Pleurostomophora ochracea, a Novel Agent of Human Eumycetoma with Yellow Grains

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The first yellow-grain fungal mycetoma, in a 60-year-old man from Central Sudan, is reported. Morphological and phylogenetic analysis of the ribosomal small subunit (SSU), large subunit (LSU), internal transcribed spacer (ITS), β-tubulin (*BT2*), actin (*ACT1*), and elongation factor (*TEF1*) genes revealed that the isolate deviated from any known agent of mycetoma; it clustered in the genus *Pleurostoma* (anamorph genus, *Pleurostomophora*) in the order *Calosphaeriales*. The novel species, here named *Pleurostomophora ochracea*, is characterized by phenotypic features. The species proved to be highly susceptible to itraconazole, ketoconazole, posaconazole, and voriconazole, but not to fluconazole. The fungus was inhibited by caspofungin at 8 μg/ml, while no inhibition was found with 5-flucytosine (MIC > 64 μg/ml). Compared to other members of the genus *Pleurostomophora*, *P. ochracea* is slow growing, with a relatively high optimum growth temperature (36 to 37°C). This is the first case of a yellow-grain fungal mycetoma; yellow grains are otherwise of bacterial nature. Our case emphasizes that identification of mycetoma agents by the color of the grain only is not sufficient and may lead to inappropriate therapy.

Mycetoma is a chronic granulomatous, progressive, subcutaneous inflammatory infection (12). It is caused by true fungi or by bacteria, and hence, it is classified as eumycetoma or actinomycetoma, respectively (1, 3). The triad of a painless subcutaneous mass, multiple sinuses, and seropurulent discharge containing grains is pathognomic of mycetoma (5, 14). Eumycetoma is more common than actinomycetoma throughout the world, and in Sudan, it accounts for 70% of all mycetoma cases (5). A variety of fungi are implicated in causing eumycetoma (Table 1). In Sudan, eumycetoma is usually caused by the fungus *Madurella mycetomatis*, which forms black grains in human tissue (1, 15). The most common fungal pathogens causing eumycetoma produce either black grains or white grains (5, 10). While yellow grains have been encountered in cases of actinomycetoma, they have never been reported before in eumycetoma. The available diagnostic tools for mycetoma are limited and are mostly of a phenotypic nature (1). A first indication is usually obtained from the color of the grain: black grains are produced by fungi and yellow grains by bacteria, while white grains can be of bacterial or fungal nature. Culture media used for diagnosis are based upon this first indication. Here, we present a case of yellow-grain mycetoma, which was initially misdiagnosed as actinomycetoma based on the color of the grain and on cytology. In this report, we show that cultural and molecular techniques, in combination with an updated taxonomy, can be successfully used to identify the yellow-grain-producing fungus *Pleurostomophora ochracea* as a new causative agent of human mycetoma.

CASE REPORT

The patient was a 60-year-old male farmer from central Sudan. In 2006, he presented to the Mycetoma Clinic at the Mycetoma Research Centre of the University of Khartoum, Khartoum, Sudan, with a massive, recurrent right-foot swelling. His condition started 20 years prior to presentation with a small, painless right-foot swelling, which had gradual onset and progress and was painful. He developed multiple sinuses, and the seropurulent discharge contained yellow grains. The patient underwent four surgical excisions under general and spinal anesthesia done elsewhere. He had neither a history of local trauma nor a family history of mycetoma. The social and geographic history and drug use were not contributory to his problem. On examination, he looked well, not pale and neither jaundiced nor cyanosed. He had a normal pulse rate (75/min), respiratory rate (16/min), blood pressure (120/80), and temperature (37°C). Also, his renal and hepatic function tests, as well as his full blood count, were within normal limits. Local examination revealed a painless massive mass involving the dorsal and plantar parts of the right foot. There were multiple active and healed sinuses, and the seropurulent discharge contained yellow grains (Fig. 1). The foot X-ray confirmed a massive soft tissue swelling and revealed a periosteal reaction and bone destruction (Fig. 1). Fine-needle aspiration for cytology was taken from the lesion and showed an intense inflammatory infiltrate with grains typical of actinomycetes. The patient was started on streptomycin (1 g per day) and cotrimoxazole (945 mg twice a day) and was followed up regularly in the outpatient clinic. However, there was no clinical response to this treatment. He was then switched to amikacin sulfate (15 mg/kg of body weight twice a

TABLE 1 Agents of mycetoma in mammals and their phylogenetic positions[a]

