ORIGINAL ARTICLE

Diagnostic implications of mycetoma derived from *Madurella pseudomycetomatis* isolates from Mexico

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Abstract

Background At the dermatology service of the General Hospital of Mexico City, Mexico, two patients, father and son, with black-grain mycetoma were seen. The grains were isolated, and the cultured fungi were identified as *Madurella mycetomatis* based on morphology. Using the *M. mycetomatis* specific PCR, amplicons of a different size than that of the *M. mycetomatis* type strain were obtained.

Objective To determine the causative agent of the two black-grain mycetoma cases and develop non-culture-based diagnostic tools to identify them to the species level.

Methods The *M. mycetomatis* specific, the internal transcribed spacer (ITS) region, β-tubulin (BT) and ribosomal binding protein 2 (RBP2) PCRs were used to confirm the identity of the isolates. Genetic variation was established by amplification fragment length polymorphisms. To determine the antifungal susceptibility profile, the Sensititre™ YeastOne™ assay was used. To develop a species-specific PCR primers were designed on the sequenced PCR amplicon from the *M. mycetomatis* specific PCR.

Results By analyzing the ITS, BT and RBP2 regions the isolates were identified as *Madurella pseudomycetomatis*. The isolates from father and son were similar but not identical to *M. pseudomycetomatis* from Venezuela and one from an unknown origin. *Madurella pseudomycetomatis* isolates were inhibited by itraconazole, posaconazole and voriconazole but showed increased MIC values for amphotericin B and fluconazole. They were not inhibited by the echinocandins and five flucytosine. The two patients were treated with itraconazole resulting in cure for the father while the son was lost to follow-up. The species-specific PCR developed for *M. pseudomyceotmatis* was discriminative and specific.

Conclusion *Madurella pseudomycetomatis* is genetically diverse with same susceptibility profile as *M. mycetomatis* and causes eumycetoma in Latin America. The *M. pseudomycetomatis* specific PCR can be used to identify this causative agent to the species level; however, this needs to be validated in an endemic setting.

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Conflicts of interest

Nothing to disclose.

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Introduction

Mycetoma is chronic, granulomatous subcutaneous infection characterized by inflammation, painless tumour-like lesions and multiple sinuses discharging grains.[1,2] It is endemic in tropical and subtropical countries and can be caused by either bacteria (actinomycetoma) or fungi (eumycetoma). In Mexico,

actinomycetoma predominates over eumycetoma but in semi-arid climate zones of Africa (Sudan, Senegal, Somalia) and India.[3–9] eumycetoma is more common. *Madurella mycetomatis*is the main causative agent globally,[10] while *Nigrograna mackinnonii* predominates in Latin America.[6] In Mexico, particularly *M. mycetomatis* has been reported.[11]

Recently, novel *Madurella* agents causing mycetoma have been recognized, i.e. *Madurella fahalii, Madurella tropicana* and *Madurella pseudomycetomatis*.[12] Morphological identification of *Madurella* species is difficult, and hence molecular techniques are necessary for differentiation.[13] Susceptibility to antifungal drugs differs between species. For instance, *M. fahalii* is not inhibited by itraconazole *in vitro* unlike the other *Madurella* species.[12] Accurate identification is thus required for optimal treatment of patients. Currently, a species-specific PCR is available for *M. mycetomatis*,[14] but the remaining agents can only be identified by sequencing the internal transcribed spacer (ITS) regions.[15]

Due to the paradigm shift in mycology, distribution, prevalence and endemism of various *Madurella* species are unclear. Here, we show the presence of black-grain mycetoma caused by *M. pseudomycetomatis* in South America. Since this species was undistinguishable from the other *Madurella* species by morphology and culture methods, a novel diagnostic PCR was developed for identification to the species level.

