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Original Article

Roussoella percutanea, a novel opportunistic pathogen causing subcutaneous mycoses

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Abstract

We report the isolation of a novel agent of subcutaneous mycosis from a 45-year-old Indian male immigrant in the United States. Phylogenetic analysis of partial small ribosomal subunit and large ribosomal subunit, internal transcribed spacer, partial translation elongation factor (*TEF1*), and RNA polymerase second largest subunit (*rPB2*) loci revealed that the strain was identical to another isolate previously reported as “*Madurella mycetomatis*.” Both strains clustered in the order *Pleosporales*, nested in the family *Arthopyreniaceae/Roussoellaceae*. The fungus differed from known species and hence a new taxon, *Roussoella percutanea*, is introduced, typified by a strain that showed delayed production of pycnidial conidiomata. Antifungal susceptibility testing suggested that the new species is resistant to echinocandins and flucytosine, with variable results with azoles and amphotericin B.

Key words: *Roussoella*, *Pleosporales*, subcutaneous mycosis, phylogeny, antifungal susceptibility.

Introduction

Subcutaneous mycoses are localized traumatic infections caused by a wide array of clinically relevant opportunistic fungi [1]. The main types of subcutaneous mycoses include

eumycetoma, phaeohyphomycosis, and hyalohyphomycosis [2]. Both phaeohyphomycosis and hyalohyphomycosis are nonulcerative and are characterized by melanized or hyaline hyphae present in the infected tissue, respectively

[3]. Eumycetoma differs from these infections by the presence of multiple draining sinuses and by formation of grains composed of dense hyphal aggregations [4]. Some of the agents tend to be responsible for a specific type of clinical presentation. However, others may cause hyphomycoses or eumycetoma, with variable clinical presentations that may be determined by the host's immune status [5,6]. This may lead to difficulties in defining the exact clinical nature of some cases. Moreover, some pathogens remain sterile or are highly degenerated when isolated from clinical samples, hampering conventional identification with methods that largely rely on morphological characteristics. With the application of modern sequencing techniques, most nonsporulating species that cause subcutaneous infections can be attributed to known taxa [7] or appear to represent undescribed species. We report an isolate from a subcutaneous nodule in an Indian immigrant to the United States. Molecular analysis revealed that the strain belonged to the order *Pleosporales* but did not match any known taxon. Therefore, a novel species is introduced. Morphology and physiological characteristics of the new species were studied as well as its antifungal susceptibility profile.

Case report

A 45-year-old immunocompetent male immigrant from India to the United States noted an enlarging mass on his left foot and ankle in the spring of 2008, predominantly on the lateral side. He did not recall trauma in the area, and it was unclear whether he had walked barefoot at any time in his native country. However, he had received a tattoo, largely linear, on that foot area in India in 2006, which was visible overlying the swelling. A biopsy for culture was performed, revealing a filamentous fungus. Diagnoses of "eumycotic mycetoma" and "Madura foot" were conferred, although sinus tracts were not seen and it is unclear whether fungal granules were observed at that time. Identification of the organism was uncertain. Three months after initial presentation, the patient experienced a groin infection caused by methicillin-resistant *Staphylococcus aureus*, which was successfully treated with trimethoprim-sulfamethoxazole; during the same month, posaconazole 400 mg twice daily was initiated for the foot infection. Although some healing of the foot infection was noted, the patient became intolerable of the drug owing to gastrointestinal side effects; therapy was switched to voriconazole 200 mg twice daily. In August there was initial improvement of the foot, but the patient developed visual side effects due to the drug and increasing pain of the foot in September, leading to a brief trial of itraconazole. In October, magnetic reso-

Figure 1. Clinical presentation of the patient showing subcutaneous swelling and nodules.

nance imaging of the foot revealed tiny malleolar fragments in the soft tissue, thickening, edema, and cystic changes in the extensor digitorum longus tendon, with surrounding soft tissue edema and fluid collections. The radiological conclusion was tenosynovitis of the extensor digitorum longus tendon. That same month the patient was taken to surgery for the dorsal and lateral left foot, in the vicinity of the prior incision site, with incision and drainage of multiple abscesses and excision of what appeared to be chondral nodules and multiple purulent nodules. Microscopic examination of the excised material revealed masses of filamentous fungi with granulation tissue and fibrosis.

