Original article

Phylogeny and typification of *Madurella mycetomatis*, with a comparison of other agents of eumycetoma

Phylogenie und Typisierung von *Madurella mycetomatis* im Vergleich mit anderen Erregern von Eumyzetomata

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Summary

The genus *Madurella*, described for non-sporulating agents of human mycetoma, is proven to be heterogeneous on the basis of rDNA small subunit (SSU) and Internal Transcribed Spacer (ITS) sequencing data. *Madurella mycetomatis*, the main agent of mycetoma in arid zones of Central and East Africa, probably belongs to the ascomycete order Sordariales. *Madurella mycetomatis*, the generic type species, is neotypified. *Madurella grisea*, with worldwide occurrence, is likely to be a member of the order Pleosporales, just as the mycetoma agents of *Leptosphaeria*, *Pseudochaetosphaeronema*, and *Pyrenochaeta*. *Neotestudina rosatii* belongs to the order Dothideales. Judging from ITS data, *M. mycetomatis* and *N. rosatii* are species complexes. The ex-type strain of *N. rosatii*, from a human mycetome, has an ITS sequence that deviates from that of environmental strains of the species.

Zusammenfassung

Die Gattung *Madurella*, die für nicht sporulierende Erreger von Myzetomata beschrieben wurde, ist phylogenetisch heterogen, basierend auf Sequenzanalysen der SSU rDNA sowie der ITS. *Madurella mycetomatis*, der wichtigste Erreger von Myzetomata in ariden Gebieten Zentral- und Ostafrikas, ist wahrscheinlich ein Askomycet aus der Ordnung der Sordariales. *Madurella mycetomatis*, die Typenart von *Madurella*, wird hier neotypisiert. Die weltweit verbreitete *M. grisea* gehört wahrscheinlich zu den Pleosporales, was auch für die Myzetom-Erreger aus den Gattungen *Leptosphaeria, Pseudochaetosphaeronema* und *Pyrenochaeta* zutrifft. *Neotestudina rosatii* gehört in die Ordnung der Dothideales. Aufgrund von heterogenen ITS-Sequenzen stellen *M. mycetomatis* und *N. rosatii* Artenkomplexe dar. Die ITS-Sequenz des Typenstammes von *N. rosatii*, der aus einem Myzetom eines Menschen isoliert wurde, unterscheidet sich von den Sequenzen anderer Stämme dieser Art, die aus Umweltproben isoliert wurden.

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Schlüsselwörter: *Madurella*, Eumyzetom, ITS rDNA Sequenzierung, RFLP, Taxonomie.

Introduction

Eumycetoma is a massive subcutaneous fungal infection from which the etiologic agent emerges in the form of more or less compact mycelial grains. The immune response is a granulomatous foreign body reaction[1] characterized by local necrosis and the formation of fistels.[2,3] The disorder is supposed to originate from accidental traumatic inoculation of plant material such as thorns or splinters contaminated with fungi. For example, Abbott[4] noted a history of thorn pricks in 10 of 62 cases of mycetoma and in two of these found thorns still embedded in infected tissue. Basset *et al.*[5] reported on two cases with thorns inside lesions from which *Leptosphaeria senegalensis* Segretain *et al.* and *Pyrenochaeta romeroi* Borelli could be isolated. Judging from these case histories it is likely that the agents of mycetoma reside in the environment in regions endemic for the disease. They are supposed to be saprotrophic fungi able to survive hostile conditions in their natural niche and able to cause mycoses in humans when inoculated by coincidence.

However, recent findings on *Madurella mycetomatis* (Laveran) Brumpt are not in line with this view. This species is preponderant in cases of eumycetoma in Sudan. Abbott[4] reported 1231 cases in a 2.5 year period. Ahmed *et al.*[6] noted that clinical isolates are genetically identical. This suggests that living human tissue provides a suitable source for growth of the species. The fungus thus must possess a specialized pathogenic potential, which is unlikely for a saprotroph on plant material.

The genus *Madurella* was defined primarily for sterile mycelia.[7] Hence, degenerate cultures may easily have been mistaken for that species. de Hoog *et al.*[8] found that strains from imported cases of mycetoma in the Netherlands and originally identified as *M. mycetomatis* actually concerned a wide diversity of degenerate fungal strains. In the present study, *M. mycetomatis* is taxonomically redefined by comparing superficially similar clinical and environmental strains[6] with reference strains from an established culture collection. Fresh isolates originated from Sudan, which is a part of a 'mycetoma-belt' with further foci in southern India, Somalia and Senegal.[9] This endemic area is characterized by an arid and hot climate with 50–500 mm rainfall concentrated in short periods between June and October.