Name	Order	Grain
Acremonium kiliense	*Hypocreales*	White
Acremonium potronii	*Hypocreales*	White
Acremonium recifei	*Hypocreales*	White
Cylindrocarpon cyanescens	*Hypocreales*	White
Cylindrocarpon destructans	*Hypocreales*	White
Fusarium falciforme	*Hypocreales*	White
Fusarium verticillioides	*Hypocreales*	White
Fusarium solani	*Hypocreales*	White
Phialemonium obovatum	*Hypocreales*	White
Aspergillus flavus	*Eurotiales*	White
Aspergillus hollandicus	*Eurotiales*	White
Aspergillus nidulans	*Eurotiales*	White
Bipolaris spicifera	*Pleosporales*	Black
Corynespora cassiicola	*Pleosporales*	Black
Curvularia geniculata	*Pleosporales*	Black
Curvularia lunata	*Pleosporales*	Black
Leptosphaeria senegalensis	*Pleosporales*	Black
Leptosphaeria thompkinsii	*Pleosporales*	Black
Madurella grisea	*Pleosporales*	Black
Pseudochaetosphaeronema larense	*Pleosporales*	Black
Pyrenochaeta mackinnonii	*Pleosporales*	Black
Pyrenochaeta romeroi	*Pleosporales*	Black
Neotestudina rosatii	*Pleosporales*	White/black
Madurella mycetomatis	*Sordariales*	Black
Madurella pseudomycetomatis	*Sordariales*	Black
Madurella fahalii	*Sordariales*	Black
Madurella tropicana	*Sordariales*	Black
Phaeoacremonium parasiticum	*Diaporthales*	White
Phaeoacremonium krajdenii	*Diaporthales*	White
Exophiala jeanselmei	*Chaetothyriales*	Black
Pseudallescheria boydii	*Microascales*	White
Trichophyton sp.	*Onygenales*	White
Microsporum canis	*Onygenales*	White

[a] Adapted from de Hoog et al. ([10]).

day) and cotrimaxozole (945 mg twice a day) with no clinical improvement. He underwent surgical debulking to improve the response to medical treatment. Numerous yellow grains were collected from discharging sinuses, which were hard in consistency and irregular in size. Culturing of the grains revealed fungal growth, indicating that the patient was suffering from a eumycetoma rather than an actinomycetoma. Unfortunately, changing the treatment accordingly was not possible; the patient was lost at follow-up and was never seen again.

MATERIALS AND METHODS

Clinical specimen. A clinical specimen was collected from the patient when he presented with yellow-grain mycetoma at the Mycetoma Research Centre, University of Khartoum, Khartoum, Sudan, on 27 October 2008. After obtaining written consent from the patient, an aspirate with visible yellow grains was taken from the sinuses. The yellow grains were collected for further identification.

Fungal isolation. Grains collected from the sinus were washed twice in physiological saline and inoculated into Lowenstein-Jensen (LJ) medium (Hi Media Laboratories, Mumbai, India). The grain was cultured for 3 to 4 weeks at 37°C. This initial inoculation on LJ medium was guided by the color of the grain: yellow grains were considered to be of bacterial origin. After a week, the color of the grains converted from yellow to black, and the grain was subcultured on Sabouraud's dextrose agar (SDA) (Hi Media). The culture was identified morphologically as a fungal species. For identification, the isolate was shipped to the Department of Medical Microbiology and Infectious Diseases, Erasmus MC, Rotterdam, Netherlands, and to the Centraalbureau voor Schimmelcultures (CBS) KNAW Fungal Biodiversity Centre, Utrecht, Netherlands, and deposited in the CBS collection under number CBS 131321.

Morphology and physiology. Colony characteristics and growth morphology were studied by inoculating the isolate onto plates of malt extract agar (MEA) (Oxoid, United Kingdom), oat meal agar (OA) (homemade at CBS, Netherlands), corn meal agar (CMA) (homemade at CBS, Netherlands), and potato dextrose agar (PDA) (Oxoid, United Kingdom) and incubating the plates in the dark at 30°C for up to 4 weeks. Microscopic mounts in lactic acid with cotton blue were made from cultures grown on a PDA plate and slide cultures after 2 weeks of incubation at 30°C. The slides were examined and measured with a light microscope (Nikon Eclipse 80i), and pictures were taken using a camera attached to the microscope (Nikon; digital-sight DS-5 M). A minimum of 10 measurements per structure were taken after processing in Adobe Photoshop CS3, and the average was calculated. Cardinal growth temperatures were determined on PDA, and the cultures were incubated in the dark for 4 weeks at temperatures ranging from 15°C to 40°C at 3°C intervals. The ability of the isolate to assimilate carbohydrate sources was determined with the API 20C AUX system (bioMérieux, Marcy l'Etoile, France). Prior to the carbohydrate assimilation test, the homogenized fungal suspension was prepared by ultrasonic treatment as published previously ([4], [36]) and then was adjusted to a 2 McFarland standard with the medium provided.
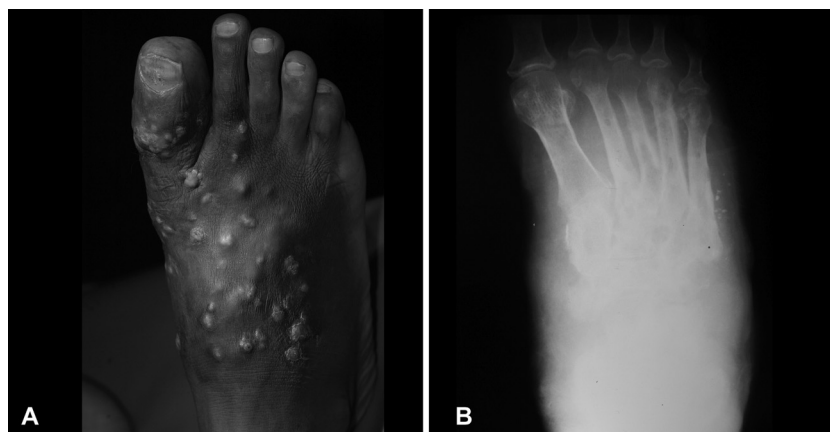

FIG 1 Clinical presentation of the patient. (A) The patient had a mycetoma on the right foot, with massive, recurrent foot swelling. (B) On the foot X-ray, a massive soft tissue swelling, a periosteal reaction, and bone destruction are seen.

