New Species of *Madurella*, Causative Agents of Black-Grain Mycetoma

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A new species of nonsporulating fungus, isolated in a case of black-grain mycetoma in Sudan, is described as *Madurella fahalii*. The species is characterized by phenotypic and molecular criteria. Multigene phylogenies based on the ribosomal DNA (rDNA) internal transcribed spacer (ITS), the partial β-tubulin gene (BT2), and the RNA polymerase II subunit 2 gene (RPB2) indicate that *M. fahalii* is closely related to *Madurella mycetomatis* and *M. pseudomykectomatosis*; the latter name is validated according to the rules of botanical nomenclature. *Madurella ikedae* was found to be synonymous with *M. mycetomatis*. An isolate from Indonesia was found to be different from all known species based on multilocus analysis and is described as *Madurella tropicana*. *Madurella* is nested within the order Sordariales, with *Chaetomium* as its nearest neighbor. *Madurella fahalii* has a relatively low optimum growth temperature (30°C) and is less susceptible to the azoles than other *Madurella* species, with voriconazole and posaconazole MICs of 1 µg/ml, a ketoconazole MIC of 2 µg/ml, and an itraconazole MIC of >16 µg/ml. Since eumycetoma is still treated only with azoles, correct species identification is important for the optimal choice of antifungal therapy.

Mycetoma is a chronic subcutaneous inflammatory disease usually found on the extremities. The infection can be caused by a variety of bacteria and fungi. In the human body, the microbe is organized in more or less compact hyphal or hypha-like structures called grains. The grains are widely diverse in shape, size, texture, and color, depending on the etiologic agent (4). Bacterial grains are usually soft and brittle, with a fine substructure and with colors ranging from off-white to pinkish, whereas fungal grains are rather firm and are composed of microscopically recognizable hyphae, which are either black or whitish. The most common agents of black-grain mycetoma are classified in the genus *Madurella*, characterized by the presence of nonconidiating hyphae. Currently, two species are accepted, viz., *Madurella mycetomatis* and *Madurella grisea* (4). The absence of sporulation has severely hampered species distinctions for nearly a half century, and most earlier names regarded as synonyms of *M. mycetomatis* have been discarded (13). However, recent molecular approaches have proven that the sterile agents of human mycetoma comprise numerous phylogenetically distinct species (7). The present paper considers agents of black-grain mycetoma related to *M. mycetomatis*.

The sterile agents of mycetoma have a long history. In 1902, Laveran (11) described *Streptothrix mycotomi* Laveran, which today is known as *Madurella mycetomatis* (Laveran) Brumpt, based only on a black grain. Because the fungus was not cultured and no type material was preserved, the species, known exclusively from human black-grain mycetoma, was neotypified with CBS 109801 (2, 8). In addition to *Madurella*, Brumpt (1906) (cited in reference 6a) also introduced the genus *Indiella* Brumpt for sterile etiologic agents of white-grain mycetoma. The type species is of doubtful identity (8), and therefore, the genus *Indiella* has been discarded. A total of 21 species have been described in the genera *Madurella* and *Indiella*, de Hoog et al. (8) considered 15 of these to be doubtful, because no authentic material was preserved. *Indiella americana* Delamarre et Gatti and *Madurella clapieri* (Catanei) Redaelli et Gif. were listed as synonyms of *Pseudallescheria boydii* on the basis of secondary material. *Madurella ikedae* Gammel and *Madurella americana* Gammel were treated as synonyms of *Madurella mycetomatis*. An authentic strain of *M. ikedae* from J. Gammel, CBS 247.48, was available for study. The type of *Madurella tabarkae* Blanc et Brun (5) is also lost, but their extensive description strongly suggests identity with *Madurella mycetomatis*. Recently, Yan et al. introduced a new species, *Madurella pseudomykectomatosis* Yan et al., partly defined by molecular data (19). *Madurella grisea* Mackinon et al. is unrelated, being a member of Pleosporales (7).

In the present article, we introduce a novel *Madurella* species differing from *M. mycetomatis*, *M. pseudomykectomatosis*, and *M. grisea* based on ribosomal DNA (rDNA) internal transcribed spacer (ITS), beta-tubulin gene (BT2), and RNA polymerase II subunit gene (RPB2) sequences and some phenotypic characteristics. The taxon is described as *Madurella fahalii*. The species is compared with existing species of *Madurella*, but also with the ascomycete genus *Chaetomium*, to which it bears sequence similarity. In the course of this study, we also encountered another strain, CBS 201.38, previously referred to as *Madurella americana*, that did not match with any of the known *Madurella* species; it is described as *Madurella tropicana*.

