ARTICLE

Polyphasic taxonomy of *Aspergillus* section *Sparsi*

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Abstract: *Aspergillus* section *Sparsi* includes species which have large globose conidial heads with colours ranging from light grey to olive-buff. In this study, we examined isolates of species tentatively assigned to section *Sparsi* using a polyphasic approach. The characters examined include sequence analysis of partial β-tubulin, calmodulin and ITS sequences of the isolates, morphological and physiological tests, and examination of the extrolite profiles. Our data indicate that the revised section *Sparsi* includes 10 species: *A. anthodesmis, A. biplanus, A. conjunctus, A. diversus, A. funiculosus, A. implicatus, A. panamensis, A. quitensis, A. sparsus,* and the new taxon *A. haitiensis.* The recently described *A. quitensis* and *A. ecuadorensis* are synonyms of *A. amazonicus* based on both molecular and physiological data. The white-spored species *A. implicatus* has also been found to belong to this section. *Aspergillus haitiensis* sp. nov. is characterised by whitish colonies becoming reddish brown due to the production of conidial heads, and dark coloured smooth stipes. The taxon produces gregatins, siderin and several unknown but characteristic metabolites.

Key words:
Aspergillus section *Sparsi*
β-tubulin
calmodulin
Eurotiales
extrolites
ITS
polyphasic taxonomy

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INTRODUCTION

The *Aspergillus sparsus* species group (*Aspergillus* section *Sparsi*; Gams *et al.* 1985) was established by Raper & Fennell (1965) to accommodate four species isolated from tropical or subtropical soils. Species assigned to this group have large globose conidial heads, which irregularly split with age, with colours ranging from light grey to olive-buff. Samson (1979) suggested that *A. gorakhpurensis* should also be placed to this section. However, phylogenetic analysis of parts of the ribosomal RNA gene cluster indicated that this species belongs to *Aspergillus* section *Cremei* (Peterson 1995, 2000). According to the recent data of Peterson *et al.* (2008) and Peterson (2008), the monophyletic section *Sparsi* belongs to subgenus *Nidulantes*, and in addition to *A. sparsus, A. biplanus, A. diversus* and *A. funiculosus,* originally placed to this section by Raper & Fennell (1965), it also includes *A. panamensis* and *A. conjunctus* previously assigned to section *Usti,* and *A. anthodesmis* which was previously placed in the *A. wentii* group (Raper & Fennell 1965).

In this study, we examined available isolates of the species proposed to belong to section *Sparsi* to clarify the taxonomic status of this section. The methods used include sequence analysis of the ITS region (including internal transcribed 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, analysis of macro- and micromorphological characters and extrolite profiles.

MATERIALS AND METHODS

Morphological examinations

The strains examined are listed in Table 1. The strains were grown for 7 d as three-point inoculations on Czapek agar, Czapek yeast autolysate agar (CYA), malt extract agar (MEA), and oatmeal agar (OA) at 25 °C and 37 °C (medium compositions in Samson *et al.* 2010).

Analysis for Extrolites

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). The secondary metabolite production was confirmed by identical UV spectra with those of standards and by comparison to retention indices and retention times for pure compound standards (Frisvad & Thrane 1993, Rahbaek *et al.* 2000).

Table 1. The *Aspergillus* section *Sparsi* isolates examined in this study.

Species	Strain No.	Origin
A. amazonicus	CBS 124228^T = E19D	Soil, Makas, Ecuador
A. anthodesmis	CBS 552.77^T = NRRL 22884	Soil, Ivory Coast
A. biplanus	CBS 468.65^T = NRRL 5071	Soil, Tilaran, Costa Rica
A. biplanus	CBS 469.65 = NRRL 5073	Soil, Tilaran, Costa Rica
A. biplanus	NRRL 5072	Soil, Tilaran, Costa Rica
A. conjunctus	CBS 476.65^T = NRRL 5080	Forest soil, Palmar, Province of Punteras, Costa Rica
A. diversus	CBS 480.65^T = NRRL 5074	Soil, Esparta, Costa Rica
A. ecuadorensis	CBS 124229^T =E19F	Soil, Makas, Ecuador
A. funiculosus	CBS 116.56^T = NRRL 4744	Soil, Ibadan, Nigeria
A. haitiensis	CBS 464.91^T = NRRL 4569	Soil under sage and cactus, Haiti
A. haitiensis	CBS 468.91 = NRRL 4568	Desert soil, Haiti
A. implicatus	CBS 484.95^T	Soil, Ivory Coast
A. panamensis	CBS 120.45^T = NRRL 1785	Soil, Panama
A. panamensis	NRRL 1786	Soil, Panama
A. quitensis	CBS 124227^T = E19C	Soil, Makas, Ecuador
A. sparsus	CBS 139.61^T = NRRL 1933	Soil, Costa Rica
A. sparsus	NRRL 1937	Soil, San Antonio, Texas, USA