Materials and methods

Culture conditions

Environmental isolates from Sudan are those listed by Ahmed *et al.*[6] Clinical isolates were obtained by deep biopsy at the Mycetoma Research Center, Khartoum, Sudan. They were maintained on malt extract agar (MEA) slants at 24 °C and transferred to fresh tubes every 6 months. Reference strains, selected from genera which in the literature were reported to comprise agents of eumycetoma (Table 1), were retrieved from the collection of the Centraalbureau voor Schimmelcultures (Utrecht, the Netherlands).

DNA extraction

Two protocols were used: (i) about 1 cm^2 of mycelium was transferred to a 2 : 1 mixture of silica gel and Celite 545 with 300 µl CTAB-buffer [Tris–HCl, 200 mmol l^{-1}, pH 7.5; Na-EDTA, 200 mmol l^{-1}; NaCl 8.2%; cetyltrimethylammonium bromide (CTAB) 2%]. The material was ground with a micropestle (Eppendorf). After adding 200 µl CTAB-buffer and vigorous shaking the sample was incubated for 10 min in a 65 °C waterbath. Chloroform (500 µl) was added, vortexed briefly and centrifuged for 5 min at 20 800 ***g***. After transferring the aqueous supernatant to a new Eppendorf tube, two volumes (*c.* 800 µl) ethanol 96%, −20 °C were added and mixed gently. The DNA was precipitated at −20 °C for at least 30 min. The pellet obtained by centrifuging 5 min at 14 000 rpm was washed twice with 500 µl ethanol 70%, −20 °C. The DNA was dried overnight at room temperature and suspended in 97.5 µl TE-buffer (10 mmol l^{-1} Tris, 10 mmol l^{-1} Na-EDTA, pH 8.0) with 2.5 µl RNase solution (10 mg pancreatic RNase 20 U mg^{-1} in 1 ml 0.01 mol l^{-1} Na-acetate). Samples were incubated for 5–30 min at 37 °C and stored in a refrigerator. (ii) The FastDNA® Kit (Bio101, Carlsbad, CA, USA) was used with 1 cm^2 of mycelium homogenized with a Lysing Matrix and Cell Lysing Solution (CLS-Y) in the FastPrep® Instrument. After centrifugation a Binding

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Table 1 Genera containing agents of mycetoma with their purported phylogenetic position, the latter taken from de Hoog *et al.*[7] and from the present paper.

Name	Order	Grain	Reference
*Fusarium falciforme**	Hypocreales	White	
Acremonium kiliense	Hypocreales	White	
Acremonium cf. *potronii*	Hypocreales	White	30
Acremonium recifei	Hypocreales	White	31
Cylindrocarpon cyanescens	Hypocreales	White	32
Cylindrocarpon destructans	Hypocreales	White	33
Fusarium verticillioides	Hypocreales	White	34
Fusarium solani	Hypocreales	White	35
Phialemonium obovatum (originally published as *Acremonium hyalinulum*) (dog)	Hypocreales	White	36
Aspergillus flavus	Eurotiales	White	37
Aspergillus hollandicus (=A. vitis)	Eurotiales	White	30, 38
Aspergillus nidulans	Eurotiales	White	9, 58
Bipolaris spicifera (dog, horse)	Pleosporales	Black	39, 40
Corynespora cassiicola	Pleosporales	Black	41
Curvularia geniculata (dog, horse)	Pleosporales	Black	42–44
Curvularia lunata	Pleosporales	Black	9, 42, 45
Leptosphaeria senegalensis	Pleosporales	Black	46
Leptosphaeria thompkinsii	Pleosporales	Black	47
Madurella grisea	Pleosporales	Black	48
Pseudochaetosphaeronema larense	Pleosporales	Black	27
Pyrenochaeta mackinnonii	Pleosporales	Black	59
Pyrenochaeta romeroi	Pleosporales	Black	
Madurella mycetomatis	Sordariales	Black	
Phaeoacremonium parasiticum	Sordariales	Black	49
Neotestudina rosatii	Dothideales	White	29
Exophiala jeanselmei	Chaetothyriales	Black	50
Phialophora verrucosa	Chaetothyriales	Black	51, 52
Rhinocladiella atrovirens	Chaetothyriales	Black	53
Pseudallescheria boydii	Microascales	White	
Polycytella hominis	Microascales	White	54
Trichophyton	Onygenales	White	
Dermatophyte	Onygenales	White	55