**CASE REPORT**

A 45-year-old male electrical engineer from Omdurman, Sudan, presented to the Mycetoma Research Centre, University of Khartoum, Khartoum, Sudan, with a nodule on his left sole (Fig. 1). His condition started 4 years prior to presentation as a small, painless nodule. The nodule gradually developed discharging sinuses.
There was no history of local trauma at the lesion site and no family history of mycetoma. His family history, drug history, and social contacts were not contributory to his present condition.

The patient was in good health. General and systemic examinations yielded results within normal limits. His blood pressure (BP) was 110/80, and his pulse was 86 beats/min. Local examination of the left foot revealed an irregular mass on his sole. The mass was 5 to 10 cm in diameter, firm in consistency, and fixed to the underlying structure; it was not tender, and its temperature was slightly elevated. The skin was stretched and hyperpigmented, with multiple healed and active sinuses. The discharge was seropurulent. No enlarged regional lymph nodes were detected.

His full blood count and renal and hepatic functions were within normal limits. Ultrasonic examination revealed multiple cavities with thick capsules containing thick fluid and numerous sharp, bright, hyperechoic echoes consistent with grains. Cytological examination of a fine needle aspirate revealed polymorphonuclear inflammatory cells consisting of neutrophils, lymphocytes, plasma cells, histiocytes, macrophages, and foreign-body giant cells in addition to grains. The grains were closely surrounded and occasionally infiltrated by neutrophils, causing their fragmentation. Grains were cultured on blood agar (BA) and Sabouraud glucose agar (SGA) (Difco Laboratories, Paris, France) by repeated subculture, and deposited in the CBS collection as CBS 129176.

**Physiology.** The growth rates of *M. fahalii* and five reference strains (*M. tropicana* CBS 201.38, *M. pseudomycetomatis* CBS 216.29, *M. mycetomatis* mm45, *M. mycetomatis* mm50, and *M. mycetomatis* mm55) at different temperatures were determined by incubation on SGA plates in the dark for 2 weeks at 4, 23, 30, 37, and 42°C.

**Antifungal susceptibility testing.** The susceptibilities of *M. fahalii* to antifungals were tested in triplicate by using the Sensititre system (Trek Diagnostic Systems, Ltd.) as described by van de Sande et al. (18). The compounds tested were amphotericin B (0.008 to 16 μg/ml), ketoconazole (0.008 to 16 μg/ml), itraconazole (0.008 to 16 μg/ml), posaconazole (0.008 to 8 μg/ml), fluconazole (0.125 to 256 μg/ml), voriconazole (0.008 to 16 μg/ml), 5-flucytosine (0.03 to 64 μg/ml), and caspofungin (0.008 to 16 μg/ml). As a reference, MICs for 36 *M. mycetomatis* isolates and 1 *M. pseudomycetomatis* isolate were also determined.

**DNA extraction.** Three-week-old *Madurella* cultures were scraped from SGA plates, frozen in liquid nitrogen, and ground with a mortar and pestle. DNA was extracted with the Promega Wizard kit (Promega) by adding a 300-μl lysis solution to the ground mycelium and mixing gently. From this step onward, the yeast protocol from this kit was followed according to the manufacturer’s instructions.

**Sequencing.** The ribosomal DNA internal transcribed spacer (ITS) was amplified using primers V9G (5′-TTAGTCCTGCGCTTTTGA-3′) and LS266 (5′-GCATTCCAAAACACTCGACT-3′) and was sequenced with primers ITS5 (5′-GAAGTAAAGTCGTAACAAGG-3′) and ITS4 (5′-TCCTCGGCTATTGATATGC-3′) (8). A portion of the RNA polymerase II subunit gene (RPB2) was amplified using primers RPBP2-5f (5′-GAYGAYMGWATCAAYTTYGG-3′) and RPBP2am-7r (5′-GAATTTGGCCATGTRTTCAT-3′) and was sequenced with the same primers and primer RPBP2fw2 (5′-TCATCGCGAGGAACATGAG-3′) (15). The beta-tubulin gene (BT2) was amplified using primers bt1819f (5′-TTCCGTCGCCACAACCTCGACT-3′) and bt2916 (5′-CTCACGCCTGAAGATCAT-3′) and was sequenced with the same primers and primer bt1819r (5′-TGACCCAGCAGATGTTCGAC-3′) (8, 15).