Isolation and analysis of nucleic acids

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 Biotechnologies) according to the instructions of the manufacturer. Fragments containing the ITS region were amplified using primers ITS1 and ITS4 as described previously (White *et al.* 1990). Amplification of part of the β-tubulin gene was performed using the primers Bt2a and Bt2b (Glass & Donaldson 1995). Amplifications of the partial calmodulin gene were set up as described previously (Hong *et al.* 2005). Sequence analysis was performed with the Big Dye Terminator Cycle Sequencing Ready Reaction Kit for both strands, and the sequences were aligned with the MT Navigator software (Applied Biosystems). All the sequencing reactions were purified by gel filtration through Sephadex G-50 (Amersham Pharmacia Biotech, Piscataway, NJ) equilibrated in double-distilled water and analyzed on the ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The unique ITS, β-tubulin, and calmodulin sequences were deposited at the GenBank nucleotide sequence database under accession numbers FJ491645–FJ491675, and FJ943936–FJ943941.

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 1000 bootstrap replications (Hillis & Bull 1993). An *A. ochraceoroseus* isolate belonging to section *Ochraceorosei* of subgenus *Nidulantes* (Peterson *et al.* 2008) was used as outgroup in these experiments. The alignments were deposited in TreeBASE (<treebase.org/treebase-web/home.html>) under accession number S11028.

RESULTS AND DISCUSSION

Phylogeny

We examined the genetic relatedness of section *Sparsi* isolates using sequence analysis of the ITS region of the ribosomal RNA gene cluster, and parts of the calmodulin and β-tubulin genes. The calmodulin data set included 566 characters, with 288 parsimony informative characters. One of the 56 MP trees is shown in Fig. 1 (tree length: 741, consistency index: 0.7247, retention index: 0.8903). During analysis of a part of the β-tubulin gene, 494 characters were analysed, among which 196 were found to be parsimony informative. The single MP tree based on partial β-tubulin genes sequences is shown in Fig. 2 (length: 507 steps, consistency index: 0.7179, retention index: 0.8938). The ITS data set included 559 characters with 58 parsimony informative characters. One of the 702 MP trees is presented in Fig. 3 (tree length: 180, consistency index: 0.8056, retention index: 0.8763).

Phylogenetic analysis of β-tubulin, calmodulin and ITS sequence data indicated that *Aspergillus* section *Sparsi* includes 10 species. *Aspergillus biplanus* and *A. diversus* are closely related to each other on all trees, while another