Case reports

Case 1 A male, 66-year-old patient weighing 65 kg, presented with a localized dermatosis from the right pelvis to the foot. The central part and metatarsal region of the right foot showed a tumorous lesion, fibrotic with several sinuses from which some purulent material was exuded (Fig. 1a). The patient complained of pain to palpation and diambulation. He was a resident of Tapachula, Chiapas (1102 km southeast of Mexico City) and worked as a farmer. He reported multiple injuries during a time period of 18 years. The presumptive clinical diagnosis was mycetoma. To confirm the diagnosis, a direct examination (KOH) of the fistula exudate was taken. Multiple large dark grains of filamentous consistency were observed, and a dark filamentous colony with brown-ocher pigment was isolated Sabouraud-dextrose agar media. Multiple brown hyphae with clamydoconidia were observed after direct examination of the cultures, which is suggestive for *Madurella* spp. On radiography, a little osteolytic activity was demonstrated in the metatarsal bones (Fig. 1b). To confirm the diagnosis, haematoxylin and eosin (H&E) staining was performed on part of the biopsy material. This demonstrated hyperkeratosis, irregular acanthosis and pseudoepitheliomatous hyperplasia. In the deep dermis and subcutaneous tissue, a suppurative granulomatous process was observed, composed of a lympho-histiocytic infiltrate, forming some microabscesses with the presence of multiple dark grains formed by thick, septate hyphae (Fig. 1c). The patient reported receiving multiple previous treatments based on sulphamethoxazole-trimethoprim, ketoconazole, as well as multiple unspecified topical treatments. The patient was treated with itraconazole 400 mg/day for 18 months; the dose was subsequently lowered to 300 mg/day for 6 months, achieving clinical and mycological cure after 2 years of treatment.

Case 2 A 28-year-old male, equally 65 kg, presented with a localized dermatosis from the left pelvis to the foot, affecting the first metatarsal and the cuboid zone to the malleolus. The lesion started two years ago as a small lesion. The patient itself referred to it as a 'pimple'. The small lesion grew slowly to a tumoral lesion with multiple sinuses from which some purulent material drained upon pressure (Fig. 1d). The patient complained of pain to palpation and itching. He was resident of Comaltitlán, Chiapas (1041 km southeast of Mexico City) and son of the patient 1. Like his father, he was a farmer. He reported multiple injuries due to his occupation, and in particular referred to a trauma with spines. He received multiple treatments with unspecified antibiotics as well as homemade herb-based treatments, without obtaining any response. To identify the causative agent direct examination (KOH) of the exudate of the lesion was performed. Multiple black grains of filamentary consistency were observed. A black colony with ocher pigment was obtained on Sabouraud dextrose agar media consisting of multiple thick septate hyphae with clamydodoconidia, suggestive of *Madurella* spp. On radiography, discrete osteolytic activity was demonstrated (Fig. 1e). A biopsy with H & E staining showed hyperkeratosis, irregular acanthosis and pseudoepitheliomatous hyperplasia. A suppurative granuloma formed by an infiltrate with lymphocytes and histiocytes, with some microabscesses and the presence of grains formed by thick and dark hyphae was observed in the deep dermis and subcutaneous tissue (Fig. 1f). The final clinical diagnosis was eumycetoma with black grains. The treatment was itraconazole at a dose of 400 mg/day for one year, reaching clinical and mycological cure but patiently was subsequently lost to follow-up

Materials and methods

Strains and growth conditions

Grains were isolated from the biopsies of the patients (Mex2a and Mex3a) above and cultured on Sabouraud's dextrose agar (SDA) at 37 °C for two weeks. Phenotypic and morphological identification was performed at the Dermatology Service of the General Hospital Mexico City, Mexico. Additional fungal strains used in the study were grown and identified in a similar way: VE1, CBS102791, CBS129177T, CBS216.29, CBS248.48, UMIP81.77, UMIP582.60, UMIP1137.76, MM55, P1, CBS 102793, CBS 201.38T, CBS 132272 and CBS 128765.