Genera containing established agents[7] are presented in bold type; occasional agents are cited with reference to case reports.
*Until recently known as *Acremonium falciforme*.[61]

Matrix containing guanidine thiocyanate was added to the supernatant. The bound DNA was washed with Salt/Ethanol Wash Solution (SEWS-M) and collected with a DNA Elution Solution (DES). When checked on 1% agarose gel, the FastDNA® Kit resulted in a better quality of DNA, but less quantity.

PCR

The rDNA Internal Transcribed Spacer (ITS) region was amplified with primers V9G and LS266.[10] Other primer combinations (V9G/ITS4, LS266/ITS5) were used in case of negative result. The near-complete SSU rDNA gene was amplified with primers NS1 and NS24-UCB[11] and sequenced with primers listed by de Hoog *et al.*[7] Of several programs used, optimal results were obtained with the following: predwell (94 °C, 5′), then 30 cycles of denaturation (94 °C, 1′), annealing (48 °C, 1′) and elongation (72 °C, 2′), followed by postdwell (72 °C, 3′). One unit Super-*Taq* polymerase (ITK Diagnostics, Leiden, the Netherlands) was used for a 50 μl reaction mixture with 2 μl raw DNA. In some cases 6% dimethylsulfoxide (Merck, Amsterdam, the Netherlands) was added. Amplicons were purified using GFX™ and Gel Band Purification Kit (Amersham Pharmacia, Roosendaal, the Netherlands). DNA was bound to the GFX-column with a Capture-buffer (containing acetate and chaotrope), washed with a wash-buffer (containing Tris–EDTA and ethanol) and collected with TE-buffer. Concentrations of amplicons were estimated by comparison with known concentrations of SmartLadder (Eurogentec, Seraing, Belgium) on a 1% agarose gel.

RFLP

Twenty-two environmental and 14 reference strains were used for a restriction fragment length polymorphism (RFLP) analysis of the ITS spacer domain amplified with primers ITS1 and ITS4.[12] Eight microlitre of the amplicon solution was incubated with two units of the endonucleases *Hha*I and *Hae*III (Amersham Pharmacia) at 36 °C overnight.

Sequencing and identification

The sequencing reaction was carried out with 15–50 ng of DNA for a 10 μl reaction including 4 pmol primer and 4 μl BigDye RR Mix (Applied Biosystems, Nieuwerkerk a.d. IJssel, the Netherlands) using primers ITS1 and ITS4[12] for the ITS region or Oli1, Oli5, Oli9, Oli10, Oli11, Oli13 and Oli14[13] for the SSU gene with 25 cycles, as follows: 96 °C, 10″; 50 °C, 5″; 60 °C, 4′. DNA was precipitated with ethanol and sequenced on an ABI Prism™ 310 Genetic Analyzer (Applied Biosystems) automated capillary sequencer. The sequences were adjusted using SeqMan of Lasergene software (DNAStar, Madison, WI, USA) and aligned using BioNumerics (Applied Maths, Kortrijk, Belgium). Sequences were compared with a set of dedicated sequences available for research purposes at CBS. This database was made on the basis of the known spectrum of agents of mycetoma (Table 1; taxonomic relationships according to de Hoog *et al.*[7]). Groups of agents with strictly uncolored hyphae were not taken into account. In case no match was found they were run against the GenBank sequences using BLASTn.[14]

Tree reconstruction

A distance tree of near-complete SSU rDNA sequences was generated using neighbor-joining algorithm with Kimura correction in the TREECON package (version 1.3b);[15] bootstrap values were calculated from 100 resampled data sets.