The patient refused further antifungal therapy but agreed to a third surgical procedure in January 2009. More debris was removed from the area, and the same fungus was isolated in cultures. However, swelling resumed thereafter, spreading adjacent to the prior incisions. He sought medical attention again in March for ankle swelling. A 15-assay chemical panel revealed only mild elevation of aspartate aminotransferase and alanine aminotransferase and of a random glucose. Hemoglobin A1c was elevated, consistent with a diagnosis of mild type II diabetes mellitus; complete blood count was normal. Plain radiographs of the foot revealed soft tissue swelling with no bony involvement.

A clinic visit in May led to an additional clinical diagnosis of acanthosis nigricans. The findings of the left lower leg are shown in Figure 1. There was no history of sinus tract formation. The upper nodule shown was aspirated, but only a small amount of bloody material was obtained, which was culture negative. Prolonged oral antifungal therapy was planned, but the patient was lost to follow-up; he may have returned to India.

Materials and methods

Fungal strains

The strain isolated from the present case seen in Santa Clara Valley Medical Center, San Jose, CA, USA, in 2009 was shipped to the Centraalbureau voor Schimmelcultures KNAW Fungal Biodiversity Centre, Utrecht, Netherlands, and deposited in the reference collection under number CBS 128203. Another isolate (CBS 868.95) was included in the analysis [8]. Additional strains analyzed are listed in Table 1.

Morphology and physiology

Macro- and micromorphology and growth characteristics of the isolates were studied on malt extract agar (MEA; Oxoid, UK) and oatmeal agar (OA; made at CBS [3]) plates. Plates were incubated at 30°C for 4 and up to 8 weeks. Cultures were examined with a dissecting microscope (Nikon C-DSD 230; Nikon, Tokyo, Japan), and lactic acid mounts were prepared for light microscopy examination with a Nikon ECLIPSE 80i instrument (Nikon). Photographs were taken using a Nikon digital sight DS-5M camera and analyzed using Adobe Photoshop CS5 (Adobe Systems, San Jose, CA, USA). Cardinal growth temperatures were determined on MEA plates incubated in the dark for 3 weeks at temperatures ranging from 10°C to 40°C at 3°C intervals. Laccase production was tested using ABTS agar medium containing 0.03% ABTS [2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt] [9]. ABTS plates were inoculated with standard inoculum from the tested strains and incubated for 1 week at room temperature. Carbohydrate assimilation was tested using the ID32 C identification system (bioMérieux, France), performed in duplicate to ensure reproducibility. The ability of the isolates to produce urease was determined by inoculation in 2% urea agar (Oxoid) and incubation at 25°C for 7 days.

Antifungal susceptibility testing

Susceptibility testing of filamentous fungi is the least standardized of the *in vitro* methods for testing antifungal susceptibility, particularly for slow-growing organisms. Antifungal susceptibility testing included three methodologies: agar dilution [10] and macrobroth dilution [11], as previously described, and the colorimetric Sensititre YeastOne method (Trek Diagnostic Systems) [12]. From the macrobroth dilution method, the minimum fungicidal concentration (MFC) was determined by transfer of 0.1 ml from tubes showing inhibition to Sabouraud glucose agar plates, with an endpoint of $\geq 96\%$ killing of the original inoculum as the MFC. For the Sensititre method, isolates were

transferred to Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with L-glutamine (0.3 g/l) and 20 mM morpholinepropanesulfonic acid and sonicated for 20 s at maximum power (Soniprep; Beun de Ronde, Abcoude, the Netherlands). Sonicated mycelia were incubated again at 30°C for 1 week. After growth, mycelia were harvested and washed once with RPMI and then sonicated again for 10 s at the same power. Final inocula were adjusted to 68%–72% at 660 nm (Novaspec II; Pharmacia Biotech, Cambridge, UK) in YeastOne broth (Trek Diagnostic Systems, East Grinstead, UK). Finally, Sensititre YeastOne plates were inoculated according to manufacturer's instructions and incubated at 30°C. The Sensititre system incorporates alamarBlue for viability detection by visual observation of color change; endpoint is defined as the lowest drug concentration that inhibits growth and results in no color change. Results were read after 48 h by visual inspection, and testing was performed in duplicate. For some readings by the latter method and by broth dilution, the endpoint was not reached in the dilution series, at the upper end, so it should be noted that the highest concentrations tested were 8–256 $\mu\text{g/ml}$, depending on drug and method, which will be obvious in the table of results.