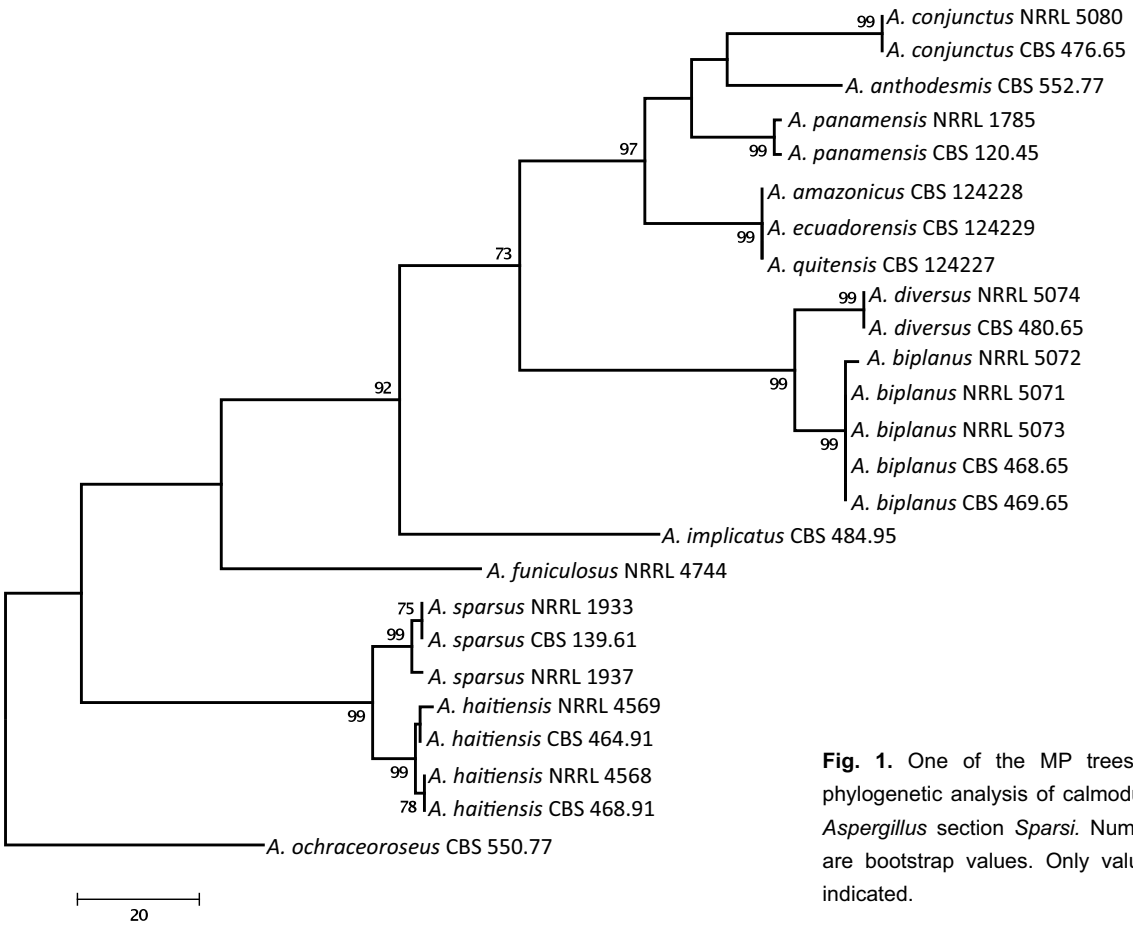

Fig. 1. One of the MP trees obtained based on phylogenetic analysis of calmodulin sequence data of *Aspergillus* section *Sparsi.* Numbers above branches are bootstrap values. Only values above 70 % are indicated.

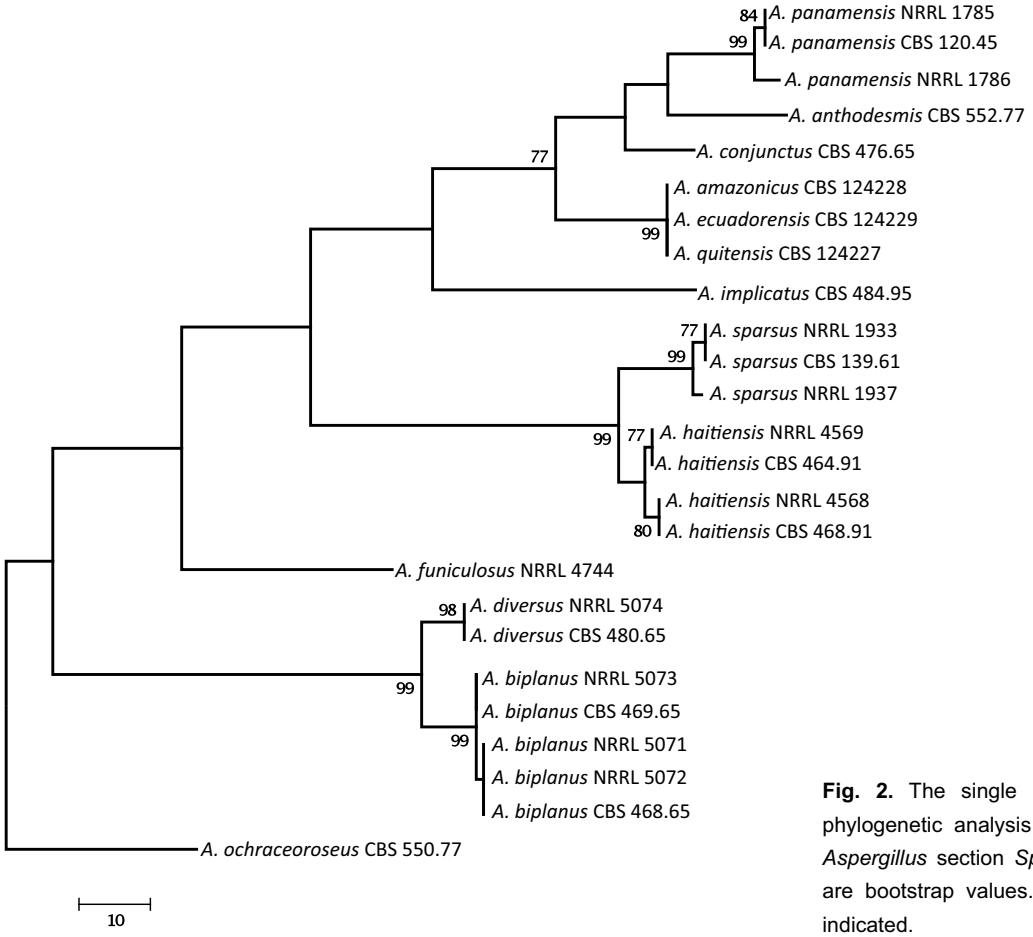

Fig. 2. The single MP tree obtained based on phylogenetic analysis of β-tubulin sequence data of *Aspergillus* section *Sparsi.* Numbers above branches are bootstrap values. Only values above 70 % are indicated.

