were similar to *A. parvisclerotigenus* CBS 121.62^T. One *A. parasiticus* isolate produced sclerotia of intermediate size (CBS 117618), while *A. flavus* IBT 27177, CBS 117622, CBS 117630 and CBS 117733 produced large sclerotia.

DNA analysis

For the molecular analysis, two regions of the genome were analysed, namely parts of the calmodulin and β-tubulin genes of the isolates. For the analysis of part of the β-tubulin gene, 510 characters were analysed. Among the 128 polymorphic sites, 74 were found to be phylogenetically informative. The NJ tree based on partial β-tubulin genes sequences is shown in Fig. 1. The topology of the tree is the same as one of the more than 10^5 MP trees constructed by the PAUP program (tree length, 173 steps; CI, 0.8844; RI, 0.9564).

The calmodulin dataset included 520 characters, with 80 parsimony informative characters. The topology of the NJ tree (Supplementary Fig. S1) was the same as one of the more than 10^5 MP trees (length, 229; CI, 0.8603; RI, 0.9290).

Most of the sequenced Argentinean isolates fell into one of two main clades, represented by *A. flavus* and *A. parasiticus*. Isolate CBS 117616 was related to *A. caelatus* (Horn, 1997), which we could also confirm by its morphology. *A. sojae*, *A. toxicarius* and *A. terricola* var. *americanus* were found to belong to the *A. parasiticus* clade. Four isolates (CBS 117610–117612 and CBS 117615) from *Arachis glabrata* leaves formed a well-defined clade related to *A. parasiticus* on the trees based on β-tubulin and calmodulin data (Fig. 1 and Supplementary Fig. S1). However, these isolates have internal transcribed spacer (ITS) sequences identical to those of *A. parasiticus* (data not shown). Another six Argentinean isolates from *Arachis hypogaea* seeds formed a well-defined clade related to *A. flavus* and *A. parvisclerotigenus* on trees based on β-tubulin and calmodulin sequence data (Fig. 1 and Supplementary Fig. S1). These results are in agreement with our morphological and extrolite results. Furthermore, these Argentinean isolates belong to the same VCG as described by Pildain *et al.* (2004, 2005). This clade also includes four isolates assigned to *A. flavus* group II by Geiser *et al.* (1998, 2000) and three isolates collected from soils from Australia, all producing small sclerotia. Our calmodulin and β-tubulin sequence data indicate that *A. oryzae*, *A.thomii*, *A. kambarensis*, *A. fasciculatus* and *A. subolivaceus* are very closely related to *A. flavus*.

Extrolites

In our extrolite study of 34 strains from Argentinean peanuts, we found that the strains which were identified as *A. flavus* produced kojic acid (100 %), aspergillic acid (100 %), cyclopiazonic acid (82 %), aflatoxins B_1 and B_2 (74 %), oryzaechlorin (44 %) and flavimine (94 %) (Table 2). A single strain of *A. tamarii* from Argentinean peanuts produced kojic acid and oryzaechlorin. Four strains of *A. parasiticus* from Argentinean peanuts produced aflatoxins B_1, B_2, G_1 and G_2, aspergillic acid, kojic acid and parasiticolides, one strain (IBT 27180) produced oryzaechlorin, one strain (IBT 27194) produced paspaline and paspalinine and one strain (CBS 117618) produced aflavinines and other sclerotial metabolites.

Sixteen strains from Argentinean peanuts with small sclerotia produced aflatoxins B_1, B_2, G_1 and G_2 (100 %), aflatrem (88 %), aflavarins (38 %), aflavinines (dihydroxyaflavinine, monohydroxyaflavinine, monohydroxyisoaflavinine and aflavinine) (100 %), aspergillic acid (100 %), cyclopiazonic acid (100 %), kojic acid (100 %), parasiticolides (100 %) and paspaline, paspalinine and emindole SB (100 %). This extrolite profile is very similar to that of *A. parvisclerotigenus*, but the Argentinean strains did not produce parasiticolides. Furthermore, *A. parvisclerotigenus* produced the compound A 30461 (oryzaechlorin). One of