TABLE 2 *Calosphaeriales* strains

Strain no.	Name	ITS	Source
STE-U 6181	*Calosphaeria africana*	EU367445	Plant
CBS 120870	*Calosphaeria africana*	EU367444	Plant
CBS 115999	*Calosphaeria pulchella*	EU367451	Plant
SM05	*Calosphaeria pulchella*	HM237300	Plant
LM06	*Calosphaeria pulchella*	HM237298	Plant
DC04	*Calosphaeria pulchella*	HM237299	Plant
SS07	*Calosphaeria pulchella*	HM237297	Plant
CBS 127681	*Jattaea discreta*	HQ878587	Plant
CBS 119343	*Jattaea leucospermi*	EU552127	Plant
STE-U 6401	*Jattaea mookgoponga*	EU367450	Plant
CBS 120867	*Jattaea mookgoponga*	EU367449	Plant
CBS 120871	*Jattaea prunicola*	EU367446	Plant
STE-U 6400	*Jattaea prunicola*	EU367448	Plant
STE-U 6399	*Jattaea prunicola*	EU367447	Plant
CBS 294.39	*Pleurostomophora repens*	AF083195	Plant
CBS 131321	*Pleurostomophora ochracea*	(JX073270)	Human
CBS 115329	*Pleurostoma ootheca*	HQ878590	Plant
IFM5 54325	*Pleurostomophora richardsiae*	AB364704	Human
CBS 302.62	*Pleurostomophora richardsiae*	AB364698	Plant
CBS 506.90	*Pleurostomophora richardsiae*	AB364702	Human
CBS 295.39	*Pleurostomophora richardsiae*	AB364697	Plant
IFM 4926	*Pleurostomophora richardsiae*	AB364694	Human
PC1	*Pleurostomophora richardsiae*	AB364693	Human
CBS 483.80	*Pleurostomophora richardsiae*	AB364701	Human
IFM 41579	*Pleurostomophora richardsiae*	AB364695	Human
CBS 270.33	*Pleurostomophora richardsiae*	AY729811	Human
CBS 406.93	*Pleurostomophora richardsiae*	AB364703	Plant
CBS 631.94	*Phaeoacremonium aleophilum*	AF266647	Plant
CBS 222.95	*Phaeoacremonium inflatipes*	AF266655	Plant
STE-U 5963	*Togninia minima*	EU128019	Plant
CBS 113648	*Togniniella acerosa*	EU367453	Plant
CBS 113726	*Togniniella acerosa*	EU367452	Plant

Finally, 100 μl of this inoculum was used to fill the cupules of the test strips as directed by the manufacturer. The ability of the isolate to hydrolyze casein, gelatin, and starch was determined by culturing the strains on selective plates according to the manufacturer's instructions. The ability to produce urease was determined by culturing on urea agar (urea base concentrate [Difco] with 1.5% [wt/vol] agar). Sodium chloride tolerance was determined by comparing the growth after 2 weeks on SDA slants containing 0, 0.5, 5, 10, and 30% NaCl.

DNA extraction, amplification, and sequencing. Genomic DNA was extracted by scraping material off Sabouraud plates, freezing it in liquid nitrogen, and grinding it with a mortar and pestle. DNA was extracted from the resulting pulp with the Promega Wizard Kit (Promega) according to the manufacturer's instructions. The ribosomal DNA (rDNA) internal transcribed spacer (ITS) gene and partial sequences of the actin (*ACT1*), β-tubulin (*BT2*), and elongation factor 1α (*TEF1*) genes, as well as the 18S rDNA gene (small subunit [SSU]) and 28S rDNA gene (large subunit [LSU]), were amplified and sequenced. The primer pairs for the genes were ITS4 and ITS5 (39), ACT-512F and ACT-783R (9), T1 and BT2b (18, 28), EF2 and EF728F (9, 23), NS1 and NS24 (17, 39), and LRoR and LR5 (38), respectively. Additional primers used for sequencing of the SSU included NS2, NS3, NS6, and NS7 (39).

Alignment and phylogenetic analysis. A consensus sequence was computed from the forward and reverse sequences with SeqMan from the Lasergene package (DNAstar, Madison, WI). Sequences retrieved from GenBank are listed in Fig. 2 and in Table 2. Sequences were aligned with Multiple Sequence Comparison by Log-Expectation (MUSCLE) using the EMBL-EBI Web server (http://www.ebi.ac.uk/Tools/msa/muscle/). An alignment was constructed for the complete ITS (ITS1-5.8S-ITS2), in-

cluding 30 strains representing 12 species. A combined 28S LSU and 18S SSU alignment for 39 strains from 36 fungal species was constructed. To investigate the phylogenetic relationship of the newly isolated fungus and related taxa, maximum-likelihood, maximum-parsimony, and Bayesian analyses were used for both alignments. Maximum likelihood with 500 bootstraps using the Tamura-Nei model and maximum-parsimony analysis were conducted in MEGA v. 5.05 (33, 34). Bayesian analysis was done using MrBayes v. 3.1.2 software. All trees were constructed by the outgroup method and edited in MEGA v. 5.05.