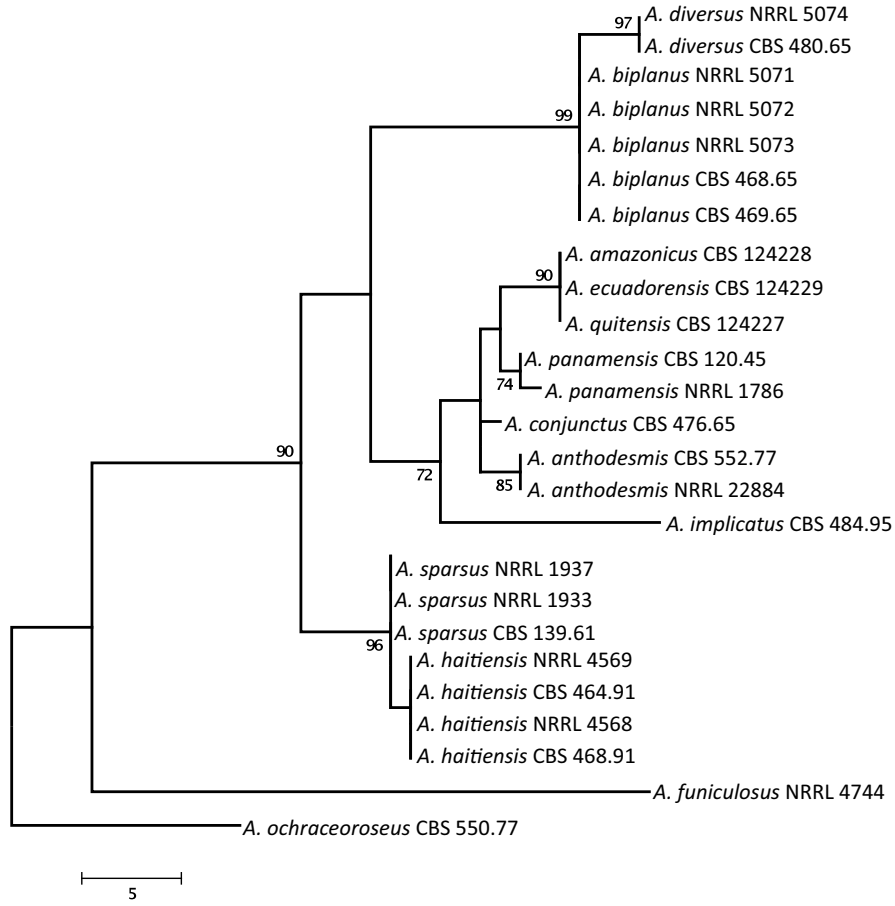

Fig. 3. One of the MP trees obtained based on phylogenetic analysis of ITS sequence data of *Aspergillus* section *Sparsi.* Numbers above branches are bootstrap values. Only values above 70 % are indicated.

clade includes *A. panamensis, A. anthodesmis, A. conjunctus,* and the recently described *A. amazonicus*, *A. quitensis* and *A. ecuadorensis* isolates on the trees based on β-tubulin and ITS sequence data (Figs 2, 3; Mares *et al.* 2008). Although Mares *et al.* (2008) found that these three isolates have identical ITS sequences, they were suggested to represent distinct species based on morphological data (length of talks, diameter of vesicles, morphology of conidia and number of phialides), and were placed in *Aspergillus* section *Wentii*. However, these three isolates could not be distinguished from each other based on molecular, morphological or extrolite data in our study, and clearly belong to section *Sparsi* (Figs 1–4). *Aspergillus amazonicus* is chosen as the correct name for the taxon and *A. quitensis* and *A. ecuadorensis* are considered synonyms. *Aspergillus implicatus*, a white-spored species originally assigned to *Aspergillus* section *Candidi* (Maggi & Persiani 1994), also belongs to this section. This species was described to produce conidiophores surrounded by sterile hyphae, not yet seen in any other species of the *Aspergillus* genus. Unfortunately the ex-type culture showed only poor sporulation and only a few conidiophores with sterile outgrowth could be observed (Fig. 5).