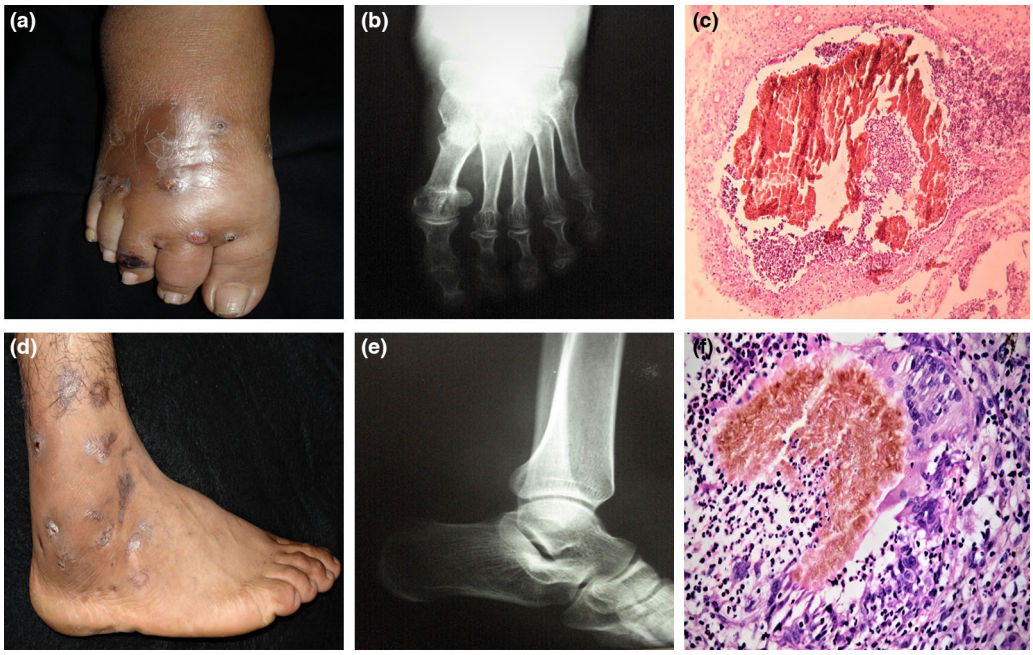

Figure 1 (a) Clinical presentation of case 1 with eumycetoma of the right foot with deformities and multiple sinuses. (b) Case 1 X-ray images showing osteolytic activity in the metatarsals**.** (c) H and E stained with characteristic grain section surrounded by cementious material (×40). (d) Clinical presentation of Case 2 with eumycetoma of the right limb. (e) Case 2 X-ray images showing discrete osteolytic activity. (f) Histological section showing grains composed of hyphal material and surrounded by cementious material (×40).

Table 1 Primers used for molecular identification of isolates[14,15,32]

Primer	Function	Sequence
V9G	ITS PCR/sequencing	5′-TTACGTCCCTGCCCTTTGTA-3′
LS266	ITS PCR/sequencing	5′-GCATTCCCAAACAACTCGACTC-3′
β-tubulinFw	β-Tubulin PCR/sequencing	5′-TTCCGTCCCGACAACTTCGT-3′
β-tubulin Rv	β-Tubulin PCR/sequencing	5′-CTCAGCCTCAGTGAACTCCAT-3′
β-tubulin Fw2	β-Tubulin sequencing	5′-TGACCCAGCAGATGTTCGAC-3′
RBP2	RBP2 PCR/sequencing	5′-GAYGAYMGWGATCAYTTYGG-3′
RBP2	RBP2 PCR/sequencing	5′-GAATRTTGGCCATGGTRTCCAT-3′
26.1a	*M. mycetomatis* specific PCR	5′-AATGAGTTGGGCTTTAACGG-3′
26.1b	*M. mycetomatis* specific PCR	5′-GCAACACGCCCTGGGCGA-3′
28.3a	*M. mycetomatis* specific PCR	5′-TCCCGGTAGTGTAGTGTCCCT-3′
28.3b	*M. mycetomatis* specific PCR	5′-TCCGCGGGGCGTCCGCCGGA-3′
MP1	*M. pseudomycetomatis* specific PCR	5′-GCGTGAAGAGTCTGCTGTTG-3′
MP2	*M. pseudomycetomatis* specific PCR	5′-TAGCCTGAATCCCACAAACC-3′

DNA extraction

DNA from Fungal isolates was extracted as described elsewhere.[16] Using the ZR Fungal/Bacterial DNA MiniPrep™ kit (Zymo Research, Irvine, CA, USA) or NucleoMag plant kit extraction (Macherey-Nagel, Dueren, Germany) in a King-FisherTM Flex system (Thermo Fisher Scientific, Basingstoke, Hants, UK).