Antifungal susceptibility. Antifungal susceptibilities for eight antifungal drugs were determined in triplicate by using the colorimetric Sensititre YeastOne method (Trek Diagnostic Systems, East Grinstead, United Kingdom) as described previously (36). In short, the isolate was cultured for 10 days in RPMI 1640 medium supplemented with L-glutamine (0.3 g/liter) and 20 mM morpholinepropanesulfonic acid at 37°C. Mycelia were harvested by 5 min of centrifugation at 2,158 × g and washed once with sterile saline. After sonication (20 s at 28-μm maximum power; Soniprep, Beun de Ronde, Netherlands) of the hyphal suspension, Tween 60 was added at 0.05% (vol/vol), and the transmissions were adjusted to 70% at 660 nm (Novaspec II; Pharmacia Biotech). The inoculated plates were incubated at 37°C for 7 days. The drug concentrations used ranged from 0.016 μg/ml to 8 μg/ml for amphotericin B, itraconazole, ketoconazole, and voriconazole; from 0.25 μg/ml to 128 μg/ml for fluconazole; and from 0.125 μg/ml to 64 μg/ml for 5-flucytosine.

Nucleotide sequence accession numbers. The GenBank accession numbers for isolate CBS 131321 amplified genes are as follows: 28S rRNA, JX073274; 18S rRNA, JX073269; β-tubulin, JX073271; actin, JX073275; and elongation factor, JX097097.

MycoBank accession number. The MycoBank accession number for *P. ochracea* is MB800514.

RESULTS

Phylogeny. A BLAST search with the ITS, 18S SSU, and 28S LSU sequences did not yield identity with any known fungus. Therefore, the sequences were used to determine the higher-order phylogeny for our isolate. The alignment of LSU and SSU consisted of 1,909 characters, of which 1,520 were constant, 62 were parsimony uninformative, and 327 were parsimony informative. Introns were found in SSUs of three strains from the data set, and they were deleted from the alignment. Maximum-parsimony analysis of the combined data set resulted in 8 most parsimonious trees (length of the tree = 1,109; consistency index [CI] = 0.500451; retention index [RI] = 0.759444; rescaled consistency index [RCI] = 0.380065). The SSU and LSU phylogenetic analyses showed that the reported pathogenic isolate was closely related to the family *Pleurostomataceae*, with high bootstrap support with all algorithms used (1.00, 100, and 100) (Fig. 2) in the order *Calosphaeriales*.

The ITS alignment consisted of 600 characters, of which 342 were constant, 242 were parsimony informative, and 16 were parsimony uninformative. Maximum-parsimony analysis resulted in 217 most parsimonious trees (length = 356; CI = 0.688202; RI = 0.904475; RCI = 0.622462). ITS analysis confirmed that the species belonged to the *Pleurostomataceae*, in which it formed a well-supported clade with the genus *Pleurostoma* (0.99, 93, and 94) (Fig. 3). Moreover, analysis of the actin, β-tubulin, and elongation factor of the newly isolated strain and a representative isolate from each species currently attributed to the *Pleurostomataceae* confirmed that the isolate was appropriately placed in this family (data not shown) as a member of the anamorph genus *Pleurostomophora*.