DNA extraction, amplification, and sequencing

Genomic DNA was extracted by scraping a 10-mm² fungal mass grown onto the agar surface in 2-ml screw-capped tubes containing 490 μl cetyl trimethyl ammonium bromide (CTAB) buffer (2% CTAB, 100 mM Tris-HCl, 20 mM ethylenediaminetetraacetic acid, 1.4 M NaCl) and acid-washed glass beads. Subsequently, 10 μl proteinase K was added and vortex-mixed for 2–5 min. Tubes were incubated at 60°C for 1 h and vortex-mixed again to ensure homogeneity of the sample. This was followed by addition of 500 μl chloroform:isoamylalcohol 24:1 to the tubes, which were inverted repeatedly for at least 2 min. Tubes were centrifuged, and the supernatant was collected in new sterile tubes with 0.55 volumes of 2-propanol at 0°C–4°C and inverted several times. The precipitated total nucleic acids were centrifuged, and the pellet was washed with 70% ethanol, air-dried, and resuspended in 100 μL Tris-EDTA buffer.

Amplification and sequencing of internal transcribed spacer (ITS), partial small ribosomal subunit (SSU), and large ribosomal subunit (LSU) were performed with primer pairs ITS4 and ITS5 for ITS [13]; NS1 and NS24 for amplification with additional primers NS2, NS3, NS6, and NS7 for sequencing of SSU [13,14]; and LR0R and LR5 for D1/D2 of LSU [15]. Partial amplification and sequencing of elongation factor 1- α (*TEF1*) and DNA-dependent RNA polymerase II second largest

Table 1. GenBank accession numbers for the species analyzed in this study.

Species name	No.	GenBank accession number				
		Large ribosomal subunit	Small ribosomal subunit	<i>rPB2</i>	<i>EF</i>	Internal transcribed spacer
<i>Roussoella percutanea</i>	CBS 128203	KF366448	KF366450	KF366453	KF407988	KF322117
<i>R. percutanea</i>	CBS 868.95	KF366449	KF366451	KF366452	KF407987	KF322118
<i>R. hysteroioides</i>	CBS 546.94	KF443381	AY642528	KF443392	KF443399	KF443405
<i>R. hysteroioides</i>	KT 1651	AB524621	AB524480	AB539101	AB539114	
<i>R. pustulans</i>	KT 1709	AB524623	AB524482	AB539103	AB539116	
<i>R. hysteroioides</i>	CBS 125434	AB524622	AB524481	AB539102	AB539115	
<i>R. tosaensis</i>	KT 1659	AB524625	AB524484	AB539104	AB539117	
<i>R. intermedia</i>	CBS 170.96	KF443382	KF443390	KF443394	KF443398	KF443407
<i>Arthopyrenia salicis</i>	CBS 368.94	AY538339	AY538333	KF443397	KF443404	KF443410
<i>Torula herbarum</i>	CBS 379.58	KF443383	KF443388	GU456362	KF443400	
<i>T. herbarum</i>	CBS 220.69	KF443384	KF443389	KF443393	KF443401	KF443406
<i>T. herbarum</i>	CBS 595.96	KF443385	KF443387	KF443395	KF443402	KF443408
<i>T. herbarum</i>	CBS 111855	KF443386	KF443391	KF443396	KF443403	KF443409
<i>Melanomma pulvis-pyrius</i>	CBS 400.97	DQ678072	DQ678020	DQ677969	DQ677916	
<i>M. pulvis-pyrius</i>	CBS 109.77	FJ201986	FJ201987	GU456359	GU456274	
<i>Herpotrichia juniperi</i>	CBS 200.31	DQ678029	DQ678029	DQ677978	DQ677925	
<i>H. diffusa</i>	CBS 250.62	DQ678071	U42484	DQ677968	DQ677915	
<i>Pleomassaria siparia</i>	CBS 279.74	DQ678078	DQ678027	DQ677976	DQ677923	
<i>Lophiostoma compressum</i>	KT 521	JN941380	JN941375	JN993490		
<i>L. compressum</i>	KT 534	JN941379	JN941376	JN993492		
<i>L. angustilabrum</i>	CBS 629.86	DQ678069	DQ678017	DQ677965	DQ677912	
<i>L. arundinis</i>	CBS 621.86	DQ782384	DQ782383	DQ782386	DQ782387	
<i>Murispora rubicunda</i>	IFRD 2017	FJ795507	GU456308		GU456289	
<i>Neomassariosphaeria grandispora</i>	CBS 613.86	GU301842	GU296172	EF165042	GU349036	
<i>Amniculicola parva</i>	CBS 123092	GU301797	GU296134		GU349065	
<i>A. lignicola</i>	CBS 123094	EF493861	EF493863	EF493862	GU456278	
<i>A. immersa</i>	CBS 123083	FJ795498	GU456295	GU456358	GU456273	
<i>Kalmusia scabrispora</i>	KT 2202	AB524594	AB524453	AB539094	AB539107	
<i>Bimuria novae-zelandiae</i>	CBS 107.79	AY016356	AY016338	DQ470917	DQ471087	
<i>Montagnula opulenta</i>	CBS 168.34	NG_027581	NG_013127	DQ677984		
<i>Karstenula rhodostoma</i>	CBS 690.94	AY787933	GU296154	GU371788	GU349067	
<i>Paraphaeosphaeria michotii</i>	CBS 652.86	GQ387581	GU456304	GU456351	GU456266	
<i>Paraconiothyrium minitans</i>	CBS 122788	EU754173	EU754074	GU371776	GU349083	
<i>Preussia terricola</i>	AFTOL-ID 282	NG_027612	NG_013151	DQ470895	DQ471063	
<i>Sporormiella minima</i>	CBS 524.50	DQ678056	DQ678003	DQ677950	DQ677897	
<i>Sporormia lignicola</i>	CBS 264.69	GU301872	GU296197	GU371765	GU349027	
<i>Dothidea sambuci</i>	AFTOL-ID 274	AY544681	NG_012432	DQ522854	DQ497606	
<i>Phaeosclera dematioides</i>	CBS 157.81	GU301858	GU296184		GU349047	