**Alignment and phylogenetic reconstruction.** Sequences were edited using Sci Ed Central software (Scientific and Educational Software, Cary, NC.). The Mega program, version 5.05 (17), was used to construct phylogenetic trees, based on the maximum-likelihood algorithm, for various members of the Sordariales, with *Pleospora herbarum* as the outgroup (Table 1). A 1,000-bootstrap replicate was used, and bootstrap values equal to or greater than 70% were considered significant. The ITS, BT2, and RPB2 genes were analyzed separately.

**Nucleotide sequence accession numbers.** ITS, β-tubulin, and RPB2 sequences were determined for *M. fahalii* CBS 129176, *M. mycetomatis* mm52, *M. mycetomatis* mm54, *M. mycetomatis* mm55, *M. mycetomatis* mm56, *M. mycetomatis* mm58, *M. mycetomatis* mm63, *M. mycetomatis* mm64, *M. mycetomatis* mm68, *M. pseudomycetomatis* CBS 129177, *M. pseudomycetomatis* CBS 216.29, and *M. tropicana* CBS 201.38. Their accession numbers can be found in Table 1.

**RESULTS**

**Morphology and physiology.** The growth rate of *M. fahalii* CBS 129176 was slow; the colony reached a diameter of 26 mm after 2 weeks at 37°C. Colonies were flat and gray-brown (Fig. 2A). No diffusible pigment was produced on SGA. Microscopically, numerous chlamydospores were observed, in addition to brownish hyphae (Fig. 2B). Chlamydospores were subglobose, thick-walled, about 10 μm in diameter, and intercalary or terminal. No conidia were detected. The fungus showed optimal growth at 30°C (Fig. 3); at this temperature, the colony reached a diameter of 29 mm.

**FIG 1** Mycetoma lesions on the left sole caused by *Madurella fahalii*. New Madurella Species

**MATERIALS AND METHODS**

**Fungal isolation.** A culture was grown from excised grains, maintained on blood agar (BA) and Sabouraud glucose agar (SGA) (Difco Laboratories, Paris, France) by repeated subculture, and deposited in the CBS collection as CBS 129176.
strain sent to CBS by G. Pollacci as EU815933.1). TMMU 3956 differed by 1 bp from CBS 216.29, a pairwise comparison of ITS sequences showed that our strain was identical to the neotype strain of Madurella mycetomatis (TMMU 3956) (GenBank accession number JN573187). A BLAST search in GenBank identified the gene of our strain was 959 bp (GenBank accession number JN573201). A BLAST search in GenBank identified the gene of our strain was 959 bp (GenBank accession number JN573201). A BLAST search in GenBank identified the gene of our strain was 959 bp (GenBank accession number JN573201). A BLAST search in GenBank identified the gene of our strain was 959 bp (GenBank accession number JN573201). A BLAST search in GenBank identified the gene of our strain was 959 bp (GenBank accession number JN573201). A BLAST search in GenBank identified the gene of our strain was 959 bp (GenBank accession number JN573201). 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**TAXONOMY**


Colonies (on SGA at 37°C) are moderately expanding, dry, yellowish brown to gray; reverse, yellowish brown to gray. Budding cells are absent. Hyphae are septate, thick-walled, pale brown. Conidiophores are absent. Many chlamydospores are present. The teleomorph is unknown. Optimal growth occurs at 30°C.

Holotype dried culture CBS-H 20690, ex-type strain CBS 129176, from human black-grain mycetoma, Khartoum, Sudan, isolated by A. Fahal.


Colonies (on SGA at 37°C) are slowly expanding, dry, brown to gray; reverse, brown to gray. An orange-brown pigment is excreted. Budding cells are absent. Hyphae are septate, thick-walled, pale brown. Conidiophores are absent. Chlamydospores are present. The teleomorph is unknown. Optimal growth occurs at 30°C.

Holotype dried culture CBS-H 20691, ex-type strain CBS
FIG 4 Consensus phylogenetic trees of the order Sordariales inferred from the ribosomal DNA internal transcribed spacer (ITS) (A), the beta-tubulin gene (BT2) (B) and the RNA polymerase II subunit gene (RPB2) (C). The trees were constructed in Mega, version 5.05, using maximum likelihood. Bootstrap support values were estimated based on 1,000 replicates and are shown above the branches. *Pleospora herbarum* was the outgroup.
129177, from human black-grain mycetoma of the lower jaw, Chongqing, China, isolated by J. Yan.