Phylogenetic analysis of sequence data indicated that the four examined *A. sparsus* isolates fall into two closely related clades. The three phylogenies were concordant, with no conflict between the topologies of the gene trees, in accordance with the phylogenetic species recognition concept detailed by Taylor *et al.* (2000). The ex-type strain of *A. sparsus* (CBS 139.61^{T}) together with an isolate from Texas, USA form one clade, while two isolates came from soil from Haiti form another clade on all trees (Figs 1–3). Both of the latter isolates were found by Raper & Fennell (1965) to differ from the ex-type strain of *A. sparsus* in producing more restrictedly growing colonies in shades of reddish brown on MEA plates, while one of the isolates (CBS 464.91 = NRRL 4569) also produced "small fragmentary sporulating structures adjacent to the agar surface that bear conidia similar to those of normal heads" (Raper & Fennell 1965). Here we describe this new species as *Aspergillus haitiensis*.

Regarding the value of the different loci for species delimitation in section *Sparsi*, all species could be distinguished using either ITS, β-tubulin or calmodulin sequence data. However, the resolving power was much higher for the protein coding genes than for the ITS region. The situation is more difficult in other sections of Aspergilli, including for example sections *Nigri* (Samson *et al.* 2007), *Clavati* (Varga *et al.* 2007), and *Cervini* (J. Varga, unpubl. observ.), where the ITS region cannot be used reliably to distinguish all species assigned to the given section.

Extrolite profiles

Among the species assigned to *Aspergillus* section *Sparsi, A. panamensis* produces cyclogregatin and gregatins (also called graminins or aspertetronins; Anke *et al.* 1980a, b,