subunit (*rPB2*) was done with primer pairs EF-983F and EF-2218R (<http://www.aftol.org/pdfs/EF1primer.pdf>) and *rPB2*-5F and *rPB2*-7 CR [16], respectively.

Alignment and phylogenetic reconstruction

A consensus of forward and reverse sequences was computed and edited with SeqMan Lasergene package (DNASTar, Madison, WI, USA). Sequences derived from this study were deposited in GenBank; accession num-

bers of deposited sequences and sequences retrieved from GenBank are listed in Table 1. Partial sequences of LSU and SSU were aligned by MUSCLE using the EMBL-EBI web server (<http://www.ebi.ac.uk/Tools/msa/muscle/>). Partial sequences of *rPB2* and *TEF1* were aligned in the RevTrans 1.4 web server [17]. Concatenated alignments of LSU, SSU, *rPB2*, and *TEF1* were prepared using Data-Convert 1.0 (<http://inbio.byu.edu/faculty/dam83/cdm>). To investigate the phylogenetic relationship of the isolated species and related taxa, maximum likelihood and Bayesian

analysis were used. Bayesian analysis was done in MrBayes v. 3.1.2 in the Cipres web server (<http://www.phylo.org/>). For the combined matrix, two parallel runs with four Markov chain Monte Carlo chains for each run was performed in MrBayes, and the number of generations was set for 20 000 000. Maximum likelihood analysis was performed using RAXML v. 7.2.8 in the Cipres web server. Trees derived from each analysis were edited in MEGA v. 5.05 [18]. ITS sequences were aligned by MUSCLE, and the phylogenetic analysis was done with maximum likelihood and Bayesian analysis, as mentioned above.