Physiology. The minimum growth temperature for the isolate was 15°C, and the maximum was above 40°C, with an optimum at 36 to 37°C (Fig. 4). Measurements were also taken after 8 days of
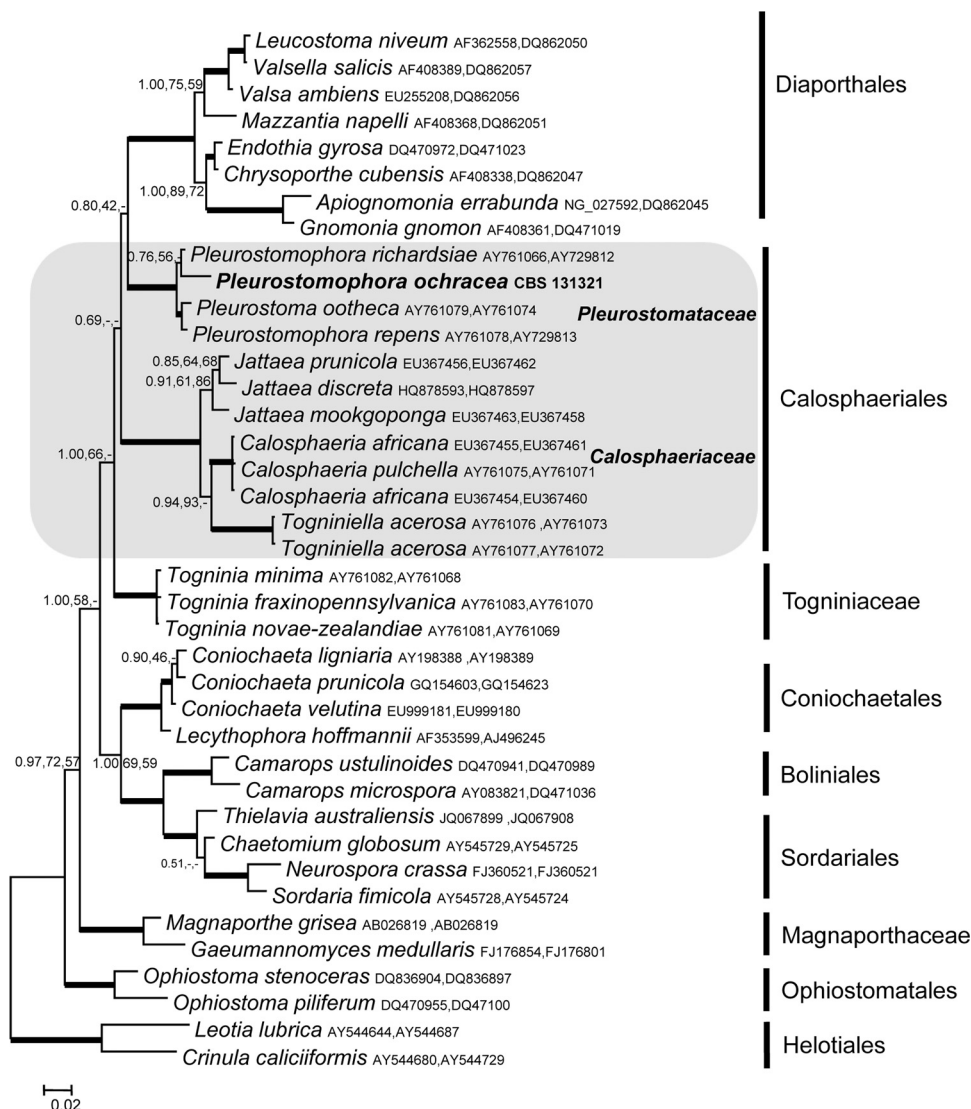
FIG 2 Phylogram of two loci (SSU and LSU) obtained by Bayesian analysis, maximum likelihood, and maximum parsimony (values of >0.8 for Bayesian probability and >80% for maximum likelihood and maximum parsimony are shown with thick branches). *Leotia lubrica* and *Crinula caliciiformis* were used as the outgroup. The portion of the phylogram relating to *Calosphaeriales* is shaded. The names of families within the order and the new isolate are shown in bold.

incubation on PDA to compare the growth pattern of the isolate with those of other *Pleurostomophora* species. The colony diameter reached only 1 to 2 mm when grown at 20°C. The isolate decomposed casein in the first week. Starch was hydrolyzed; some plates showed a narrow clear zone around the colony, and in others, a zone extending up to 2.4 cm was observed. Gelatin hydrolysis was positive using both the tube and plate techniques. Urease was strongly positive within 3 days. The isolate showed some sensitivity to cycloheximide and was sensitive to salt, exhibiting some inhibition with 0.5%, poor or no growth with 5%, and no growth with 10% and 30% NaCl.

Antifungal susceptibility. The MICs of the strain tested are shown in Table 3. Our strain was highly susceptible to the azoles itraconazole, ketoconazole, posaconazole, and voriconazole. Low MICs were also found for amphotericin B. The only azole for which a high MIC was found (128 µg/ml) was fluconazole. The

fungus was inhibited by caspofungin at 8 µg/ml, but not by 5-flucytosine (MIC > 64 µg/ml).

Taxonomy. *Pleurostomophora ochracea* Mhmoud, Abdalla Ahmed, Fahal, de Hoog, van de Sande, sp. nov. MycoBank accession number MB800514. Etymology: named after its formation of yellow grains in human tissue.