Colonies (on SGA at 37°C) are slowly expanding, dry, white-brown to gray; reverse, white-brown to gray. An orange-brown pigment is excreted. Budding cells are absent. Hyphae are septate, thick-walled, pale brown. Conidiophores are absent. Many chlamydospores are present. The teleomorph is unknown. Optimal growth occurs at 30°C.

Holotype dried culture CBS-H 20692, ex-typus strain CBS 201.38, from human black-grain mycetoma, Medan, Indonesia, 1938.

**DISCUSSION**

Several nonsporulating fungal species are known as etiologic agents of black-grain mycetoma, but their identification has been hampered by a lack of diagnostic criteria. Melanized agents that fail to produce conidia or ascospores by definition belong to the genus Madurella. Species identification traditionally has relied on colony characteristics. During the first half of the 20th century, multiple Madurella names were in use. The first agent described was Madurella mycetomi Laveran (1902), later renamed Madurella mycetomatis (Laveran) Brummitt by McGinnis et al. (11, 14). Mackinnon et al. (1954) suggested that all names referred to a single species and considered the name *M. mycetomatis* a nomen dubium (13). Machado et al. (1992) restored *M. mycetomatis*, with the older names as synonyms (12). de Hoog et al. (2000) considered the majority of the names for Madurella species to be doubtful, because no material is known to be preserved for molecular confirmation (8). Only Madurella grisea and *M. mycetomatis* were considered to be valid species. Recently, Yan et al. (2010) added a novel species, Madurella pseudomycetomatis (19), but this name was considered a nomen invalidum because the nomenclatural type was not indicated and no Latin description was given (16). We corrected this here, and the name *M. pseudomycetomatis* was reintroduced.

With the introduction of molecular phylogenetic tools such as ITS sequencing, taxonomic questions in Madurella can be addressed with more precision. Some of the species have been mentioned in the literature as Madurella species and have been given numbers, such as Madurella species 1 (7), but were never properly described (6, 7, 9). Madurella pseudomycetomatis differed from *M. mycetomatis* in several properties, e.g., the ITS sequence and the absence of exuded pigment. The ITS sequence of *M. pseudomycetomatis* differs by only 1 bp from that of CBS 216.29, sent to CBS by G. Pollacci as M. ikedae (Gammel) Pollacci et Nannizzi, and the BT2 and RPB2 sequences are also almost identical. The ex-type strain of M. *ikedae*, sent by J. Gammel to the CBS in 1948 and maintained as CBS 247.48, was found to be identical to Madurella mycetomatis. Based on these data, CBS 216.29 should be considered a misidentification. In the course of our study, we found that CBS 201.38, from a human mycetoma in Medan, Indonesia, ap-

### TABLE 2 \*In vitro*  antifungal susceptibilities of *Madurella* species

<table>
<thead>
<tr>
<th>Antifungal agent</th>
<th><em>M. mycetomatis</em>&lt;sup&gt;a&lt;/sup&gt;</th>
<th><em>M. pseudomycetomatis</em>&lt;sup&gt;a&lt;/sup&gt;</th>
<th><em>M. tropicana</em>&lt;sup&gt;a&lt;/sup&gt;</th>
<th><em>M. fahalii</em>&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphotericin B</td>
<td>1 (&lt;0.03–4)</td>
<td>0.5 (0.03)</td>
<td>0.125</td>
<td>0.5</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>0.25 (&lt;0.008–2)</td>
<td>0.03</td>
<td>0.01</td>
<td>2</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>0.25 (&lt;0.008–0.5)</td>
<td>0.03 (0.06)</td>
<td>0.01</td>
<td>&gt;16</td>
</tr>
<tr>
<td>Posaconazole</td>
<td>0.125 (&lt;0.03–0.125)</td>
<td>0.01</td>
<td>0.03</td>
<td>1</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>32 (&lt;0.125–256)</td>
<td>128 (&gt;32)</td>
<td>4</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Voriconazole</td>
<td>0.5 (&lt;0.008–2)</td>
<td>0.06 (0.03)</td>
<td>0.03</td>
<td>1</td>
</tr>
<tr>
<td>5-Flucytosine</td>
<td>&gt;64</td>
<td>&gt;64 (&gt;64)</td>
<td>&gt;64</td>
<td>&gt;64</td>
</tr>
<tr>
<td>Caspofungin</td>
<td>&gt;16</td>
<td>&gt;16</td>
<td>&gt;16</td>
<td>&gt;16</td>
</tr>
</tbody>
</table>

<sup>a</sup> One *M. fahalii* strain, 36 *M. mycetomatis* strains, 1 *M. tropicana* strain, and 2 *M. pseudomycetomatis* strains were tested.