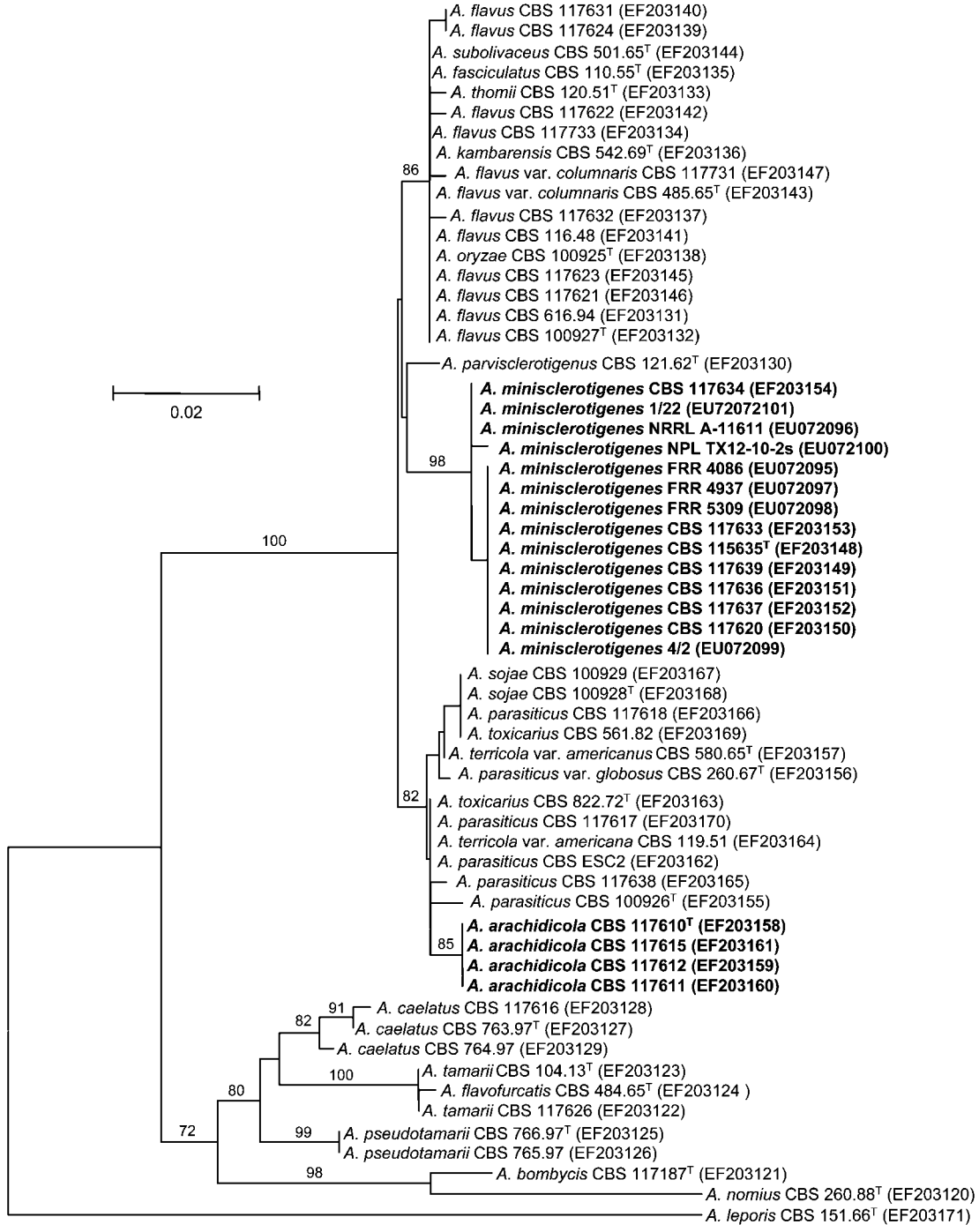

Fig. 1. Neighbour-joining tree based on β-tubulin sequence data of *Aspergillus* section *Flavi*. Numbers above branches are bootstrap values. Only values above 70 % are indicated. Bar, 2 substitutions per 100 nucleotide positions.

the strains listed by Hesseltine *et al.* (1970), NRRL A-11611 (=NRRL 6444), also produced aflatoxin B_1, B_2, G_1 and G_2, aflatrem, aflavinines, aspergillic acid, cyclopiazonic acid, parasiticolides, kojic acid, aspergillic acid, paspaline, paspalinine and emodin SB and is very similar to the eight Argentinean strains (Table 2).

Four isolates (CBS 117610–117612 and CBS 117615) produced aflatoxins B_1, B_2, G_1 and G_2 (100 %), aspergillic acid (33 %), chrysogine (67 %), oryzaechlorin (17 %), parasiticolide (50 %), an extrolite with parasiticolide chromophore (50 %), extrolite NO2 (100 %) and extrolite EPIF (100 %). All strains had a floccose colony texture and

Table 2. Production of mycotoxins and other extrolites by selected species in *Aspergillus* section *Flavi* based on HPLC-DAD analyses

Domesticated species, *A. oryzae* and *A. sojae*, and species with yellow conidia, *Petromyces alliaceus* and *A. lanosus*, are not included. A, Kojic acid; B, aflatoxin B_1; C, aflatoxin G_1; D, cyclopiazonic acid; E, aspergillic acid; F, asperfuran; G, parasiticolides; H, chrysogine; I, aflavarins; J, paspalinin and paspaline; K, aflatrems and aflavinines; L, nominine.