Results

Selected strains analyzed are listed in Table 2. As many of them failed to produce reproductive structures *in vitro*, strains potentially matching *M. mycetomatis* were screened by using RFLP of the rDNA ITS region. The Sudanese strains from cases of mycetoma had profiles identical to those of five *M. mycetomatis* reference strains from the CBS collection (CBS247.48, CBS216.29, CBS201.38, CBS217.55) and of *Madurella* sp. CBS248.48. The set of remaining mycetoma strains compared, comprising *Pseudochaetosphaeronema*, *Pyrenochaeta*, *Leptosphaeria*, *Neotestudina*, as well as *M. grisea* MacKinnon *et al.* and CBS868.95 of *M. mycetomatis*, were consistently different. One strain of *M. mycetomatis*, CBS246.66, appeared to be misidentified, as it was found to be identical with *M. grisea* (Table 2).

The ITS sequence data (not shown) of *M. mycetomatis*, CBS247.48 was found to be nearly identical (1–7 bp difference) to five strains from cases of mycetoma in Sudan and two in Mali. CBS247.48 is possibly Gammel's[16] strain from the USA, which may be the ex-type strain of *M. ikedae* Gammel; we were unable to ascertain the history of this strain in the CBS archives. Four further clinical strains, CBS201.38, from Indonesia, CBS248.48, from New Mexico, USA, CBS216.29, from Italy, and CBS217.55, from Argentina, clustered at significant distance (average 5.3%) but could be aligned with confidence. The differences were not detected by *Hae*III and *Hha*I digestion. None of these isolates was a type strain of any described species. The distance from *M. mycetomatis* involved 38 bp. Variability within the group was noted at eight positions, none of which was phylogenetically informative. Distances to remaining species compared were too large to allow meaningful tree reconstruction. Nearest neighbors found in GenBank were members of the genera *Thielavia*, *Corynascus*, and *Chaetomium*; all are classified in the ascomycete order Sordariales. Only partial ITS alignment was possible with corresponding sequences of *M. mycetomatis*, as they deviated at least 9%. *Madurella mycetomatis* CBS868.95, isolated in the Netherlands from a patient originating from the Caribbean, was very different and could not be aligned to any of the fungi mentioned above.

Sequencing of the total SSU rDNA gene of *M. mycetomatis*, CBS247.48 revealed that *Chaetomium elatum* Kunze:Fr. (GenBank M83257) was its nearest neighbor (Ascomycetes: Sordariales), at 6 bp difference (Fig. 1). Further genera found close to CBS247.48 were *Neurospora*, *Sordaria* and *Podospora*, which all belong to the order Sordariales.

Sequencing of the total SSU rDNA gene of an authentic strain of *M. grisea*, CBS331.55, revealed that it clustered in the middle of a clade with species of the genera *Leptosphaeria*, *Cucurbidothis*, *Westerdykella*, *Sporormia* and related genera (Fig. 1), with *L. bicolor* D. Hawksworth *et al.* as its nearest neighbor, although at 89 bp difference. All these genera are members of ascomycetes known to belong to the order Pleosporales.

The ITS sequences of species of *M. mycetomatis*, *Chaetomium*, and other members of Sordariales could

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Table 2 Identity of strains of *Madurella*.