### TABLE 3 Summary of diagnostic parameters of *Madurella* species

<table>
<thead>
<tr>
<th>Characteristic</th>
<th><em>M. mycetomatis</em></th>
<th><em>M. grisea</em></th>
<th><em>M. pseudomycetomatis</em></th>
<th><em>M. tropicana</em></th>
<th><em>M. fahalii</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Grain</td>
<td>Black</td>
<td>Black</td>
<td>Black</td>
<td>Black</td>
<td>Black</td>
</tr>
<tr>
<td>Brown pigment on SGA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+ (at 23, 30, and 37°C)</td>
<td>–</td>
<td>+ (at 23 and 30°C)</td>
<td>+ (at 23 and 30°C)</td>
<td>–</td>
</tr>
<tr>
<td>Optimal growth temp (°C)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>37</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Susceptibility to itraconazole</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Ordinal affiliation</td>
<td>Sordariales</td>
<td>Pleosporales</td>
<td>Sordariales</td>
<td>Sordariales</td>
<td>Sordariales</td>
</tr>
</tbody>
</table>

<sup>a</sup> SGA, Sabouraud glucose agar.

<sup>b</sup> When grown for 2 weeks on SGA.

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peared to be different from \textit{M. mycetomatis} as well, with considerable sequence variation in all three sequences. This strain was deposited in CBS in 1938 as \textit{Madurella americana} Gammel. Because no type material is known to exist for \textit{M. americana}, this name cannot be applied. Therefore, CBS 201.38 is introduced as a new species, \textit{M. tropicana}.

The fungi described in this report differ from \textit{M. mycetomatis}, \textit{M. pseudomyxomatis}, and \textit{M. grisea} on the basis of multilocus phylogenetic data. The sequence data also reveal that \textit{M. fahalii}, \textit{M. tropicana}, and \textit{M. pseudomyxomatis}, like \textit{M. mycetomatis}, belong to the order Sordariales, as stated previously by de Hoog et al. (7). These fungi are closely related to the genera \textit{Chaetomium} and \textit{Chaetomidium}. The criteria for generic attribution were primarily tissue morphology and overall sterility on mycological media. As noted by Mackinnon et al. (13) and confirmed by sequencing of the total small-subunit (SSU) rDNA gene of \textit{M. grisea} strain CBS 331.55 by de Hoog et al. (7), \textit{M. grisea} differs considerably from the remaining \textit{Madurella} species and appears to be nested within the Pleosporales (Table 3). Proper generic attribution will await further molecular data on pleosporalean agents of black-grain mycetoma, such as \textit{Pyrenochaeta}.

Species belonging to \textit{Madurella} in the restricted sense (i.e., belonging to the Sordariales) yielded similar physiological profiles. Optimal growth occurred at 37°C for \textit{M. mycetomatis} but at a slightly lower temperature (30°C) for \textit{M. tropicana}, \textit{M. pseudomyxomatis}, and \textit{M. fahalii}, while the latter species did not exude brown pigments into the agar (Table 3). This isolate of \textit{Madurella fahalii} differed from the remaining \textit{Madurella} species in being more resistant to the azole group of antifungal agents. The MIC of ketoconazole, currently the drug of choice for mycetoma therapy in Sudan (1), was very high for this isolate. Furthermore, \textit{M. fahalii} strain CBS 129176 proved to be resistant to itraconazole, a drug recently studied for its safety and efficacy in patients with mycetoma caused by \textit{M. mycetomatis} in Sudan (10). Since only a single isolate of \textit{M. fahalii} was available for analysis, whether all strains of \textit{M. fahalii} will display resistance to the azoles remains questionable. This question can be answered only when more isolates of \textit{M. fahalii} are discovered and tested for their antifungal susceptibilities. Unlike this isolate of \textit{M. fahalii}, \textit{M. mycetomatis} is highly susceptible to itraconazole \textit{in vitro} (3, 18). \textit{In vivo}, \textit{M. mycetomatis}-infected patients showed good clinical response to 400 mg itraconazole daily, but the fungal grain itself remained viable (10). Therefore, the most appropriate therapy for patients infected with \textit{M. fahalii} remains problematic.

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