Species	A	B	C	D	E	F	G	H	I	J	K	L
A. arachidicola	+	+	+	−	+	−	+	+	−	−	−	−
A. avenaceus	−	−	−	−	−	−	−	−	−	−	−	−
A. bombycis	+	+	+	−	±	−	−	−	−	−	−	−
A. caelatus	+	−	−	±	−	−	−	−	−	−	−	−
A. flavus	+	±	−	±	+	±	−	−	−	±	−	−
A. leporis	+	−	−	−	−	−	−	−	−	−	−	−
A. minisclerotigenes	+	+	+	+	+	−	+	−	+	+	+	−
A. nomius	+	+	+	−	+	−	−	−	−	−	−	+
A. parasiticus	+	+	+	−	+	−	+	−	−	±	−	−
A. parvisclerotigenus	+	+	+	+	+	−	−	−	+	+	+	−
A. pseudotamarii	+	+	−	±	−	−	−	−	−	−	−	−
A. tamarii	+	−	−	±	−	−	−	−	−	−	−	−

a conidium colour similar to that of *A. flavus* but, except for the production of chrysogine by most isolates, they exhibited extrolite profiles characteristic of *A. parasiticus*. Chrysogine production was also observed in *A.* cf. *nomius* NRRL 3353, a strain that had formerly been characterized as being an atypical *A. flavus* (Hesseltine *et al.*, 1970). These strains had a conidial ornamentation between *A. parasiticus* and *A. flavus*, in agreement with isolates determined as 'o-type' by Feibelman *et al.* (1998) and Kumeda *et al.* (2003).

DISCUSSION

The isolates representing two new taxa are related to either *A. parasiticus* or *A. parvisclerotigenus*. The isolates proposed here as *Aspergillus arachidicola* sp. nov. are not as dark green (Raper & Fennell, 1965) as *A. parasiticus*. The conidiophores are regularly biseriate, but uniseriate conidial heads are also produced. However, conidium shape and ornamentation and other microscopical characteristics of *A. arachidicola* overlap with those of *A. parasiticus*. The production of aflatoxins B and G and lack of CPA production are similar in these two species, but the production of chrysogine and the negative results on AFPA of *A. arachidicola* are valuable characters for distinguishing these two species.

A. flavus is different from *A. arachidicola* by its yellowish-green colony colour (Raper & Fennell, 1965) and the inability to produce type-G aflatoxins. Typical *A. flavus* isolates produce aflatoxin B_1, CPA and aspergillic acid (Samson *et al.*, 2004). Both species have roughened stipes, but *A. flavus* usually has longer stipes (more than 1000 μm), and also have smooth or finely roughened conidia.

Aspergillus minisclerotigenes sp. nov. is proposed as a new taxon for isolates with typical small sclerotia which came from peanut plants or peanut fields from Argentina, Australia, Nigeria and Texas. Some of these isolates have been described as *A. flavus* group II by Geiser *et al.* (1998, 2000). These isolates resemble *A. parvisclerotigenus* (CBS 121.62^T) on the basis of morphological characteristics and extrolite production, but differ by producing parasiticolide, while *A. parvisclerotigenus* produces the compounds A 30461 and speradine A, not detected in *A. minisclerotigenes*. Large amounts of parasiticol, sterigmatocystin and *O*-methylsterigmatocystin were also detected in *A. parvisclerotigenus*, but not in *A. minisclerotigenes*. On the other hand, *A. minisclerotigenes* was more effective than *A. parvisclerotigenus* in producing sclerotial metabolites (aflavinines, aflatrems, paspalinine, paspaline, aflavarins). However, all microsclerotial strains previously allocated to *A. flavus* appear to produce all these sclerotial indole metabolites (Tanaka *et al.*, 1989). It has been shown that the biosynthesis of sclerotial metabolites and aflatoxin is regulated by the gene *veA*, which is necessary for sclerotial formation in an isolate producing small sclerotia (Duran *et al.*, 2007). A more detailed phenotypic study of more isolates representing *A. parvisclerotigenus* is necessary to determine whether there are more phenotypic differences between the two taxa that produce small sclerotia. However, our sequence data clearly show that the two microsclerotial species are genetically different.

Taxonomy

Latin diagnosis of *Aspergillus arachidicola* Pildain, Frisvad & Samson sp. nov. MB 505189

Coloniae in agaro MEA *dicto post* 7 *dies* 25 °C 6–6.5 cm *diametro*, *velutinae*, *olivaceae vel olivaceo-brunneae*; *reversum viridi-luteum*. *Conidiophora uniseriata*, *stipes hyalinus*,

asperulatus, (250–)400–600(–1000) × (6.5–)9–10 μm; *vesiculae globosae vel subglobosae*, (23–)28–50 μm *diametro*; *metulae* 9.5–13.5 × 5–6.5 μm; *phialides* 7–11 × 3–6.5 μm. *Conidia globosa vel subglobosa*, *echinulata*, *viridula*, (3.5–)4.5–5(–6.5) μm. *Sclerotia absentia.*

Typus siccus in herb. CBS 117610 *et ex-typus vivus*, *isolatus Arachis glabrata*, Corrientes *provincia* Argentina.

Description of *Aspergillus arachidicola* Pildain, Frisvad & Samson sp. nov. MB 505189

Aspergillus arachidicola (a.ra.chi.di.co′la. N.L. n. *arachidicola* inhabitant of *Arachis*).