Results

Phylogeny

The ITS sequence of the isolated strain CBS 128203 did not show identity with any known species with a BLAST search

in GenBank. However, in the CBS database, 100% identity was found with strain CBS 868.95 listed as “*Madurella mycetomatis*.” Phylogenetic analysis of SSU, LSU, *rPB2*, and *TEF1* was done to determine the higher ordinal and familial positions of the isolates. The combined dataset consisted of 38 taxa with 3233 total alignment characters. The cladogram derived from Bayesian analysis is presented in Figure 2; *Dothidea sambuci* and *Phaeosclera dematioides* were used as the outgroup. Phylogenetic analysis showed that the isolates were clustered in the order *Pleosporales* and in the family *Arthopyreniaceae/Roussoellaceae* with high Bayesian inference posterior probability and bootstrap support (1.00, 100%), respectively (Fig. 2). The unknown species was found in the basal lineage of the genera *Arthopyrenia*, *Roussoella*, and *Roussoellopsis*.

ITS alignment at the familial level was constructed including 16 taxa comprising 561 alignment characters. The

Figure 2. Phylogram of the combined dataset small ribosomal subunit, large ribosomal subunit, *EF*, and *rPB2* obtained by Bayesian analysis and maximum likelihood (values > 0.8 Bayesian posterior probability, and >80% maximum likelihood are shown with bolded branches).

Figure 3. Phylogenetic tree of internal transcribed spacer resulting from Bayesian analysis and maximum likelihood (values at the nodes are Bayesian inference posterior probability/bootstrap support).

ITS tree contained representative strains of *Arthopyrenia*, *Roussoella*, and *Torula herbarum*, and the tree was rooted with *Lophiostoma arundinis* and *L. compressum*. The ITS analysis was in accordance with the combined genes tree and the tested species clustered in the family *Arthopyreniaceae/Roussoellaceae* in a position paraphyletic to *Arthopyrenia* and *Roussoella* (Fig. 3).

Physiology

The optimum growth temperature for the isolates was 30°C, maximum was 40°C, and minimum was below 10°C (Fig. 4). The isolates were urease and laccase positive with a halo for laccase reaching 40–45 mm. The results of carbohydrate assimilation for our strain CBS 128203 and CBS 868.95 are shown in Table 2. Both tested strains assimilated

a large diversity of carbohydrates in ID32 C strips but remained negative for LAT (DL-lactate), RIB (D-ribose), and GRT (sodium glucuronate). Results were variable with LAC (D-lactose; Table 2).

Antifungal susceptibility

The two isolates were tested using three methods, which produced some disparate results (Table 3). The 2008 isolate from our patient had been tested by agar dilution, with minimal inhibitory concentrations (MICs) of ≤ 0.03 µg/ml at 72 and 96 h to posaconazole and of 0.06 µg/ml and 0.125 µg/ml to voriconazole, respectively. However, those results cannot be compared with the results in Table 3 for methodological discrepancies because the intervening treatments that are described involving those and other drugs could have altered susceptibility, in addition to the methodological differences. Despite the differences in results in Table 3 with respect to methods and between CBS 868.95 and CBS 128203, there are some preliminary conclusions that might be useful to guide future therapy. Very little fungicidal activity was demonstrated. Echinocandins and flucytosine appear to have no useful role; of the azoles, fluconazole appears to be the least promising. Both of the testing methods that were applied to both isolates show that CBS 868.95 is more resistant to amphotericin B than is CBS 128203. Even when either method was examined alone in Table 3, there were differences between the two isolates (eg, voriconazole, posaconazole), which suggest that testing of individual future isolates could give isolate-specific useful information. There is considerable methodological variability, however, in many of the results with the other azoles, which obscures drawing conclusions about this class of drugs.

Figure 4. Colony diameters of CBS 128203 and CBS 868.95 after 2 weeks of incubation at temperatures ranging from 10°C–40°C with 3°C intervals.

Table 2. Carbohydrate assimilation test for CBS 128203 and CBS 868.95.