Madurella mycetomatis specific PCR

Madurella mycetomatis specific PCR was performed as previously described.[14] Briefly, the PCR was performed in 25 μL reaction volume containing 0.2 U of Supertaq DNA polymerase (Eurogentec Seraing, Liege, Belgium), Supertaq buffer, 10 mmol/L dNTP mix (Thermo fisher), 50 pmol of each primer (Table 1) and 2.5 of DNA (40 ng/μL). Forty cycles amplification

was performed using the T100 Thermocycler (Biorad, Veenendaal, The Netherlands) with the following conditions: 94 °C for 4 min, 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s and a final extension at 72 °C for 7 min. Two *M. mycetomatis* isolates were included as positive controls (MM55 and P1) and isolates of *M. fahalii* (CBS 102793) and *M. tropicana* (CBS 201.38T) were included to evaluate cross-reaction.

Multilocus sequencing

Amplification and sequencing of rDNA ITS, β-tubulin (BT) and Ribosomal Binding Protein 2 (RBP2) genes amplification and sequencing were performed as described elsewhere[15,17] using the BigDye Terminator v3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Waltham, MA, USA) according to the manufacturer's instructions. The sequences were analyzed using Chromas software (Technelysium Pty Ltd, South Brisbane, Australia) and obtained sequences were deposited in GenBank (Mex2a ITS accession number: MN545597, Mex3a ITS accession number: MN545598).

Amplification fragment length polymorphisms

Amplification fragment length polymorphisms (AFLP) analysis was performed as described previously.[18–20] Following amplification, the AFLP fragments were ran by capillary electrophoresis. The capillary electrophoresis 20 μL reaction consisted of a 2 μL (1:10 dilution) of the AFLP product, 0.1 μL of Genescan 600LIZ size marker (Thermo Fisher, Landsmeer, The Netherlands) and 17.9 μL HiDi Formamide (Thermo Fisher). Data were analyzed using Bionumerics software (Applied Maths, Sint-Martens-Latem, Belgium).

Antifungal susceptibility profile

Minimum inhibitory concentrations (MICs) were determined using the Sensititre™ YeastOne™ Alamar Blue assay (Thermo Fisher Scientific) as previously described.[21] Briefly, isolates were grown in RPMI 1640 medium (Lonza, Verviers, Belgium) supplemented with L-glutamine (0.3 g/L) and 20 mmol/L morpholinepropanesulphonic acid (MOPS) for 7 days and the mycelia were harvested by 5 min centrifugation (Rotanta 46R, Tuttlingen, Germany) at 2158 ***g*** and washed with RMPI. The inoculum was homogenized, by sonicating the mycelia for 10 s at 28 μm (Soniprep, Healthfield, East Sussex, UK). A fungal suspension was made in YeastOne Broth (Thermo Scientific™, Santa Fe, NM, USA) with a transmission range between 68–72% (Novaspec II, Pharmacia Biotech, Uppsala, Sweden). One hundred microlitre of the fungal suspension was used to rehydrate the YO10 Sensititre® YeastONE plate and incubated for 7 days at 37 °C. The YO10 panel contained serial two-fold dilutions of anidulafungin (0.015–8 μg/mL), micafungin (0.008–8 μg/mL), caspofungin (0.008–8 μg/mL), 5-flucytosine (0.06–64 μg/mL), amphotericin B (0.12–8 μg/mL), posaconazole (0.008–8 μg/mL), voriconazole (0.008–8 μg/mL), itraconazole (0.015–16 μg/mL) and fluconazole (0.12–256 μg/mL). The MICs of this slow-growing filamentous fungus were read at day 7 following incubation at 37 °C.

Madurella pseudomycetomatis specific PCR

The sequence obtained (Accession number: MN384980) from *M. mycetomatis* specific PCR of *M. pseudomycetomatis* was used to design primers for a species-specific PCR for *M. pseudomycetomatis* (see Table 1). The PCR consisted of 12.5 μL of Fastart Master mix (Roche diagnostics, Almere, The Netherlands), 0.5 μm of forward primer and reverse primer, 8 μL H_2O and add 40 ng of genomic DNA in a 25 μL reaction volume. The PCR conditions were as follows: 4 min 94 °C, 30 s at 94 °C, 30 s at 55 °C and 1 min at 72 °C (35 cycles) followed by final extension time of 4 min at 72 °C. The PCR products were visualized on 1% agarose gel electrophoresis.