After 1 week of growth on all media tested (PDA, MEA, OA, and CMA), the colony surface and reverse were typically creamy white to pale yellow and felty, initially with some mycelial tufts. On OA and CMA, colonies turned dark olivaceous brown to nearly black after 1 month of incubation. No diffusible pigment was produced in any medium (Fig. 5). Vegetative hyphae were branched, septate, and hyaline to pale brown, turning dark brown after longer incubation. Hyphae were 1.2 to 3.2 µm wide. Hyphal walls were smooth or verruculose. Conidiophores were absent. Conidiogenous cells were variable and poorly differentiated, and phialidic. Three distinct types of phi-
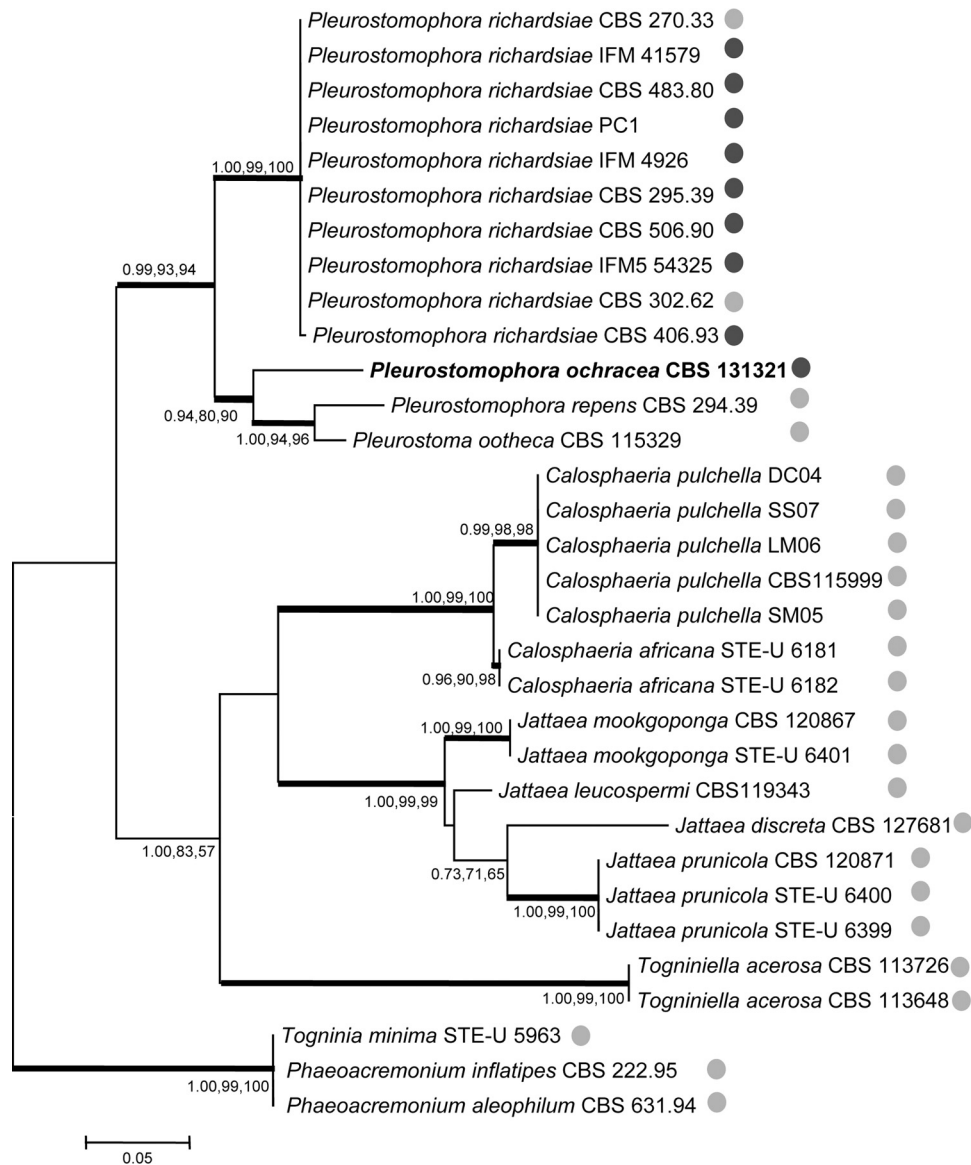
FIG 3 Phylogenetic tree resulting from Bayesian analysis, maximum likelihood, and maximum parsimony for the ITS gene (values of >0.8 for Bayesian probability and >80% for maximum likelihood and maximum parsimony are shown with thick branches). *Togninia minima*, *Phaeoacremonium inflatipes*, and *Phaeoacremonium aleophilum* were used as the outgroup. Light-gray circles, strains isolated from plants; dark-gray circles, human-pathogenic strains.
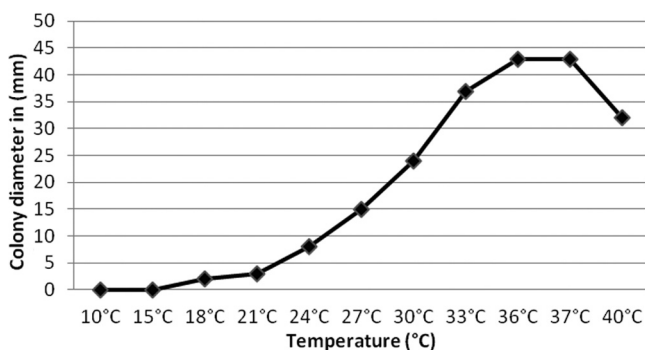
alides were observed. Type I phialides were short adelophialides without basal septa, occasionally wider at the base, and were (0.4) 1.0 to 5.0 (6.5) μm (where the parenthetical values are the minimum and maximum sizes, respectively) by (1) 1.0 to 2.0 (3) μm. Type II phi-

FIG 4 Colony diameters after 1 month of incubation at various temperatures ranging from 15 to 36°C at 3°C intervals, including 10°C, 37°C, and 40°C.