Original name	Number	Source	Locality	ITS differences %	ITS differences Inform.	ITS differences Var.	Final ident
Sordariales							
M. mycetomatis	CBS109801NT	Foot	Sudan	–	0	8	*M. mycetomatis*
M. mycetomatis	CBS247.48	Foot	USA	–	0	8	*M. mycetomatis*
M. mycetomatis	CBS110359	Foot	Mali	–	0	8	*M. mycetomatis*
M. mycetomatis	P2	Foot	Mali	–	0	8	*M. mycetomatis*
M. mycetomatis	DH11854	Foot	Sudan	–	0	8	*M. mycetomatis*
M. mycetomatis	DH11849	Foot	Sudan	–	0	8	*M. mycetomatis*
M. mycetomatis	DH11850	Foot	Sudan	–	0	8	*M. mycetomatis*
M. mycetomatis	CBS110087	Foot	Sudan	–	0	8	*M. mycetomatis*
M. mycetomatis	CBS216.29	?	Italy	5.3	38	8	*M.* spec. 1
M. mycetomatis	CBS201.38	Foot	Indonesia	5.3	38	8	*M.* spec. 1
M. mycetomatis	CBS217.55	Hand	Argentina[56]	5.3	38	8	*M.* spec. 1
M. sp.	CBS248.48	?	New Mexico	5.3	38 ↓	8	*M.* spec. 1
Dothideales							
N. rosatii	CBS331.78	Seed	India	39.5	23 ↑	1	*N.* spec. 1
N. rosatii	CBS628.74	Soil	Egypt	39.5	23	1	*N.* spec. 1
N. rosatii	CBS271.75	Soil	India	39.5	23	1	*N.* spec. 1
N. rosatii	CBS690.82	Seed	Japan	39.5	23	1	*N.* spec. 1
N. rosatii	CBS427.62T	Foot	Somalia	38.5 ↓	0	0	*N. rosatii*
U. bilgramii	DH11974	Thorn	Sudan	38.6	37	0	*U. bilgramii*
U. bilgramii	CBS110020	Thorn	Sudan	38.6	37	0	*U. bilgramii*
U. bilgramii	DH11978	Thorn	Sudan	38.6	37	0	*U. bilgramii*
U. bilgramii	DH11979	Thorn	Sudan	38.6	37	0	*U. bilgramii*
U. bilgramii	DH11980	Thorn	Sudan	38.6 ↓	37 ↓	0	*U. bilgramii*
Pleosporales							
M. grisea	CBS331.50AUT	Foot	Argentina	–	0	0	*M. grisea*
M. grisea	CBS332.50AUT	Foot	Chile	–	0	0	*M. grisea*
M. mycetomatis	CBS246.66	Jaw	India	–	0	0	*M. grisea*
Pseudo. larense	CBS640.73T	Man	Venezuela	27.5	–	–	*Pseudo. larense*
Py. mackinnonii	CBS674.75T	Man	Venezuela	30.3	–	–	*Py. mackinnonii*
Py. mackinnonii	CBS110022	Foot	Mexico	30.4	–	–	*Py. mackinnonii*
Py. romeroi	CBS252.60T	Man	Venezuela	29.0	–	–	*Py. romeroi*
L. thompkinsii	CBS200.79	Man	Senegal	29.6	–	–	*L. thompkinsii*
L. thompkinsii	CBS201.79	Man	Senegal	29.6	–	–	*L. thompkinsii*
L. senegalensis	CBS196.79T	Man	Senegal	31.2 ↓	–	–	*L. senegalensis*
Phylogenetic position doubtful							
M. mycetomatis	CBS868.95	Foot	Aruba[57]	–	–	–	*M.* spec. 2

Internal Transcribed Spacer (ITS) differences: Inform. = informative positions (mutations and indels) compared with flanking species (direction of comparison indicated by arrows); Var = intra-group single nucleotide polymorphisms (mutations and indels) listed as variance within the species; if not alignable with confidence, a percentage difference over the ITS domain is alone listed.

L., Leptosphaeria; *M., Madurella*; *N., Neotestudina*; *Pseudo., Pseudochaetosphaeronema*; *Py., Pyrenochaeta*; *U., Ulospora.*

not be aligned to any of the above species with purported affinity to the order Pleosporales including *M. grisea*; even partial alignment did not produce acceptable levels of confidence. A separate comparison was therefore made for *M. grisea* with the coelomycetous agents of eumycetoma *Py. romeroi*, *Py. mackinnonii* Borelli and *Pseudo. larense* (Borelli & Zamora) Punithalingam, plus the ascoma-forming agents classified in *Leptosphaeria*. The mutual distances were large (Table 2), with percentages difference between 27.5 and 31.2. Only very small parts of the ITS regions of the extremes of the cluster, i.e. *L. senegalensis* and *L. thompkinsii* El-Ani on the one hand and *M. grisea* on the other, could be aligned with confidence. Two strains of *M. grisea*, CBS331.50, CBS332.50 (both authentic strains from J.E. MacKinnon), and one of *M. mycetomatis*, CBS246.66, were found to be identical to each other. This species took a rather isolated position. ITS regions of other members of the Pleosporales, such as *Alternaria*, *Bipolaris*, and *Curvularia*, could not