Results

The initial diagnosis of both patients was mycetoma caused by *M. mycetomatis,* which was based on the hairy aspect of the colonies isolated from the grains. Microscopically dark hyphae with chlamydospores and absence of conidia were noted. For verification, the isolates were tested using currently applied *M. mycetomatis* specific PCRs.[14] With primer pair 26.1b and 28.3b, a 370 bp amplicon was obtained for both Mexican isolates as well as for the two *M. mycetomatis* control isolates (Fig. 2a). When primer pair 26.1a and 28.3a was used, a 640 bp amplicon was obtained, which was 150 bp larger than the expected 490 bp amplicon obtained with the two *M. mycetomatis* control isolates (Fig. 2b, Lanes 1–2). The 640 bp amplicon (GenBank: MN384980) had no homology to any known sequence. Partial ITS, BT and RBP2 genes were sequenced for identification. Upon BLAST analysis, the ITS sequences of isolates Mex2a and Mex3a were found to be 100% identical to the ITS sequence of *M. pseudomycetomatis* (JX280868.1). The RBP2 and BT sequences were 100% identical to the RBP2 (JN573211.1) and BT (JN573199.1) sequences of *M. pseudomycetomatis* strain. We, therefore, concluded that both Mex2a and Mex3a are *M. pseudomycetomatis* isolates and that the currently used specific PCR for *M. mycetomatis* gave an amplicon with *M. pseudomycetomatis*.

We evaluated if *M. mycetomatis* specific PCR also cross-reacted with other *Madurella* species. Amplicons of 370 bp were obtained from all *Madurella* species other than *M. pseudomycetomatis* using primer pair 26.1b and 28.3b. This result was confirmed by *in silico* analysis of the deposited ITS sequences. With primer pair 26.1a and 28.3a, no amplification was obtained for *M. tropicana* or *M. fahalii* (Fig. 2b).

The sequence of the 640 bp amplicon of *M. pseudomycetomatis* was then used to develop a species-specific PCR which generated a 325 bp amplicon (Fig. 2c). Specificity was tested using the primers in *M. mycetomatis, M. fahalii* and