TABLE 3 Antifungal susceptibility of *P. ochracea*

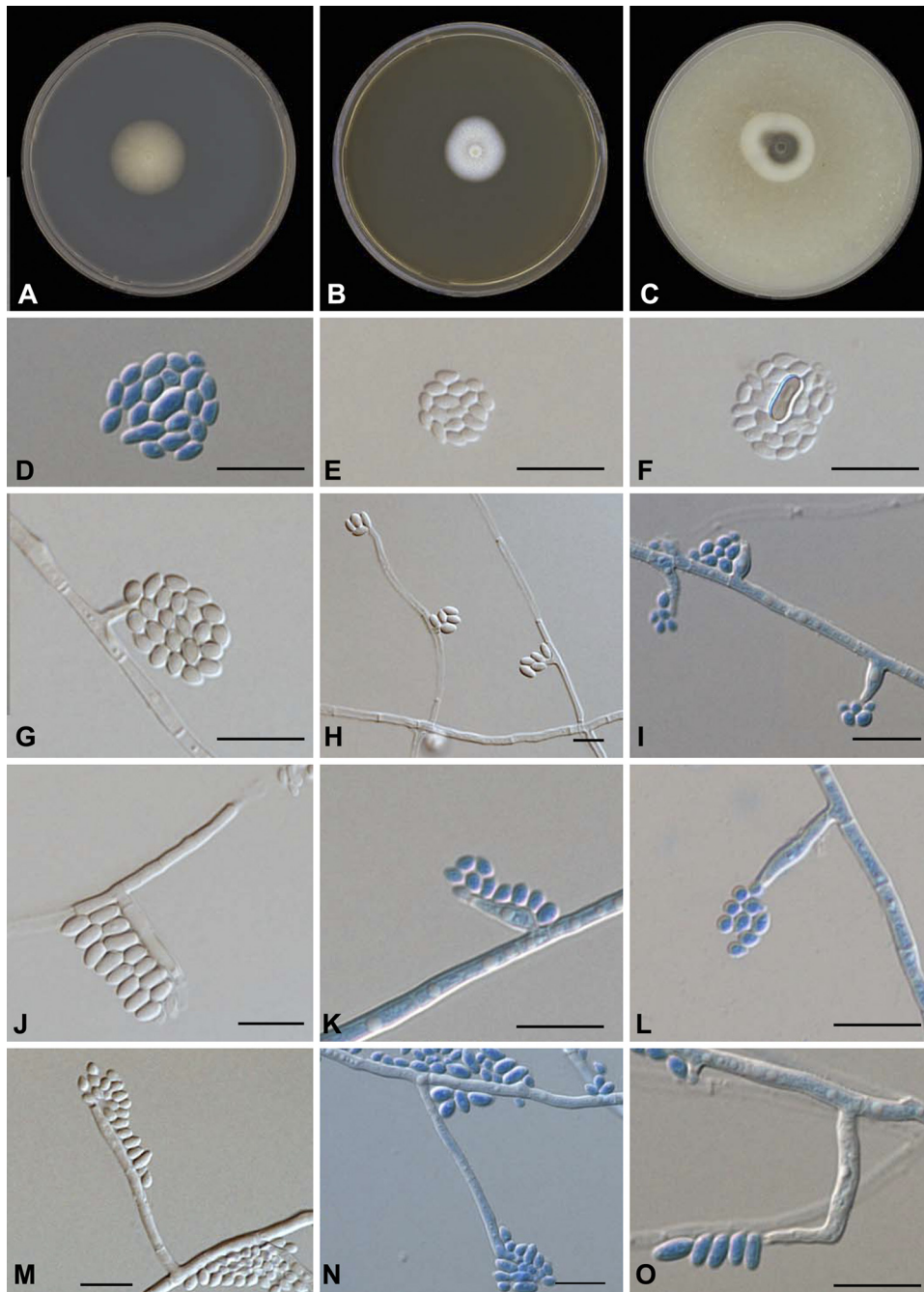| Antifungal agent	MIC (μg/ml)
Amphotericin B	1
Itraconazole	0.25
Ketoconazole	0.25
Fluconazole	128
Voriconazole	0.5
Posaconazole	1
5-Flucytosin	>64
Caspofungin	8

FIG. 5. *P. ochracea* growth and morphology. Shown are colonies after 2 weeks of incubation at 37°C. (A) PDA. (B) MEA. (C) OA. (D, E, and F) Different-shape conidia. (G, H, and I) Type I phialides. (J, K, and L) Type II phialides. (M, N, and O) Type III phialides. All scale bars, 10 μm.

alides were elongate-ampulliform, swollen at the base and tapered toward the apex, (6.2) 7 to 8.6 (8.7) by (1.3) 1.0 to 2.0 (2.5) μm. Type III phialides were (10.7) 11.6 to 17.9 (20.0) by (1.3) 1.7 to 2.4 (2.6) μm, (sub)cylindrical to elongate-ampulliform (Fig. 5). Conidia were smooth-walled and aggregate at the tip of the phialides; most conidia were hyaline, and some were brown. Two types were observed: either small and subspherical to ellipsoidal, (2.0) 2.2 to 3.0 (3.5) by (1.3) 1.6 to 2.0 (2.3) μm, or larger and allantoid, (3.5) 4 to 6 (6.2) by (1.2) 1.7 to 2.5 (2.6) μm (Fig. 5). Teleomorph unknown. Holotype: dried culture in CBS Herbarium H-20972; ex-type strain CBS 131321, isolated

by N. A. Mhmoud and A. H. Fahal from human yellow-grain mycetoma, Khartoum, Sudan.