Carbohydrate	CBS 128203	CBS 868.95	Carbohydrate	CBS 128203	CBS 868.95
GAL (D-galactose)	[+]	[+]	SOR (D-sorbitol)	[+]	[+]
ACT (Actidione)	[+]	[+]	XYL (D-xylose)	[+]	[+]
SAC (D-saccharose)	[+]	[+]	RIB (D-ribose)	[−]	[−]
NAG (N-acetyl-glucosamine)	[+]	[+]	GLY (glycerol)	[−]	[−]
LAT (DL-lactate)	[−]	[−]	RHA (L-rhamnose)	[+]	[+]
ARA (L-arabinose)	[+]	[+]	PLE (palatinose)	[+]	[+]
CEL (D-cellobiose)	[+]	[+]	ERY (erythritol)	[+]	[+]
RAF (D-raffinose)	[+]	[+]	MEL (D-melibiose)	[+]	[+]
MAL (D-maltose)	[+]	[+]	GRT (sodium glucuronate)	[−]	[−]
TRE (D-trehalose)	[+]	[+]	MLZ (D-melezitose)	[+]	[+]
2KG (potassium 2-keto-gluconate)	[+]	[+]	GNT (potassium gluconate)	[−]	[−]
MDG (methyl-D-glucopyranoside)	[+]	[+]	LVT (levulinate)	[−]	[−]
MaN	[+]	[+]	GLU (D-glucose)	[+]	[+]
LAC (D-lactose)	[−]	[+]	SBE (L-sorbose)	[+]	[+]
INO (inositol)	[+]	[+]	GLN (glucosamine)	[W]	[W]
ESC (esculin)	[−]	[−]			

[−], negative; [+], positive; [W], weak.

Table 3. Antifungal susceptibility test results for CBS 128203 and CBS 868.95 done by Sensititre YeastOne and broth macrodilution methods.

Drug	Isolate					
	CBS 128203			CBS 868.95		
	Sensititre MIC	Macrobroth dilution MIC	MFC	Sensititre MIC	Macrobroth dilution MIC	MFC
Amphotericin B	0.5	1	1	4	>8	>8
Flucytosine	>64	>32	>32	>64	>64	>64
Ketoconazole	0.5	nd	nd	0.5	nd	nd
Itraconazole	0.5	8	>8	0.25	>8	>8
Fluconazole	>256	4	>64	16	16	>64
Voriconazole	2	8	>8	0.25	>8	>8
Posaconazole	1	1	1	0.25	>8	>8
Caspofungin	>16	25	>50	>16	>50	>50
Micafungin	nd	>16	>16	nd	>16	>16
Anidulafungin	nd	>16	>16	nd	8	>16

All results in micrograms per liter.

MFC, minimum fungicidal concentration; MIC, minimal inhibitory concentration; nd, not determined.

Taxonomy

Roussioella percutanea: S. A. Ahmed, D. A. Stevens, W. van de Sande, and S. de Hoog, sp. nov. (Fig. 5), MB 804857

Colonies on MEA cottony, creamy white, turning dark gray with age. Colonies on OA floccose, dark grayish green, with pale gray margin. Hyphae branched, septate, hyaline becoming dark brown with age. Pycnidia observed after 8 weeks, 59–102 × 54–96 µm, black, solitary, spherical to subspherical, with thin wall of *textura angularis*. Conidiophores hyaline, phialidic, obclavate. Conidia 1.2–2.0 × 0.7–0.9 µm, hyaline to pale brown, unicellular, ellipsoidal.

Teleomorph unknown. Minimum growth temperature below 10°C, optimum 30°C, maximum 40°C (Fig. 4).

Holotype: dried culture in CBS herbarium H-21351; type strain CBS 868.95, from subcutaneous lesion on lateral right foot of patient originated from Curacao, J. F. Meis.

Discussion

Subcutaneous mycoses are a heterogeneous group of infections that occur after traumatic inoculation of etiologic agents into cutaneous or subcutaneous tissue [2]. The

Figure 5. *Roussoella percutanea*, CBS 868.95. Colonies after 2 weeks of incubation on (A) malt extract agar and (B) oatmeal agar. C–D. Pycnidia. E. Conidiophores. F. Pycnidium wall. G. Conidia. All scale bars = 10 µm.

infection remains localized and may expand slowly to the adjacent tissue and deep structures [19]. Infections are either quickly resolved or chronic. Complete clinical pictures of some of the infections take years to develop, and most patients are unable to recall any incidence of trauma at the site of infection [20]. Our immunocompetent patient denied any history of injury to the infected foot, and the previously reported case [8] also did not recall any trauma. However, our patient had a tattoo adjacent to the lesion site, which may have been the cause of trauma and subsequent infection, especially if it was made using unclean equipment. In some rare cases of mycetoma, sinuses may be poorly demarcated. In such cases, the presence of fungal granules in tissue is the only diagnostic feature to distinguish mycetoma from phaeohyphomycosis with local necrosis [21]. After reviewing our patient's clinical history, we judged the infection to be different from mycetoma because of the absence of sinuses. As mentioned, hyphal masses with granulation tissue and fibrosis were noted. Also, in the previously reported case [8], only hyphae with necrosis and inflammation and without sinus tracts and granules were noted. Recurrences were common in our patient and in the previously reported patient [8].