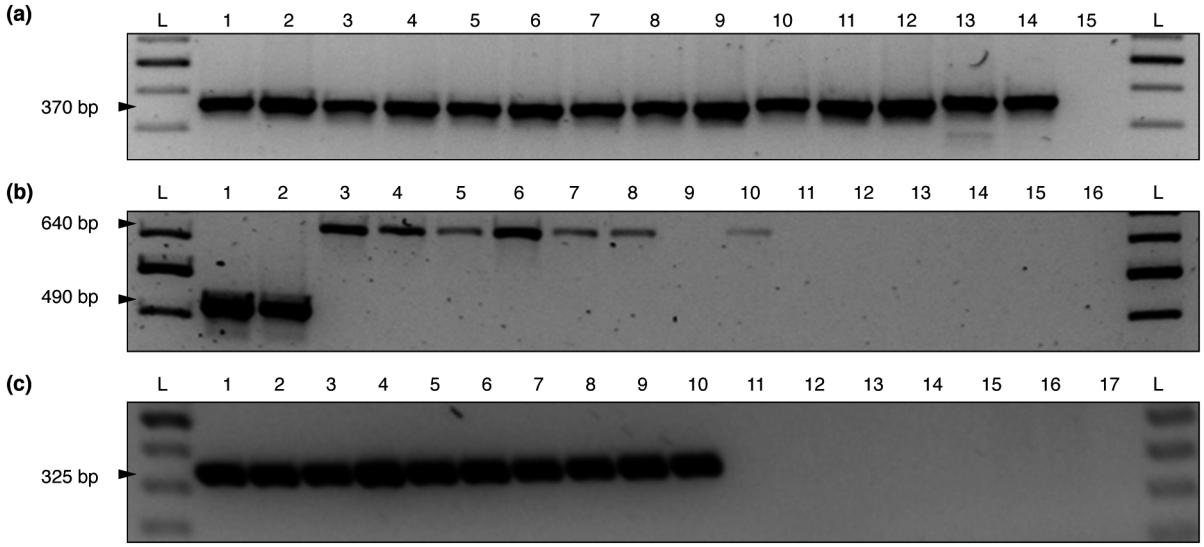

Figure 2 (a) *Madurella mycetomatis* specific PCR (26.1b and 28.3b) cross reacts with other *Madurella* species with identical bands. Lanes 1–2: *M. mycetomatis* isolates, Lanes 3–9: *Madurella pseudomycetomatis*, lanes 10–12: *M. fahalii*, *M. tropicana* lanes 13-14: *M. tropicana* and 15: Negative control, L: 100 bp plus ladder. (b) Primer pair 26.1a and 28.3a Lanes 1–2: *M. mycetomatis,* Lanes 3–10: *Madurella pseudomycetomatis*, Lanes 11–13: *M. fahalii* Lanes 14–15: *M. tropicana* and 16: negative control. (c) *Madurella pseudomycetomatis* specific PCR. Lanes 1–10: *Madurella pseudomycetomatis*, Lanes 11–12: *M. mycetomatis*, Lanes 13–14 *M. fahalii*, Lanes: 15–16 *M. tropicana* and 17: negative control.

M. tropicana as well as in other agents of eumycetoma such as *Medicopsis romeroi* and *Falciformispora senegalensis*. Amplification was obtained only in *M. pseudomycetomatis* which confirmed the specificity of the PCR within this group of tested species.

Since the isolates were obtained from father and son, we evaluated if these isolates could have been acquired from the same environmental source. AFLP analysis revealed that the two isolates were genetically similar but not identical. Furthermore, they were similar but not identical to two isolates VE1 (from Venezuela) and UMIP 1137.76 (unknown origin), respectively (Fig. 3). They were, however, genetically distinct from the other isolates obtained from other geographical regions. Moreover, these Mexican isolates were phylogenetically distinct from *M. mycetomatis* MM55 (CBS 131320) and *M. fahalii* CBS102793.

Since *M. fahalii* is known to be less susceptible to itraconazolethan *M. mycetomatis*,[12] we determined the *in vitro* susceptibility of *M. pseudomycetomatis* isolates against nine commonly used antifungal agents. *Madurella pseudomycetomatis* isolates were susceptible to the azole class of antifungals (itraconazole, posaconazole and voriconazole), and to amphotericin B as seen in Table 2. The MIC50 for the antifungals were as follows: itraconazole (0.03 μg/mL), posaconazole (0.015 μg/mL), voriconazole (0.06 μg/mL) fluconazole (16 μg/mL) and amphotericin B (0.5 μg/mL). The isolates were not inhibited by the echinocandins and 5-flucytosine (MIC50 ≥8 μg/mL and ≥64 μg/mL, respectively). Both patients were treated with itraconazole and patient Mex2a had mycological and clinical cure after two years, while patient Mex3a showed clinical improvement but was lost for follow-up.

Discussion

The two cases of eumycetoma in father and son caused by *M. pseudomycetomatis* were originally identified as *M. mycetomatis* by conventional techniques and using *M. mycetomatis* specific primers 26.1b and 28.3b.[14] The primers were developed prior to the description of additional *Madurella* species,[12,14] and thus it is not surprising that these are not recognized. Only when primers 26.1a and 28.3a correct identity of the etiologic agent was revealed. However, in another study using the same *M. mycetomatis* primers PCR, no cross-reactivity with *M. Pseudomycetomatis* was reported.[22]

Madurella mycetomatis, M. fahalii, M. tropicana and *M. pseudomycetomatis* are indistinguishable by culture.[23] The few isolates from South and Central America identified by molecular techniques were found to be *M. pseudomycetomatis* instead of *M. mycetomatis*.[24] Therefore, it cannot be excluded that *M. pseudomycetomatis* is the dominant *Madurella* species in Latin-America,[11] rather than *M. mycetomatis*. Our specific molecular assay was able to discriminate *M. pseudomycetomatis* from the other *Madurella* species with a high degree of specificity. The ITS-based PCR respects intra-specific variability as shown, for example in AFLP pattern. Since