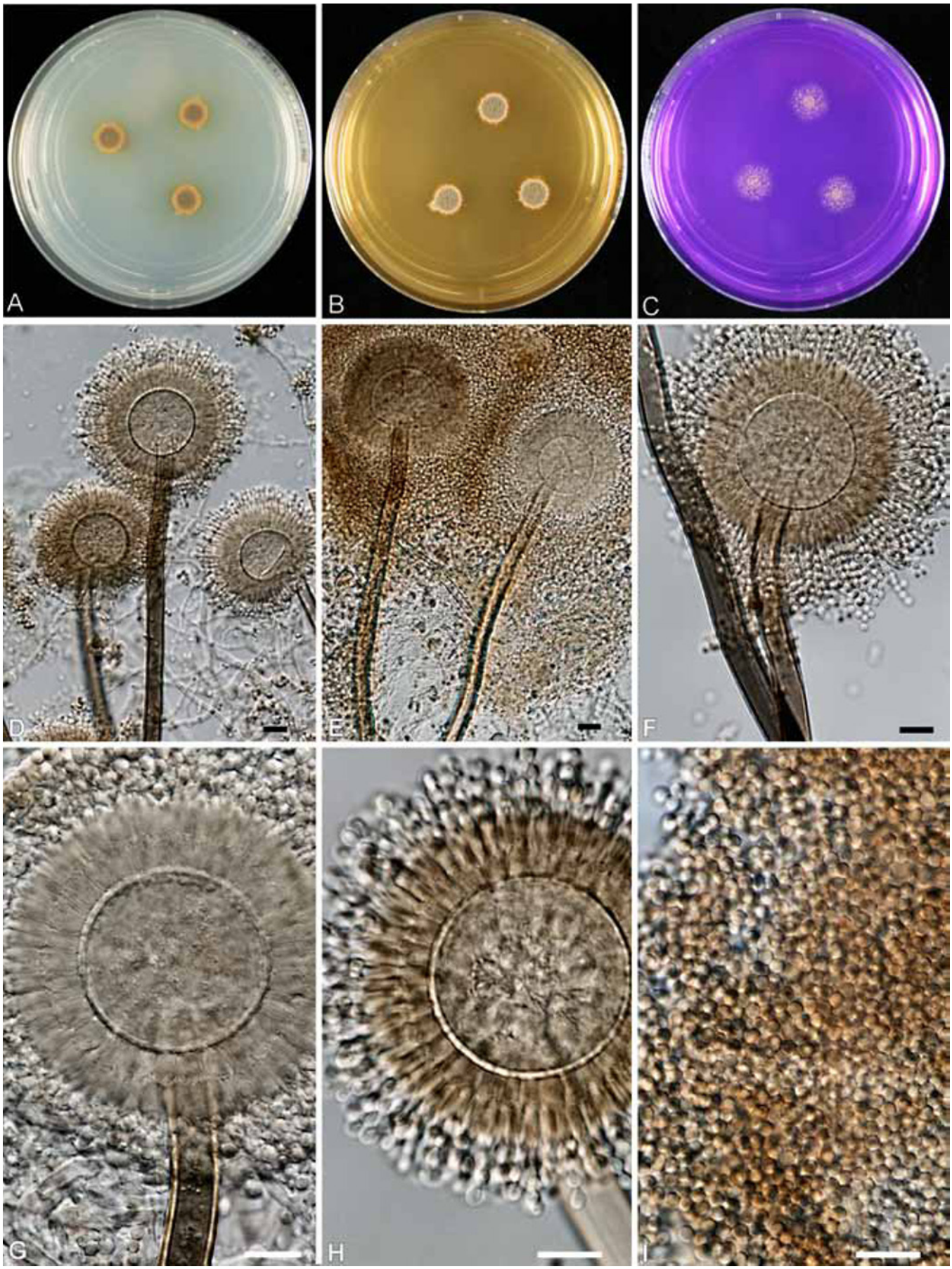

Fig. 4. *Aspergillus amazonicus* (CBS 124228). **A–C.** Colonies of 7 d grown at 25 °C; A on CYA, B on MEA, C on CREA. **D–I.** Conidiophores and conidia. Bars = 10 µm.

Table 2. Extrolites produced by species of *Aspergillus* section *Sparsi*. The structures of the extrolites in brackets have not yet been elucidated.

Species	Extrolites
A. amazonicus	an aszonalenin, (dob-indol, fot, Vurs1, vurs2, stan)
A. anthodesmis	gregatins, siderin (alk-769gl; AMF1, AMF2, AMF3, ANTW, kota, met k, tidmyco1, tidmyco2, senmyco1, senmyco2, senmyco3, UNTW)
A. biplanus	auroglaucin, (BLØDO, CUR-678, KONI, OKSI-1121, RAI-701, RAI-843, SKOT, VERN-652, VERN-655, VERN-661, VERN-673, vers-965, vers-979, vers-1049, vers-1107)
A. conjunctus	auroglaucin, siderin?, (alk-1538, alk-1756, blæam, CONJ1, CONJ2, CONJ3, DUTS, INSUX,JON1, JON2, JON3, JON4, kola, kola2, SVIF1, SVIF2, UT, verruc1, verruc2, vers-1049, vers-1107), a falconensin (? by *A. conjunctus* SRRC 423)
A. diversus	auroglaucin, mycophenolic acid?, (alka-704, CONJ1, kola2, OKSI-1, OKSI-2, vers-965, vers-979, vers-1049, vers-1107; OKSI-3, OKSI-4, OKSI-5, OKSI-6 by NRRL 5075)
A. funiculosus	arugosin E, ethericin A, funicin = ethericin B, terrein?, (AQ-798, AQ-1456, bianthron-1396, DERH, DRI, emon, hæms, NOL, RAI-921, RAI-972, storå, SULTI-1, SULTI-2, vers-818, vers-856)
A. haitiensis NRRL 4568	(ATROV, GYLA, NIDU, tidmyco1, tidmyco2, tidmyco3, spar1, spar2, spar3)
A. haitiensis NRRL 4569	gregatins, siderin, (AMF1, AMF2, AMF3, senmyco1, senmyco2, senmyco3, tidmyco1, tidmyco2, tidmyco3)
A. implicatus	a versicolorin, an austalide derivative (?)
A. panamensis	gregatins, siderin, (AQ-1456, OTTO),
A. sparsus	(NIDU, senmyco1, senmyco2, senmyco3, spar1, spar2)

