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 \(\beta\)-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. funicolosus*, *A. implicatus*, *A. panamensis*, *A. quitensis*, *A. sparsus*, and the new taxon *A. haitiensis*. The recently described *A. quitensis* and *A. ecuadoresensis* 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*  
\(\beta\)-tubulin  
calmodulin  
Eurotiales  
extrolites  
ITS  
polyphasic taxonomy

**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. funicolosus*, 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 \(\beta\)-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).

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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 (Swoford 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 bicanus and A. diversus are closely related to each other on all trees, while another

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

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain No.</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. amazonicus</td>
<td>CBS 124228° = E19D</td>
<td>Soil, Makas, Ecuador</td>
</tr>
<tr>
<td>A. anthodesmis</td>
<td>CBS 552.77° = NRRL 22884</td>
<td>Soil, Ivory Coast</td>
</tr>
<tr>
<td>A. bicanus</td>
<td>CBS 468.65° = NRRL 5071</td>
<td>Soil, Tilaran, Costa Rica</td>
</tr>
<tr>
<td>A. biplanus</td>
<td>CBS 469.65 = NRRL 5073</td>
<td>Soil, Tilaran, Costa Rica</td>
</tr>
<tr>
<td>A. bicanus</td>
<td>NRRL 5072</td>
<td>Soil, Tilaran, Costa Rica</td>
</tr>
<tr>
<td>A. conjunctus</td>
<td>CBS 476.65° = NRRL 5080</td>
<td>Forest soil, Palmar, Province of Punteras, Costa Rica</td>
</tr>
<tr>
<td>A. diversus</td>
<td>CBS 480.65° = NRRL 5074</td>
<td>Soil, Esparta, Costa Rica</td>
</tr>
<tr>
<td>A. eucadoriensis</td>
<td>CBS 124229° = E19F</td>
<td>Soil, Makas, Ecuador</td>
</tr>
<tr>
<td>A. funiculosus</td>
<td>CBS 116.56° = NRRL 4744</td>
<td>Soil, Ibadan, Nigeria</td>
</tr>
<tr>
<td>A. haitiensis</td>
<td>CBS 464.91° = NRRL 4569</td>
<td>Soil under sage and cactus, Haiti</td>
</tr>
<tr>
<td>A. haitiensis</td>
<td>CBS 468.91 = NRRL 4568</td>
<td>Desert soil, Haiti</td>
</tr>
<tr>
<td>A. implicatus</td>
<td>CBS 484.95°</td>
<td>Soil, Ivory Coast</td>
</tr>
<tr>
<td>A. panamensis</td>
<td>CBS 120.45° = NRRL 1785</td>
<td>Soil, Panama</td>
</tr>
<tr>
<td>A. panamensis</td>
<td>NRRL 1786</td>
<td>Soil, Panama</td>
</tr>
<tr>
<td>A. quitensis</td>
<td>CBS 124227° = E19C</td>
<td>Soil, Makas, Ecuador</td>
</tr>
<tr>
<td>A. sparsus</td>
<td>CBS 139.61° = NRRL 1933</td>
<td>Soil, Costa Rica</td>
</tr>
<tr>
<td>A. sparsus</td>
<td>NRRL 1937</td>
<td>Soil, San Antonio, Texas, USA</td>
</tr>
</tbody>
</table>
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 *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) 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.
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 (Fremin 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,* 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. Extralites produced by species of *Aspergillus* section *Sparsi.* The structures of the extrolites in brackets have not yet been elucidated.**

<table>
<thead>
<tr>
<th>Species</th>
<th>Extralites</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. amazonicus</em></td>
<td>an aszonalenin, (dob-indol, fot, Vurs1, vurs2, stan)</td>
</tr>
<tr>
<td><em>A. anthodesmis</em></td>
<td>gregatins, siderin (alk-769gl; AMF1, AMF2, AMF3, ANTW, kota, met k, tidmyco1, tidmyco2, senmyco1, senmyco2, senmyco3, UNTW)</td>
</tr>
<tr>
<td><em>A. conjunctus</em></td>
<td>auroglaucin, siderin?, (alk-1538, alk-1756, bla,am, CONJ1, CONJ2, CONJ3, DUTS, INSUX, JON1, JON2, JON3, JON4, kola, kola2, SVIF1, SVIF2, UT, verruc1, verruc2, vers-1049, vers-1107), a falconenin (? by <em>A. conjunctus</em> SRRC 423)</td>
</tr>
<tr>
<td><em>A. diversus</em></td>
<td>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)</td>
</tr>
<tr>
<td><em>A. funiculosus</em></td>
<td>arugosin E, ethericin A, funicin = ethericin B, terrein?, (AQ-798, AQ-1456, bianthron-1396, DERH, DRI, emon, hæms, NOL, RAI-921, RAI-972, storø, SULTI-1, SULTI-2, vers-818, vers-856)</td>
</tr>
<tr>
<td><em>A. haitiensis</em> NRRL 4568</td>
<td>(ATROV, GYLA, NIDU, tidmyco1, tidmyco2, tidmyco3, spar1, spar2, spar3)</td>
</tr>
<tr>
<td><em>A. haitiensis</em> NRRL 4569</td>
<td>gregatins, siderin, (AMF1, AMF2, AMF3, senmyco1, senmyco2, senmyco3, tidmyco1, tidmyco2, tidmyco3)</td>
</tr>
<tr>
<td><em>A. implicatus</em></td>
<td>a versicolorin, an austalide derivative (?)</td>
</tr>
<tr>
<td><em>A. panamensis</em></td>
<td>gregatins, siderin, (AQ-1456, OTTO)</td>
</tr>
<tr>
<td><em>A. sparsus</em></td>
<td>(NIDU, senmyco1, senmyco2, senmyco3, spar1, spar2)</td>
</tr>
</tbody>
</table>

**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 similes, sed coloniis porphyreis et stipitibus fuscatis, laevisibus 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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