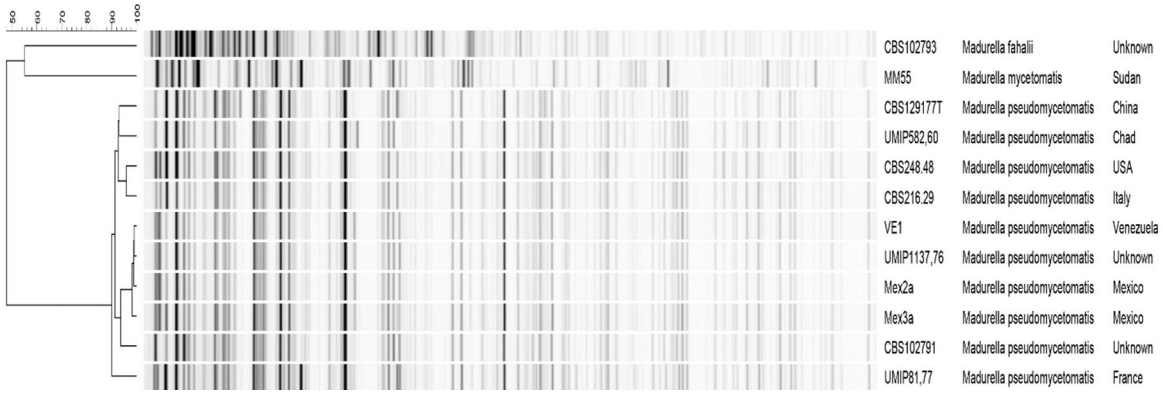

Figure 3 Amplification fragment length polymorphisms analysis of *Madurella pseudomycetomatis* and related species. *Madurella mycetomatis* strain MM55 and *M. mycetomatis* and *M. fahalii* strain CBS102793 used as outliers.

Table 2 MIC50 for seven *Madurella pseudomycetomatis* isolates determined by the Sensititre™ YeastOne™ assay

Antifungal agent	MIC range (μg/mL)	MIC50 (μg/mL)	GM MIC (μg/mL)
Itraconazole	0.015–0.06	0.03	0.03
Posaconazole	0.008–0.06	0.015	0.017
Voriconazole	0.008–0.25	0.06	0.08
Fluconazole	0.12–32	16	6.525
Amphotericin B	0.12–1	0.5	0.45
5-Flucytosine	>64	>64	>64
Caspofungin	>8	>8	>8
Micafungin	>8	>8	>8
Anidulafungin	>8	>8	>8

M. pseudomycetomatis might cause a considerable portion of eumycetoma cases in Latin-America, it is important to know the antifungal susceptibility profile and the clinical response to antifungal agents. Therefore, we evaluated the susceptibility of seven *M. pseudomycetomatis* isolates to the commonly used antifungals using the Sensititre™ YeastOne™ system. *Madurella pseudomycetomatis* isolates were susceptible *in vitro* to the azoles and amphotericin B with the lowest MIC for posaconazole. However, they were not inhibited by fluconazole. The echinocandins and 5-flucytosine did not also inhibit the growth of *M. pseudomycetomatis*.[25–28] This susceptibility pattern was similar to that reported in a previous study[22] and did not differ from that of *M. mycetomatis* isolates.[25,28,29] It is, therefore, to be expected that eumycetoma caused by *M. pseudomycetomatis* maybe treated with similar antifungal agents as patients with eumycetoma caused by *M. mycetomatis*. Indeed, the cases from father and son were both treated with itraconazole and both showed clinical improvement. The *M. pseudomycetomatis* case from Venezuela was treated for 72 months with itraconazole and was also cured.[24] This is similar to the response rate for *M. mycetomatis* in which 56.2%[30] to 69%[31] cases responded to azole treatment.

In conclusion, we here demonstrated that *M. pseudomycetomatis* causes eumycetoma in Latin-America. We developed a species-specific PCR which can be used to identify this causative agent to the species level. *Madurella pseudomycetomatis* has an antifungal susceptibility profile similar to that of *M. mycetomatis* and medical treatment of *M. pseudomycetomatis* eumycetoma can be similar to that recommended for *M. mycetomatis* eumycetoma.

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