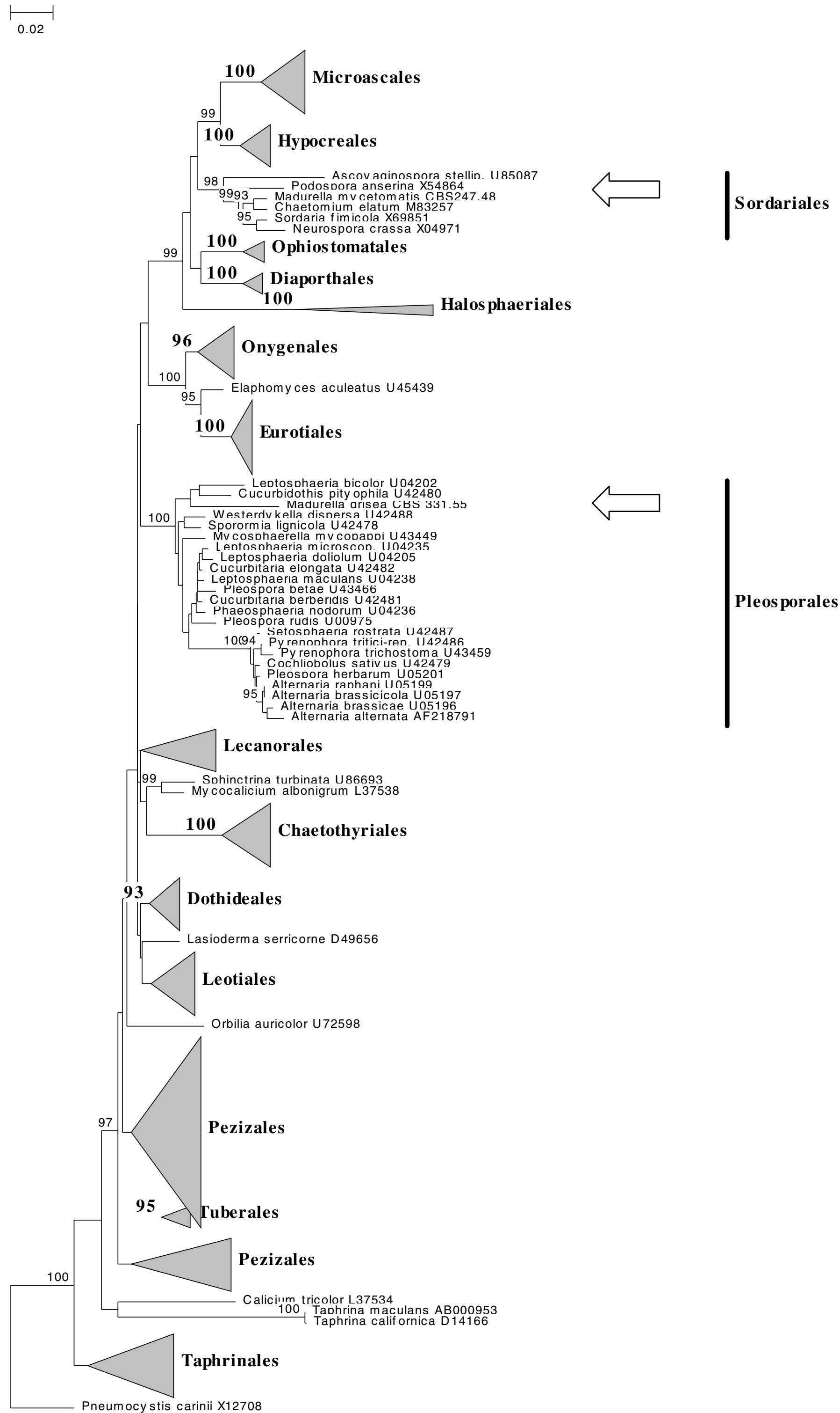

Figure 1 Distance tree of the Ascomycota based on 157 near-complete SSU rDNA sequences, constructed with the neighbor-joining algorithm with Kimura correction in the TREECON package. Bootstrap values >90 from 100 resampled data sets are shown. *Pneumocystis carinii* was selected as outgroup.

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be aligned to the remaining pleosporalen fungi (data not shown).

Five strains from Sudanese thorns, isolated during a search for environmental strains of *M. mycetomatis*,[6] were morphologically identified as *Ulospora bilgramii* (D. Hawksworth *et al.*), a species with close morphologic similarity to the known agent of mycetoma, *N. rosatii* Segretain & Destombes (Dothideales). The ITS sequences differed in 37 bp from the ex-type strain of *N. rosatii* (Table 2), CBS427.62, but could be aligned with confidence. CBS427.62 originated from a case of human mycetoma in Somalia, a country located in the dry climate zone of eastern Africa. Four environmental strains in the CBS collection, morphologically identified as *N. rosatii*, proved to differ from CBS427.62 in 23 positions (Table 2). The deviating strains all originated from (sub)tropical countries outside Africa. ITS sequences of neither *Ulospora* nor *Neotestudina* were alignable to *M. mycetomatis* or *M. grisea*.