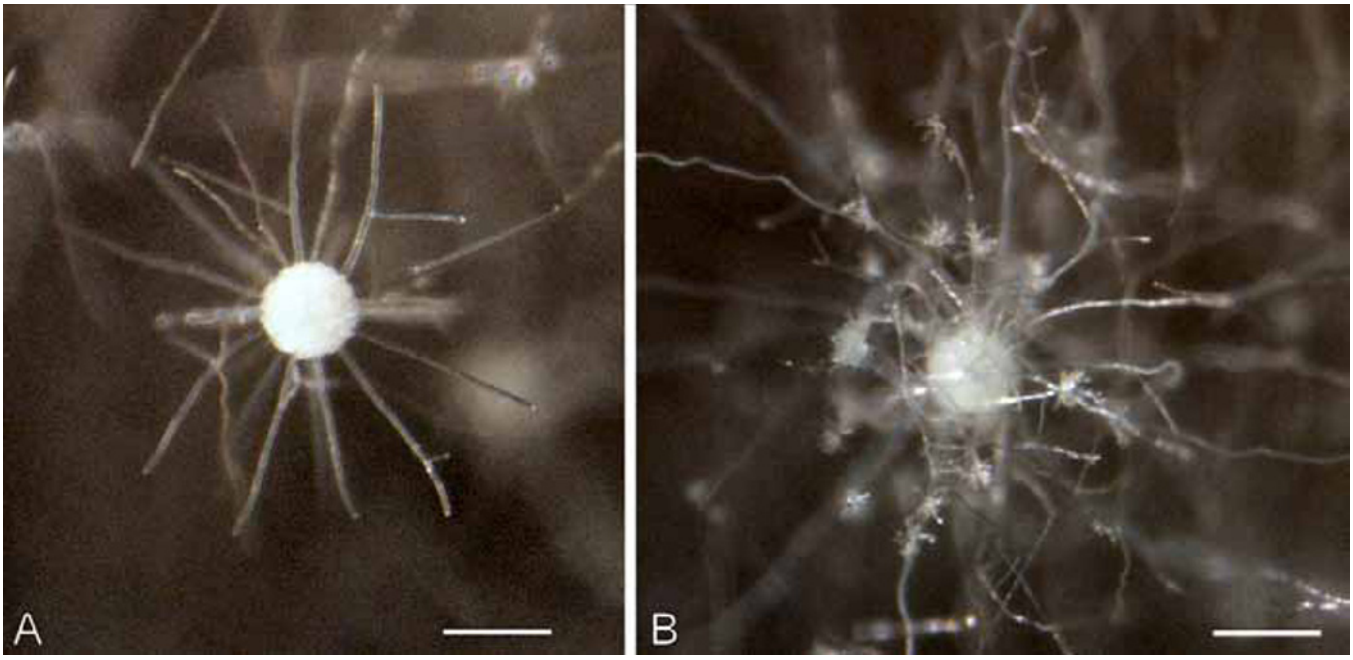

Fig. 5. *Aspergillus implicatus* (CBS 484.95). **A–B.** Conidal heads showing sterile outgrowths. Bars = 100 µm.

1988), while *A. funiculosus* has been found to produce ethericin A (also called violaceol I or aspermutarubrol), and ethericin B (or funicin; König *et al.* 1978, 1980, Nakamura *et al.* 1983) (Table 2). Ethericin A was first isolated and called aspermutarubrol from *A. sydowii*, causing the red colouration of the medium, as this unstable compound will turn into a red dye by oxidation (Shibata *et al.* 1978). The ethericins (or violaceols) are also produced by *A. versicolor* and several *Emericella* species (Fremlin *et al.* 2009). Gregatins are also produced by *A. anthodesmis* and one of the *A. haitiensis* isolates (Table 2). Siderin is related to kotanins produced by some black Aspergilli and *A. clavatus* (Samson *et al.* 2007, Varga *et al.* 2007), and is also produced by *A. panamensis, A. anthodesmis, A. conjunctus* and by an *A. haitiensis* isolate (NRRL 4569). Auraglaucin production is shared by *A. biplanus*, *A. conjunctus* and *A. diversus*, and is also produced by some *Eurotium* species (Gould & Raistrick 1934, Quilico *et al.* 1949). *Aspergillus implicatus* (Fig. 5) has been found to produce a versicolorin derivative. The two *A. haitiensis* isolates produced quite distinct extrolite profiles, but shared the production of several unknown compounds including those tentatively named tidmyco1-3. Several of the other extrolites produced by species assigned to *Aspergillus* section *Sparsi* have also been detected in other species assigned to sections *Nidulantes*, *Usti* and *Versicolores*, justifying the assignment of section *Sparsi* to *Aspergillus* subgenus *Nidulantes* (Peterson *et al.* 2008).

