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. funculosus*, *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

**Article info:** Submitted: 4 November 2010; Accepted: 22 November 2010; Published: 26 November 2010.

**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. funculosus*, 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).
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 \( \beta \)-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, \( \beta \)-tubulin, and calmodulin sequences were deposited at the GenBank nucleotide sequence database under accession numbers FJ491645–FJ491675, and FJ943936–FJ943941.

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

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

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 \( \beta \)-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 \( \beta \)-tubulin gene, 494 characters were analysed, among which 196 were found to be parsimony informative. The single MP tree based on partial \( \beta \)-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 \( \beta \)-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

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.
Polyphasic taxonomy of *Aspergillus* section *Sparsi*

**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.
clade includes \textit{A. panamensis}, \textit{A. anthodesmis}, \textit{A. conjunctus},
and the recently described \textit{A. amazonicus}, \textit{A. quitensis} and
\textit{A. ecuadorensis} isolates on the trees based on $\beta$-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 \textit{Aspergillus} section \textit{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 \textit{Sparsi} (Figs 1–4).
\textit{Aspergillus amazonicus} is chosen as the correct name for the
taxon and \textit{A. quitensis} and \textit{A. ecuadorensis} are considered
synonyms. \textit{Aspergillus implicatus}, a white-spored species
originally assigned to \textit{Aspergillus} section \textit{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 \textit{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 \textit{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 \textit{A. sparsus} (CBS 139.61$^\dagger$) 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 \textit{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 \textit{Aspergillus haitiensis}.

Regarding the value of the different loci for species
delimitation in section \textit{Sparsi}, all species could be
distinguished using either ITS, $\beta$-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 \textit{Aspergilli},
including for example sections \textit{Nigri} (Samson et al.
2007), \textit{Clavati} (Varga et al. 2007), and \textit{Cervini} (J. Varga, unpubl.
observ.), where the ITS region cannot be used reliably to
distinguish all species assigned to the given section.

**Extralite profiles**

Among the species assigned to \textit{Aspergillus} section \textit{Sparsi},
\textit{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.
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).

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 azsonalenin, (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-943, 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, storà, 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.
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* similres, sed coloniis porphyreis et stipitibus fuscatis, laevis distinguuntur.

**Typus:** H: 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. *Conidigenous 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.

**ACKNOWLEDGEMENTS**

We are grateful to Tineke van Doorn who helped with the morphological data, Uwe Braun with the Latin diagnosis, and our referees.

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Polyphasic taxonomy of Aspergillus section Sparsi

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