Colonies on YES, MEA, OA and CYA attain a diameter of 6–6.5 cm in 7 days at 25 °C; growing rapidly on CYA at 37 °C, with a diameter of 6–7 cm (Fig. 2; a colour version

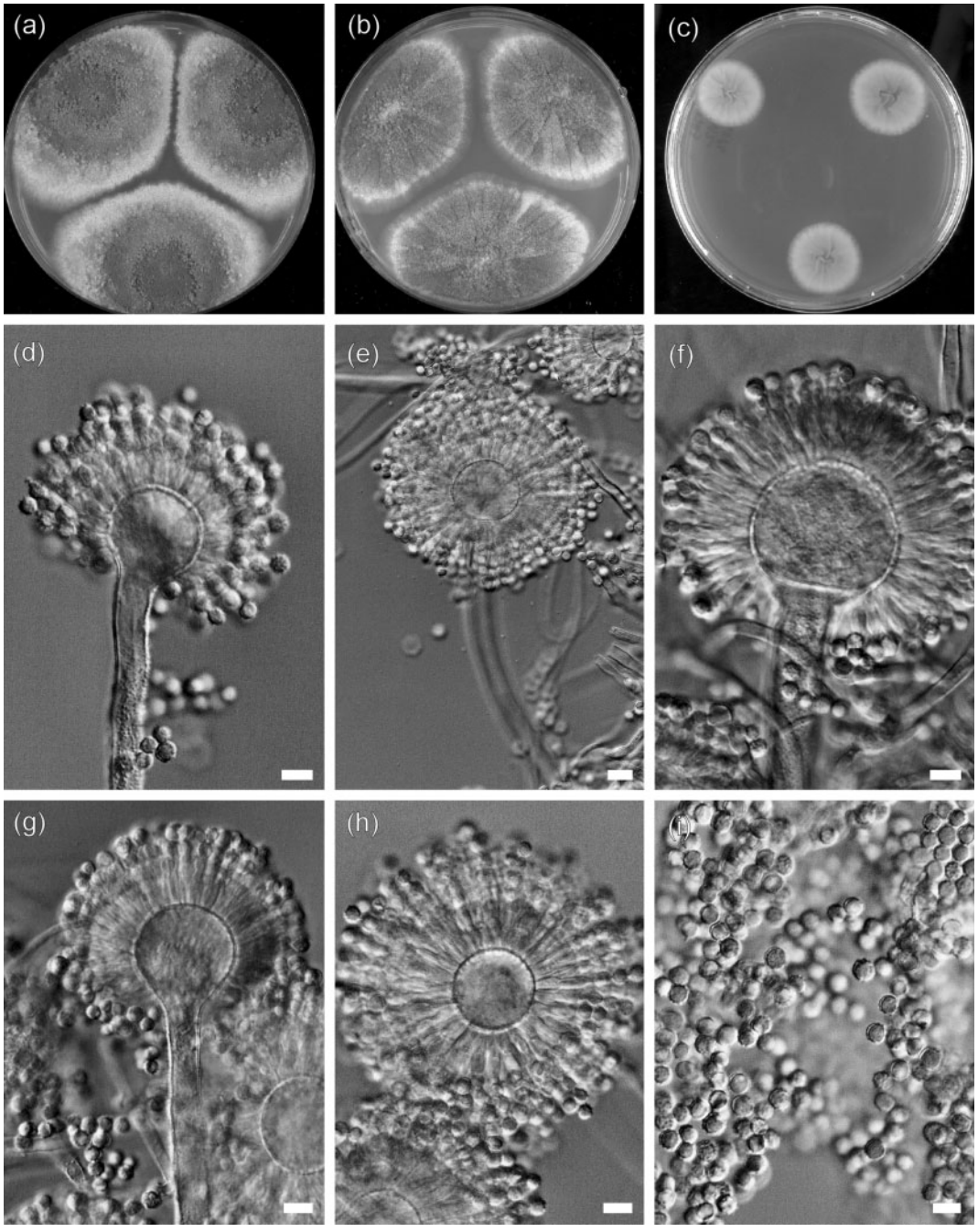

Fig. 2. *Aspergillus arachidicola* sp. nov. CBS 117610^T. (a–c) Colonies on CYA at 25 °C (a), CYA at 37 °C (b) and AFPA (c) after 7 days. (d–h) Conidiophores; (i) conidia. Bars, 10 μm. A colour version of this figure is available as Supplementary Fig. S2.

of this figure is available as Supplementary Fig. S2). Colony surface velvety with abundant conidial heads, olive to olive brown en masse (Kornerup & Wanscher, 1978). Reverse greenish yellow without diffusible pigments. Sclerotia not observed. Conidial heads uniseriate or biseriate. Stipes hyaline, finely roughened, variable in length, mostly (250–)400–600(−1000) μm; diameter just below vesicles (6.5–)9–10 μm. Vesicles globose to subglobose, (23–)28–50 μm in diameter, fertile upper 75 % of their surface; metulae 9.5–13.5 × 5–6.5 μm; phialides 7–11 × 3–6.5 μm. Conidia globose to subglobose, echinulate, greenish, (3.5–)4.5–5(–6.5) μm. Isolates grow well at 25, 37 and 42 °C.