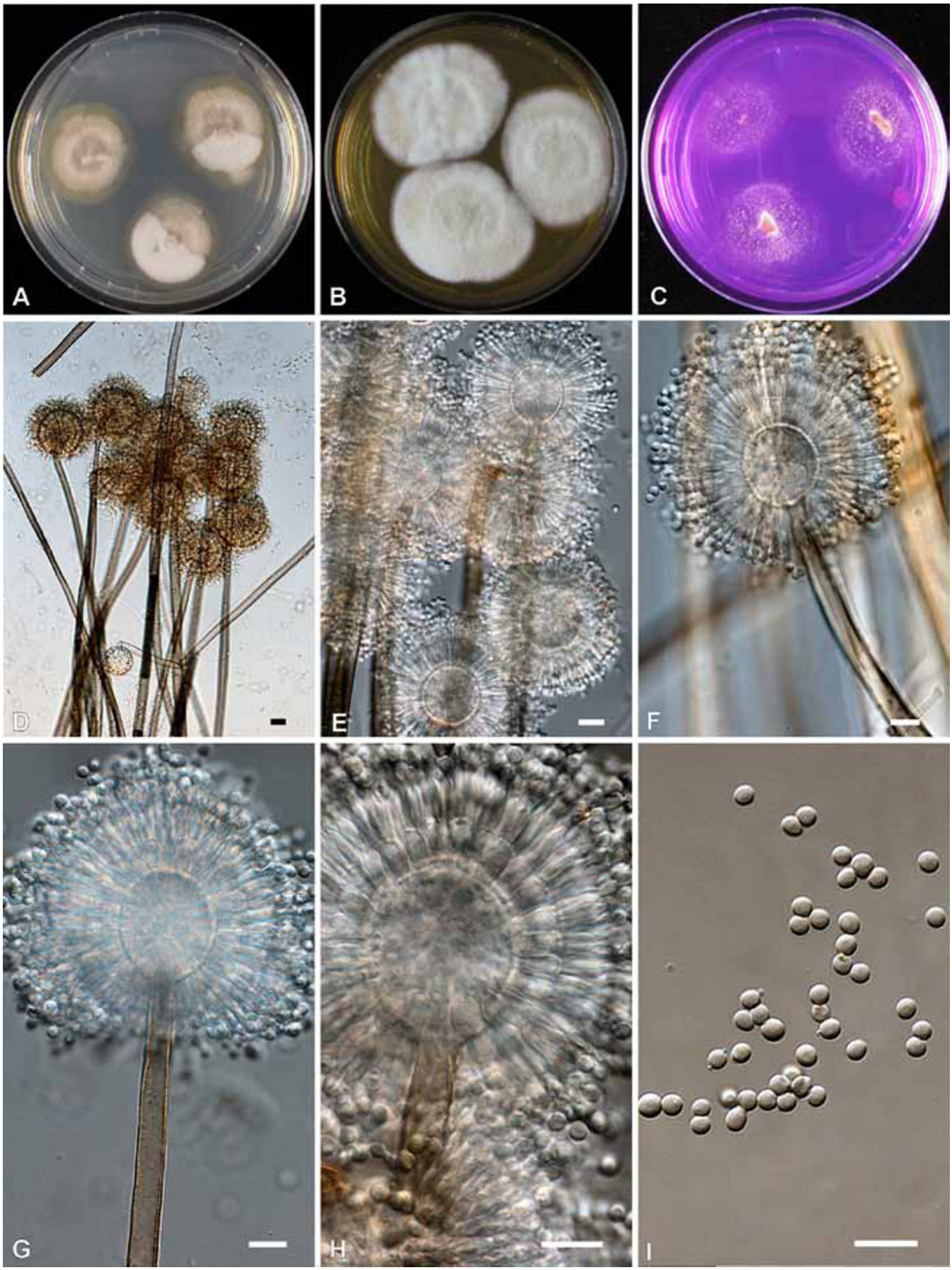

Fig. 6. *Aspergillus haitiensis* (CBS 464.91). **A–C.** Colonies of 7 d grown at 25 °C; A on CYA, B on MEA, C on CREA. **D–I.** Conidiophores and conidia. Bars = 10 µm.

Taxonomy

Aspergillus haitiensis Varga, Frisvad & Samson, **sp. nov.**
MycoBank MB517384
(Fig. 6)

Speciebus *Aspergilli* sect. *Sparsi* similes, sed coloniis porphyreis et stipitibus fuscatis, laevibus distinguitur.

Typus: Haiti: isolated from soil under sage and cactus, *W. Scott* (as 113a) (CBS H-20503 -- holotypus, cultures ex-holotype CBS 464.91 = NRRL 4569).

Colonies on MEA 50–60 mm, on CYA 30–35 mm, after 14 d at 25 °C, moderate growth on MEA after 7 d at 37 °C. Conidial heads produced sparsely on CYA, colony colour first white then reddish brown, colony texture floccose, reverse creamish to light brown. Conidial heads radiate; stipes 5–9 µm, thick-walled, dark brown in colour; vesicles 10–25 µm wide, biseriate; metulae covering the whole vesicle, measuring 2.5–4 × 5–7 µm. *Conidiogenous cells* (phialides) 2–2.5 × 7–8 µm. *Conidia* globose to ellipsoidal 4–5.6 × 5–6 µm, smooth. Fragmentary sporulating structures in addition to the normal conidial heads are also present.

Additional isolate studied: Haiti: Port de Paix, from desert soil, *W. Scott* (as 103b) (CBS 468.91 = NRRL 4568).

Diagnostic features: Thin whitish colonies turning to reddish brown colour on CYA, brown-coloured smooth stipes, and production of unknown extrolites tentatively called tidmyco1-3.

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