DISCUSSION

Mycetoma is a chronic progressive disease characterized by suppurative, swollen lesions and sinuses and can be caused by both bacteria and fungi (2, 15). Based on the color of the grain, fungal mycetomata can be divided into two large groups: those causing black or white grains. Black-grain mycetoma is mainly associated with *M. mycetomatis*, *Madurella grisea*, *Leptosphaeria senegalensis*,

Pyrenochaeta romeroi, and *Exophiala jeanselmei*; white-grain mycetoma is usually caused by *Pseudallescheria boydii*, *Acremonium kiliense*, and other, occasional agents (5) (Table 1). Yellow-grain eumycetoma, to our knowledge, has never been reported. In areas of high endemicity, like the Sudan, 70% of all mycetoma cases are eumycetoma, caused by *M. mycetomatis*, a species producing black grains (5, 15). The remaining 30% of mycetoma cases are of bacterial origin, and many of these are characterized by the presence of yellow grains. With the limited availability of diagnostic techniques in routine laboratories in areas of high endemicity, the color of grains has decisive diagnostic value (15). In this communication, we report on the first authenticated case of human eumycetoma infection caused by a yellow-grain-producing fungus.

We identified the causative agent by multigene phylogenetic analysis. The novel pathogenic isolate clustered in the ascomycete order *Calosphaeriales*. The small order *Calosphaeriales* comprises only four genera separated into two families: the family *Calosphaeriaceae* contains three genera, *viz.*, *Calosphaeria*, *Jattaea*, and *Togniniella*, while our fungus clustered in the *Pleurostomataceae*, containing only a single teleomorph genus, *Pleurostoma*, and a single anamorph genus, *Pleurostomophora* (30). Our fungus was paraphyletic to *Pleurostoma ootheca* and *Pleurostomophora repens* (Fig. 3). Because of the absence of sexual sporulation, we prefer to classify the new species in the anamorph genus *Pleurostomophora*.

The majority of causative agents of mycetoma belong to the orders *Sordariales* (particularly the genus *Madurella*) and *Pleosporales* (particularly coelomycetes) (Table 1). The order *Calosphaeriales* is a new addition to the list of orders harboring potential agents. The order was described by Barr in 1983 (6, 7); recent taxonomic revision showed that the *Togniniaceae* telemorph of *Phaeoacremonium* had some affinity to *Diaporthales* (27), but in our study, the family was found as a separate cluster (Fig. 2), which is in agreement with the results of Réblová (29). Based on SSU and LSU data, and unlike previous publications (21, 29), the *Pleurostomataceae* showed affinity to the *Diaporthales*, whereas the *Calosphaeriaceae* formed a separate, well-supported clade (Fig. 2). The order *Calosphaeriales*, comprising only 10 species, partly at large mutual phylogenetic distances, is still poorly defined. Supposedly related fungi, such as *Phaeoacremonium*, were found to be unalignable for the species-level genes used in this study, i.e., with degrees of variability equal to or larger than that of the ITS.

Most members of the cluster *Pleurostoma-Pleurostomophora* are wood-inhabiting fungi (8, 37). Two cases of phaeohyphomycosis caused by *P. repens* have been reported (20, 26). However, reexamination of both pathogenic strains revealed misidentifications, with the correct name being *Phaeoacremonium krajdenii* (26). *Pleurostomophora richardsiae* is the only confirmed human-pathogenic species in the order *Calosphaeriales*. It is known as an environmental fungus but has been reported as the causative agent of subcutaneous granuloma, phaeohyphomycosis, a chromoblastomycosis-like infection, and a bone infection (19, 22, 24, 32, 35).

Pleurostomophora ochracea and *P. richardsiae* both produce different types of phialides and conidia, but in *P. richardsiae* it is noticeably more pronounced (37). In *P. ochracea*, all three types of phialides are able to produce differently shaped conidia. All species of *Pleurostomophora* and the teleomorph genus *Pleurostoma* grow at temperatures ranging between 10 and 40°C, but *P. ochracea* has a relatively high optimum growth temperature (36°C versus 30°C) and a low growth rate. This is expressed after 8 days at 20°C, where colony diameters of *P. ochracea* are only 1 to 2 mm, while *P. richardsiae* reaches 18 to 19 mm, *P. ootheca* 30 to 32 mm, and *P. repens* 59 to 61 mm (37).

The clinical isolate of *P. ochracea* in this study appeared to be highly susceptible, *in vitro*, to ketoconazole and itraconazole, compounds that are commonly used in the treatment of subcutaneous infections, including mycetoma by pigmented fungi (16, 21, 25, 31). Of all azole antifungals tested, fluconazole was the least effective. Once it was established that the causative agent was a fungus, our patient was advised to take ketoconazole at 400 mg/day, according to the guidelines of the Mycetoma Research Centre in Khartoum, Sudan. However, the patient refused treatment and did not return to the center, and follow-up was impossible.

As noted in most cases of mycetoma, it is difficult to explain how our patient acquired his infection. He did not report any type of injury. Since the patient is a farmer by occupation, he is in daily contact with soil, thorns, wood, and other trauma-causing objects; even minor trauma might have facilitated the introduction of the fungus into his foot. The present case report shows that proper species identification in the diagnosis of mycetoma remains troublesome and that identification based exclusively on the color of the grains may be erratic (11, 13). Molecular identification is recommended to ascertain appropriate therapy.

ACKNOWLEDGMENT

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