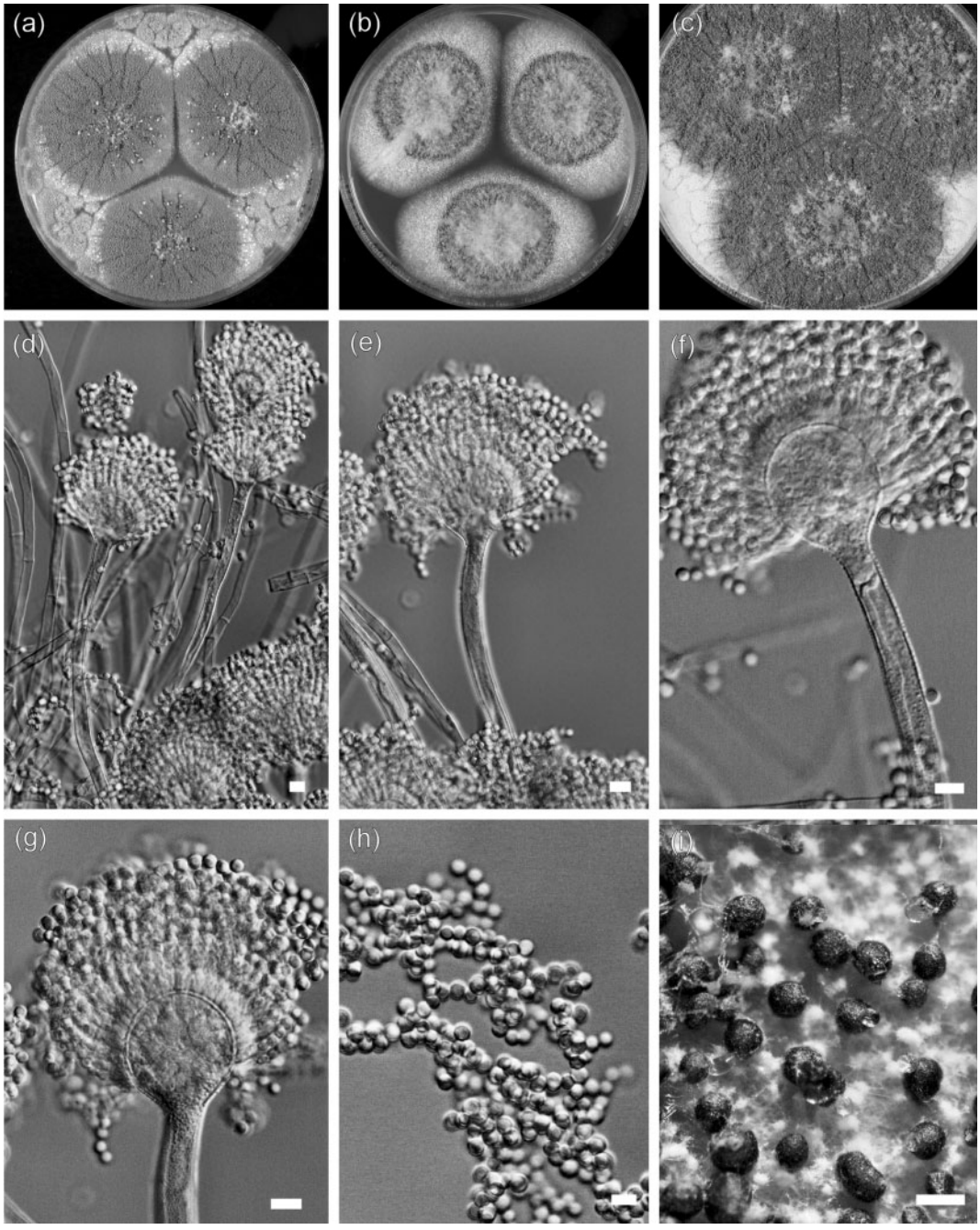

Fig. 3. *Aspergillus minisclerotigenes* sp. nov. CBS 117635^T. (a–c) Colonies on CYA at 25 °C (a), MEA (b) and YES agar (c) after 7 days. (d–g) Conidiophores; (h) conidia; (i) sclerotia. Bars, 10 μm (a–h) and 200 μm (i). A colour version of this figure is available as Supplementary Fig. S3.

Extrolites: strains of *A. arachidicola* produce kojic acid, aflatoxins B_1, B_2, G_1 and G_2 and parasiticolide, and some strains produce chrysogine.

The type strain, CBS 117610^T =IBT 25020^T, was isolated from an *Arachis glabrata* leaf, Corrientes province, Argentina.

Latin diagnosis of *Aspergillus minisclerotigenes* Vaamonde, Frisvad & Samson sp. nov. MB 505188

Coloniae in agaro MEA *dicto* 6–7 cm *diametro post* 7 *dies* 25 °C, *velutinae vel floccosae*, *mycelium vegetatovum album et conidiophora sparsa et densum stratum sclerotiorum fuscorum formantes*. *Conidia aggregata dilute viridia vel griseo-viridia*; *reversum aurantiacum vel brunneum*. *Sclerotia copiosa*, *obscure brunnea vel atra*, 150–300 μm *diametro*. *Conidiophora biseriata*, *stipes hyalinus*, *asperatus*, 1200–2000 × 11–21 μm; *vesiculae globosae vel subglobosae*, 35–50 μm *diametro*, *metulae* 11–14 × 3–5 μm; *phialideas* 6–10 × 3–5 μm. *Conidia subglobosa vel ellipsoidea*, (2–)3–4(–6) μm *diametro*, *dilute viridia*, *levia vel echinulata*.