Discussion

Two diametrically opposing theories on the etiology of human eumycetoma have appeared in the literature. The classical view (*hypothesis 1*) is that a relatively limited list of causative agents exists, which contains established pathogens typically causing mycetoma. A widely used list is that of Rippon.[17] The view is supported by the over-representation of a small number of species involved in the numerous reported cases of eumycetoma, such as *Acremonium* spp., *Pseudallescheria boydii* (Shear) McGinnis *et al.* and *Madurella* spp.

Alternatively (*hypothesis 2*) it has been suggested that mycetoma is an unspecific response to the subcutaneous inoculation of a wider range of principally saprotrophic agents. There are three arguments supporting this view. The first of these is theoretical: it is difficult to imagine how a fungus can be successful in its evolution when it is dependent on the unnatural situation of being traumatically introduced into its host. Accidental infections ecologically must be a 'spill-over', because no transmission to another host is possible.[7,18] Secondly, when more ecologic data become available on agents of mycetoma such as *Ps. boydii* or *A. kiliense* Grütz, it is realized that even the most common species on Rippon's list of agents of mycetoma actually have another ecologic behavior as mainstay, in which subcutaneous growth in humans is insignificant.[19–21] Thirdly and perhaps most importantly, there are large problems with the correct identification of agents of mycetoma. Most agents show poor morphologic differentiation or are sterile, and hence, given Rippon's[17] restricted list of well-known etiologic agents, a clinician can easily pick one of these names for the isolate at hand. This was underpinned by de Hoog *et al.*,[8] who showed that only a small portion of the agents from imported cases in the Netherlands actually concerned recognized members from Rippon's[17] list. It was therefore surmised that anything cultivated from a subcutaneous grain and showing reduced growth without reproduction *in vitro* might incorrectly be identified as '*Madurella*'.

Recent molecular data[6,22] suggest that both suppositions are partly correct. The spectrum of potential agents is larger than anticipated and includes a number of undescribed taxa, favouring *hypothesis 2*. On the contrary, in geographically limited areas a marked preponderance is noted of one or a few fungal species only, suggesting correctness of *hypothesis 1*. Strains identified as *Madurella* indeed represent a delimited taxonomic entity, rather than an amalgam of poorly differentiated strains. Below the currently known species of *Madurella* and their relatives, and *Neotestudina*-like fungi isolated from the *M. mycetomatis* endemic zone, are discussed.

Madurella mycetomatis

Thirumalachar and Padhye[23] obtained *M. mycetomatis* from soil, although at an extremely low recovery rate. Ahmed *et al.*[6] found that *M. mycetomatis* was detectable from culture-negative environmental samples by nested-polymerase chain reaction (PCR). When the species is extremely refractory to direct isolation, subcutaneous inoculation of *M. mycetomatis* into living mammal tissue apparently enhances growth of the fungus. A comparable situation is known in black yeast-like fungi, where *Cladophialophora bantiana* (Sacc.) de Hoog *et al.*[10] and *Fonsecaea pedrosoi* (Brumpt) Negroni[24] were shown to contain only clinical strains, environmental isolation being possible only by enrichment in living mammal tissue. Interestingly, *F. pedrosoi* is one of the agents of chromoblastomycosis, which is also a post-traumatic (sub)cutaneous mycosis. The supposition that traumatically inoculated species are likely to be strict saprotrophs is falsified for at least some fungi.

Madurella mycetomatis is likely to be restricted to Sudan and adjacent countries for climatic reasons. Ahmed *et al.*,[22] using ITS RFLP, found all clinical strains from Sudan to be identical to CBS247.48. This was confirmed by sequence data of the same domain.[6] Proven cases caused by this species originated from Sudan and Mali (Table 2), both countries having a desert-like climate. CBS247.48 is possibly (judging from correspondence of J.A. Gammel in the CBS archives) the ex-type strain of *M. ikedae* Gammel,

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currently a synonym of *M. mycetomatis*.[7] In that case the strain would originate from a case of eumycetoma in Texas, USA,[16] a region also having a dry climate. The CBS collection holds a number of strains from other climatic zones which showed more than 5% ITS difference and thus are likely to be a separate species (Table 2). A precise taxonomic definition of *M. mycetomatis* (*s. str.*) is overdue.