Definitive diagnosis of agents of subcutaneous mycosis and correct identification of the etiologic agent are required to implement appropriate therapy [22]. In the recent past, this was difficult because many isolates from subcutaneous locations remain sterile and lack characteristic sporulation, while some may produce conidiomata or ascomata only

with much delay [7]. In the present case, strain 128203 remained sterile without any characteristic sporulation, interfering with identification by morphology. With BLAST search in GenBank of the ITS sequence, no match with any known fungus was obtained. However, in the CBS database, 100% ITS identity was obtained with strain CBS 868.95 deposited as *Madurella mycetomatis*. The latter isolate was recovered from a pedal swelling of a 63-year-old renal transplant recipient from Curacao; the clinical details of that case are described elsewhere [8]. The Curacao strain was identified as *Madurella* because of the absence of sporulation. However, at extended incubation, the strain formed characteristic conidiomata and conidia. The identity of CBS 868.95 with coelomycetous morphology and CBS 128203 was confirmed in all partitions analyzed. Sporulating strain CBS 868.95 was selected as the type of the new species.

Sequencing of conserved gene fragments showed that the strains belonged to the order *Pleosporales* and were nested in a group containing *Arthopyrenia salicis*, CBS 368.94 (Fig. 2), and several *Roussoella* species. The exact family relations of this clade are not well resolved [23]. The genus was first affiliated with the family *Amphisphaeriaceae* in (*Xylariales*) and later with *Didymosphaeriaceae* (*Pleosporales*). Zhang et al. [24] tentatively referred to the clade as *Massariaceae* because of the relative vicinity of *Massaria inquinans*. However, in our tree, *M. inquinans* was found to be distant and was excluded from our analysis. Tanaka et al. [25] used the family name *Arthopyreniaceae*, a large

polyphyletic group of lichenized fungi. The remaining relatives (Figs. 2 and 3) are *Roussoella* and *Roussoellopsis* that both contain bambusicolous species that produce ascomata with septate ascospores [25,26]. The type species of the genus *Roussoella* described by Saccardo [27] is *R. nitidula*, which was later redescribed by Hyde et al. [28], who assigned *R. hysterioides* as the type species of the genus. Furthermore, they described the anamorph of the genus as *Cytoplea hysterioides*, which was characterized by production of pycnidial conidiomata [26]. The relationship of *Arthopyrenia* species with *Roussoella* in a clade of *Pleosporales* is unexpected, and a new family *Roussoellaceae* will be introduced [A. Aptroot, personal communication]. Our new species is unlikely to have a close relationship with the lichenized species of *Arthopyrenia* and is therefore described in the genus *Roussoella* as *R. percutanea*. The species showed morphological similarity with the type species of the genus *Roussoella* by forming characteristic conidiomata and conidia. However, conidia produced by *C. hysterioides* are two-celled, unlike those of *R. percutanea*, which are continuous. Strain CBS 170.96 of *R. intermedia* showed pycnidia-producing single-celled conidia similar to those of *R. percutanea*. *Roussoella percutanea* is the first opportunistic pathogen described in the genus *Roussoella*. Given the geographic sites where the disease was contracted in the two patients, it is noteworthy that this newly described pathogen appears to have a widespread geographic distribution.

In vitro susceptibility testing revealed that the MICs of echinocandins and flucytosine would not be attained by clinically achievable concentrations of these drugs, and results with azoles and amphotericin B are unpredictable. Until further clinical experience is gained with treatment courses, it is suggested that the clinician initially be guided by the results of *in vitro* antifungal susceptibility testing of a patient's isolate [22]. Given the observed intermethod variability in test results between isolates and the small number of strains available for testing, it would be most desirable to test several *in vitro* methodologies on new patient isolates, so that clinical outcome can then be compared with *in vitro* results and methods so as to learn which method is most clinically useful for this pathogen.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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