Typus siccus in herb. CBS 117635, *et ex-typus vivus*, *isolatus Arachis hypogaea*, Córdoba *provincia* Argentina.

Description of *Aspergillus minisclerotigenes* Vaamonde, Frisvad & Samson sp. nov. MB 505188

Aspergillus minisclerotigenes (mi.ni.scle.ro.ti′ge.nes. N.L. part. adj. *minisclerotigenes* producing small sclerotia).

Colonies on YES, MEA, OA and CYA attain a diameter of 6–7 cm after 7 days at 25 °C and also on CYA at 37 °C. Colony surface velvety and, on OA and MEA, colony surface floccose, consisting of white vegetative mycelium and sparse conidial heads and dense felt of dark sclerotia (Fig. 3; a colour version of this figure is available as Supplementary Fig. S3). Conidial structures light-greyish green en masse (Kornerup & Wanscher, 1978). Colony reverse greyish orange to brownish orange on YES, yellowish brown to light brown on MEA and OA and brown on CYA. Exudate droplets are not observed. Sclerotia 150–300 μm in diameter. Conidial heads normally biseriate, but uniseriate heads sometimes occur. Conidiophore stipes 1200–2000 × 11–21 μm, hyaline, coarsely roughened. Vesicles globose to subglobose, 35–50 μm in diameter. Metulae 11–14 × 3–5 μm, phialides 6–10 × 3–5 μm. Conidia ellipsoidal, subglobose (2–)3–4(–6) μm diameter, pale green, smooth walled to echinulate. Isolates grow well at 25, 37 and 42 °C.

Extrolites: aspergillic acid, kojic acid, cyclopiazonic acid, aflatoxins B_1, B_2, G_1 and G_2, parasiticolides, paspaline and paspalinine, aflavarin, aflavinines and aflatrem. Aspergillic acid produced on AFPA.

The type strain CBS 117635^T =IBT 25032^T (dried culture) was isolated from *Arachis hypogaea*, Córdoba province, Argentina.

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REFERENCES

Applegate, K. L. & Chipley, J. R. (1973). Increased aflatoxin G_1 production by *Aspergillus flavus* via gamma irradiation. *Mycologia* **65**, 1266–1273.

Austwick, P. K. C. & Ayerst, G. (1963). Groundnut microflora and toxicity. *Chem Ind* **1963**, 55–61.

Bayman, P. & Cotty, P. J. (1993). Genetic diversity in *Aspergillus flavus*: association with aflatoxin production and morphology. *Can J Bot* **71**, 23–31.

Bennett, J. W. & Papa, K. E. (1988). The aflatoxigenic *Aspergillus* spp. *Adv Plant Pathol* **6**, 263–280.

Chang, P.-K., Ehrlich, K. C. & Hua, S.-S. T. (2006). Cladal relatedness among *Aspergillus oryzae* isolates and *Aspergillus flavus* S and L morphotype isolates. *Int J Food Microbiol* **108**, 172–177.

Codner, R. C., Sargeant, K. & Yeo, R. (1963). Production of aflatoxin by the culture of strains of *Aspergillus flavus-oryzae* on sterilized peanuts. *Biotechnol Bioeng* **5**, 185–192.

Cole, R. A. & Cox, R. H. (1981). *Handbook of Toxic Fungal Metabolites*. New York: Academic Press.

Cotty, P. J. (1989). Virulence and cultural characteristics of two *Aspergillus flavus* strains pathogenic on cotton. *Phytopathology* **79**, 808–814.

Cotty, P. J. & Cardwell, K. F. (1999). Divergence of West African and North American communities of *Aspergillus* section *Flavi*. *Appl Environ Microbiol* **65**, 2264–2266.

Dorner, J. W., Cole, R. J. & Diener, U. L. (1984). The relationship of *Aspergillus flavus* and *Aspergillus parasiticus* with reference to production of aflatoxins and cyclopiazonic acid. *Mycopathologia* **87**, 13–15.

Duran, R. M., Cary, J. W. & Calvo, A. M. (2007). Production of cyclopiazonic acid, aflatrem, and aflatoxin by *Aspergillus flavus* is regulated by *veA*, a gene necessary for sclerotial formation. *Appl Microbiol Biotechnol* **73**, 1158–1168.

Egel, D. S., Cotty, P. J. & Elias, K. S. (1994). Relationships among isolates of *Aspergillus* sect. *Flavi* that vary in aflatoxin production. *Phytopathology* **84**, 906–912.

Ehrlich, K. C., Chang, P.-K., Yu, J. & Cotty, P. J. (2004). Aflatoxin biosynthesis cluster gene *cypA* is required for G aflatoxin formation. *Appl Environ Microbiol* **70**, 6518–6524.