No holotype material of *M. mycetomatis* is known to be preserved. Laveran[25] described the first fungal isolate from a case of mycetoma from Djibouti, at about 1000 km east of Khartoum (Sudan), as *Streptothrix mycetomi* Laveran. *Streptothrix* is a genus of Actinomycetes. Therefore, Brumpt[26] erected the genus *Madurella* for fungal agents of mycetoma producing grains in tissue, *in vitro* exuding a brownish pigment into the medium and remaining sterile. He renamed Laveran's species as '*M. mycetomi*' (correct Latin is *M. mycetomatis*). Clinical strains tend to produce sclerotium-like bodies *in vitro* on casein-agar. Brumpt's[26] description exactly matches the prevalent genotype isolated from clinical cases around Khartoum,[22] and therefore we have no doubt that this is indeed the taxon that was described by Laveran.[25] We indicate one of the Sudanese strains, CBS109801, from a mycetoma of the foot of a human patient, as **neotype** of *M. mycetomatis*. Using SSU and ITS rDNA sequence data, the ascomycete genus *Chaetomium* (Sordariales)[26] was found among the closest relatives of *M. mycetomatis*. This suggests that *M. mycetomatis* is a sterile member of the order Sordariales.

Four CBS strains, originally identified as *M. mycetomatis*, showed significant ITS deviations from *M. mycetomatis*, and are hence likely to represent a separate species. None of these strains was of African origin. Strain CBS868.95 (from the Netherlands Antilles, as *M. mycetomatis*) could not be aligned with confidence and must represent a very different fungus. It had whitish-grey rather than yellowish-brown colonies, grew faster on Sabouraud glucose agar (SGA) and did not produce any extracellular pigment. The SSU rDNA gene had an intron between the ITS1- and ITS5-primer regions. A sterile isolate of *Ps. boydii* was surmised, but no match could be found in GenBank. These data prove that '*Madurella*' is currently applied as an umbrella term covering partly unrelated species. The large number of obsolete names in the literature[7] necessitate a taxonomic study into the genus *Madurella*.

Madurella grisea

Madurella grisea has been encountered in South America and India, but not in the dry climate zones of Africa (Table 2). ITS sequences of two authentic strains were found adjacent to *L. thompkinsii* and could largely be aligned with this species. *Madurella grisea* may thus be a member of Pleosporales and hence unrelated to *M. mycetomatis*. This supposition was confirmed by SSU rDNA sequencing data, where the species was found close to *L. bicolor* and *Cucurbidothis pityophila* Petrak, although at 54 bp distance from the latter. Among the species currently most similar to *M. grisea* based on ITS data are *Pseudo. larense*, known from a single case of human mycetoma[22] and several *Leptosphaeria* species from mycetomata (Table 2). Reported clinical cases in Senegal, occasionally showing thorns embedded in granulomata,[5] concerned *L. senegalensis* and *Py. romeroi*, rather than *M. mycetomatis*. The climate in Senegal is more humid than in Central and East Africa, where plant remains are strongly desiccated.

Neotestudina rosatii

The ex-type strain of *N. rosatii*, CBS427.62, a member of the order Dothideales, originated from a case of human mycetoma.[28] The remaining strains morphologically identified as *N. rosatii* deviated significantly in ITS sequence profiles by 14 substitutions in ITS 1 and 9 in ITS2, and seven Indels in ITS1 and 2 in ITS2, and thus are likely to represent a cryptic species. These strains came from soil and from seeds of *Coriandrum* and *Cuminum*. *Neotestudina rosatii* is an extremely rare species on which very few reports are available, neither clinical nor environmental. In contrast, *U. bilgramii*,[29] a close relative of *N. rosatii*, was isolated repeatedly from thorns of a *Balanitis egyptica* tree in Sudan (Table 2). This species differs from *N. rosatii* including the cryptic species by having furrows on the surface of the ascospores while *N. rosatii* ascospores are smooth-walled.[29] The ITS rDNA *Ulospora*/*Neotestudina* cluster could not be aligned with any of the remaining species analyzed in this study. In GenBank no sequences with a close match were found.

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