Feibelman, T. P., Cotty, P. J., Doster, M. A. & Michailides, T. J. (1998). A morphologically distinct strain of *Aspergillus nomius*. *Mycologia* **90**, 618–623.

Frisvad, J. C. & Samson, R. A. (2004a). *Emericella venezuelensis*, a new species with stellate ascospores producing sterigmatocystin and aflatoxin B_1. *Syst Appl Microbiol* **27**, 672–680.

Frisvad, J. C. & Samson, R. A. (2004b). Polyphasic taxonomy of *Penicillium* subgenus *Penicillium*. A guide to identification of food and air-borne terverticillate Penicillia and their mycotoxins. *Stud Mycol* **49**, 1–174.

Frisvad, J. C. & Thrane, U. (1993). Liquid column chromatography of mycotoxins. In *Chromatography of Mycotoxins: Techniques and Applications*, pp. 253–372. Edited by V. Betina. Amsterdam: Elsevier.

Frisvad, J. C., Houbraken, J. & Samson, R. A. (1999). *Aspergillus* species and aflatoxin production: a reappraisal. In *Food Microbiology*

and Food Safety into the Next Millennium, pp. 125–126. Edited by A. C. J. Tuijtelaars, R. A. Samson, F. M. Rombouts & S. Notermans. Zeist: Foundation Food Micro '99.

Frisvad, J. C., Samson, R. A. & Smedsgaard, J. (2004). *Emericella astellata*, a new producer of aflatoxin B_1, B_2 and sterigmatocystin. *Lett Appl Microbiol* **38**, 440–445.

Frisvad, J. C., Skouboe, P. & Samson, R. A. (2005). Taxonomic comparison of three different groups of aflatoxin producers and a new efficient producer of aflatoxin B_1, sterigmatocystin and 3-*O*-methylsterigmatocystin, *Aspergillus rambellii* sp. nov. *Syst Appl Microbiol* **28**, 442–453.

Geiser, D. M., Pitt, J. I. & Taylor, J. W. (1998). Cryptic speciation and recombination in the aflatoxin-producing fungus *Aspergillus flavus*. *Proc Natl Acad Sci U S A* **95**, 388–393.

Geiser, D. M., Dorner, J. W., Horn, B. W. & Taylor, J. W. (2000). The phylogenetics of mycotoxin and sclerotium production in *Aspergillus flavus* and *Aspergillus oryzae*. *Fungal Genet Biol* **31**, 169–179.

Glass, N. L. & Donaldson, G. C. (1995). Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Appl Environ Microbiol* **61**, 1323–1330.

Hesseltine, C. W., Shotwell, O. L., Ellis, J. J. & Stubblefield, R. D. (1966). Aflatoxin formation by *Aspergillus flavus*. *Microbiol Mol Biol Rev* **30**, 795–805.

Hesseltine, C. W., Shotwell, O. L., Smith, M., Ellis, J. J., Vandegraft, E. & Shannon, G. (1970). Production of various aflatoxins by strains of the *Aspergillus flavus* series. In *Toxic Microorganisms: Mycotoxins, Botulism*, pp. 202–210. Edited by M. Herzberg. Washington DC: UJNR Joint Panels on Toxic Micro-Organisms and the US Department of the Interior.

Hillis, D. M. & Bull, J. J. (1993). An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. *Syst Biol* **42**, 182–192.

Hong, S. B., Cho, H. S., Shin, H. D., Frisvad, J. C. & Samson, R. A. (2006). Novel *Neosartorya* species isolated from soil in Korea. *Int J Syst Evol Microbiol* **56**, 477–486.

Horn, B. W. (1997). *Aspergillus caelatus*, a new species in section *Flavi*. *Mycotaxon* **61**, 185–191.

Houbraken, J., Due, M., Varga, J., Meijer, M., Frisvad, J. C. & Samson, R. A. (2007). Polyphasic taxonomy of *Aspergillus* section *Usti*. *Stud Mycol* **59**, 107–128.

Ito, Y., Peterson, S. W., Wicklow, D. T. & Goto, T. (2001). *Aspergillus pseudotamarii*, a new aflatoxin producing species in *Aspergillus* section *Flavi*. *Mycol Res* **105**, 233–239.

JECFA (1997). *Evaluation of certain food additives and contaminants*. Forty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives 1996. WHO Technical Report Series 868. Geneva: World Health Organization.

Klich, M. A. & Pitt, J. I. (1985). Differentiation of *Aspergillus flavus* from *A. parasiticus* and other closely related species. *Trans Br Mycol Soc* **91**, 99–108.

Klich, M. A., Mullaney, E. J., Daly, C. B. & Cary, J. W. (2000). Molecular and physiological aspects of aflatoxin and sterigmatocystin biosynthesis by *Aspergillus tamarii* and *A. ochraceoroseus*. *Appl Microbiol Biotechnol* **53**, 605–609.

Kornerup, A. & Wanscher, J. H. (1978). *Methuen Handbook of Colour*, 3rd edn. London: Eyre Methuen.

Kumar, S., Tamura, K. & Nei, M. (2004). MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* **5**, 150–163.

Kumeda, Y., Asao, T., Takahashi, H. & Ichinoe, M. (2003). High prevalence of B and G aflatoxin-producing fungi in sugarcane field soil in Japan: heteroduplex panel analysis identifies a new genotype within *Aspergillus* section *Flavi* and *Aspergillus nomius*. *FEMS Microbiol Ecol* **45**, 229–238.

Kurtzman, C. P., Horn, B. W. & Hesseltine, C. W. (1987). *Aspergillus nomius*, a new aflatoxin-producing species related to *Aspergillus flavus* and *Aspergillus parasiticus*. *Antonie van Leeuwenhoek* **53**, 147–158.

Murakami, H. (1971). Classification of the koji mold. *J Gen Appl Microbiol* **17**, 281–309.

Murakami, H., Hayashi, K. & Ushijima, S. (1982). Useful key characters separating three *Aspergillus* taxa: *A. flavus*, *A. parasiticus* and *A. flavus*. *J Gen Appl Microbiol* **28**, 55–60.

Novas, M. V. & Cabral, D. (2002). Association of mycotoxin and sclerotia production with compatibility groups in *Aspergillus flavus* from peanut in Argentina. *Plant Dis* **86**, 215–219.

Peterson, S. W., Ito, Y., Horn, B. W. & Goto, T. (2001). *Aspergillus bombycis*, a new aflatoxigenic species and genetic variation in its sibling species, *A. nomius*. *Mycologia* **93**, 689–703.

Pildain, M. B., Vaamonde, G. & Cabral, D. (2003). Compatibilidad vegetativa y diversidad genética en *Aspergillus flavus* de la provincia de Salta, Argentina. *Bol Soc Argent Bot* **38** (Suppl.), 275 (in Spanish).

Pildain, M. B., Vaamonde, G. & Cabral, D. (2004). Analysis of population structure of *Aspergillus flavus* from peanut based on vegetative compatibility, geographic origin, mycotoxin and sclerotia production. *Int J Food Microbiol* **93**, 31–40.

Pildain, M. B., Cabral, D. & Vaamonde, G. (2005). Poblaciones de *Aspergillus flavus* en maní cultivado en diferentes zonas agroecológicas de la Argentina, caracterización morfológica y toxigénica. *Rev Invest Agropec* **34**, 3–19 (in Spanish).

Pitt, J. I., Hocking, A. D. & Glenn, D. R. (1983). An improved medium for detection of *Aspergillus flavus* and *A. parasiticus*. *J Appl Bacteriol* **54**, 109–114.

Raper, K. B. & Fennell, D. I. (1965). *The Genus Aspergillus*. Baltimore: Williams & Wilkins.

Saito, M. & Tsurota, O. (1993). A new variety of *Aspergillus flavus* from tropical soil in Thailand and its aflatoxin productivity. *Proc Jpn Assoc Mycotoxicol* **37**, 31–36.

Samson, R. A., Hoekstra, E. S. & Frisvad, J. C. (editors) (2004). *Introduction to Food- and Airborne Fungi*, 7th edn. Utrecht: Centraalbureau voor Schimmelcultures.

Samson, R. A., Noonim, P., Meijer, M., Houbraken, J., Frisvad, J. C. & Varga, J. (2007a). Diagnostic tools to identify black aspergilli. *Stud Mycol* **59**, 129–145.

Samson, R. A., Hong, S., Peterson, S. W., Frisvad, J. C. & Varga, J. (2007b). Polyphasic taxonomy of *Aspergillus* section *Fumigati* and its teleomorph *Neosartorya*. *Stud Mycol* **59**, 147–203.

Schroeder, H. W. (1966). Effect of corn steep liquor on mycelial growth and aflatoxin production in *Aspergillus parasiticus*. *Appl Microbiol* **14**, 381–385.

Smedsgaard, J. (1997). Micro-scale extraction procedure for standardized screening of fungal metabolite production in cultures. *J Chromatogr A* **760**, 264–270.

Swofford, D. L. (2000). PAUP* 4.0: phylogenetic analysis using parsimony. Sunderland, MA: Sinauer Associates.

Tanaka, T., Hasegawa, A., Aoki, N., Yamamoto, S., Udagawa, S., Sekita, S., Harada, M., Nozawa, K. & Kawai, K. (1989). Production of aflatrem and its related indoloditerpenes by microsclerotium-producing strains of *Aspergillus flavus*. *Proc Jpn Assoc Mycotoxicol* **30**, 19–23.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.

Vaamonde, G., Degrossi, C., Comerio, R. & Fernandez Pinto, V. (1995). *Aspergillus flavus* y *A. parasiticus* en maní cultivado en la provincia de Córdoba (Argentina): Características diferenciales y capacidad aflatoxicogénica. *Bol Soc Argent Bot* **30**, 191–198 (in Spanish).

Varga, J., Due, M., Frisvad, J. C. & Samson, R. A. (2007). Taxonomic revision of *Aspergillus* section *Clavati* based on molecular, morphological and physiological data. *Stud Mycol* **59**, 89–106.