



## Revision of agents of black-grain eumycetoma in the order *Pleosporales*

S.A. Ahmed<sup>1,2,3</sup>, W.W.J. van de Sande<sup>4</sup>, D.A. Stevens<sup>5</sup>, A. Fahal<sup>6</sup>,  
A.D. van Diepeningen<sup>2</sup>, S.B.J. Menken<sup>3</sup>, G.S. de Hoog<sup>2,3,7</sup>

### Key words

*Madurella*  
mycetoma  
*Pleosporales*  
taxonomy  
*Trematosphaeriaceae*

**Abstract** Eumycetoma is a chronic fungal infection characterised by large subcutaneous masses and the presence of sinuses discharging coloured grains. The causative agents of black-grain eumycetoma mostly belong to the orders *Sordariales* and *Pleosporales*. The aim of the present study was to clarify the phylogeny and taxonomy of pleosporalean agents, viz. *Madurella grisea*, *Medicopsis romeroi* (syn.: *Pyrenochaeta romeroi*), *Nigrograna mackinnonii* (syn. *Pyrenochaeta mackinnonii*), *Leptosphaeria senegalensis*, *L. tompkinsii*, and *Pseudochaetosphaeronema larense*. A phylogenetic analysis based on five loci was performed: the Internal Transcribed Spacer (ITS), large (LSU) and small (SSU) subunit ribosomal RNA, the second largest RNA polymerase subunit (*RPB2*), and translation elongation factor 1-alpha (*TEF1*) gene. In addition, the morphological and physiological characteristics were determined. Three species were well-resolved at the family and genus level. *Madurella grisea*, *L. senegalensis*, and *L. tompkinsii* were found to belong to the family *Trematosphaeriaceae* and are reclassified as *Trematosphaeria grisea* comb. nov., *Falciformispora senegalensis* comb. nov., and *F. tompkinsii* comb. nov. *Medicopsis romeroi* and *Pseudochaetosphaeronema larense* were phylogenetically distant and both names are accepted. The genus *Nigrograna* is reduced to synonymy of *Biatrispora* and therefore *N. mackinnonii* is reclassified as *B. mackinnonii* comb. nov. Mycetoma agents in *Pleosporales* were phylogenetically quite diverse despite their morphological similarity in the formation of pycnidia, except for the ascospore-forming genus *Falciformispora* (formerly in *Leptosphaeria*). Most of the species diagnosed from human mycetoma were found to be related to waterborne or marine fungi, suggesting an association of the virulence factors with oligotrophism or halotolerance.

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### INTRODUCTION

Mycetoma is a chronic progressive, destructive inflammatory disease that initially infects subcutaneous tissues and extends slowly to eventually invade the bone. It is mainly characterised by multiple sinuses discharging white or coloured grains at the site of infection (McGinnis 1996, Fahal & Suliman 1994). As both bacteria and fungi are able to cause mycetoma, this disease is classified into two types, actinomycetoma and eumycetoma, which are of bacterial and of fungal nature, respectively (Chalmers & Archibald 1918). Both types have similar clinical presentations, with some differences in virulence and colour of the discharged grains (Fahal & Suliman 1994). Black-grain mycetoma is caused by fungi only, but the etiological agents belong to different orders (mainly to *Sordariales* and *Pleosporales*) (de Hoog et al. 2004). The most frequent agents belong to the genus *Madurella*, which exclusively comprises human-pathogens.

*Madurella* was first described by Brumpt (1905) to accommodate *Streptothrix mycetoma*, which was later renamed as *Madurella mycetomatis* (Kwon-Chung & Bennett 1992). The genus was defined as exclusively producing melanised hyphae without sporulation, and hence prior to the use of molecular methods species were difficult to distinguish (de Hoog et al. 2000). During the 20th century several species were introduced to the genus by colony characteristics, but most of these proved to be synonyms of *M. mycetomatis*. *Madurella pseudomycetomatis*, *M. tropicana*, and *M. fahalii* were recently recognised as different species of *Madurella* on the basis of DNA sequence data (Yan et al. 2010, de Hoog et al. 2012).

For many decades, *Madurella grisea* (Mackinnon et al. 1949) was treated as the sister species of *M. mycetomatis*; it is distinguished by grey cultures without soluble pigments being exuded into the medium. In contrast, cultures of *M. mycetomatis* are blackish brown and produce a dark brown halo of diffusible melanin-like compounds. Physiological differences are found in assimilation of carbon sources and in thermotolerance: *M. grisea* assimilates sucrose but not lactose, and is mostly unable to grow at 37 °C, whereas *M. mycetomatis* assimilates lactose but not sucrose, and grows optimally at 37 °C (de Hoog et al. 2000). *Madurella mycetomatis* occurs at rather high frequency in arid climate zones of East Africa and India, whereas for *M. grisea* only occasional records from South America and India are available (Mackinnon et al. 1949, McGinnis 1996, de Hoog et al. 2007). A recent phylogenetic analysis showed the two species to belong to different orders: *M. mycetomatis* (as well as the related *M. pseudomycetomatis*, *M. tropicana*, and *M. fahalii*) belong to the *Sordariales*, whereas *M. grisea* is a member of the *Pleosporales* (de Hoog et al. 2004). The

<sup>1</sup> Faculty of Medical Laboratory Sciences, University of Khartoum, Khartoum, Sudan; corresponding author e-mail: s.ahmed@cbs.knaw.nl.

<sup>2</sup> CBS-KNAW Fungal Biodiversity Centre, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands.

<sup>3</sup> Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, The Netherlands.

<sup>4</sup> Erasmus MC, Department of Medical Microbiology and Infectious Diseases, Rotterdam, The Netherlands.

<sup>5</sup> California Institute for Medical Research, San Jose, California.

<sup>6</sup> Mycetoma Research Centre, University of Khartoum, Khartoum, Sudan.

<sup>7</sup> Peking University Health Science Center, Research Center for Medical Mycology, Beijing, China; Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University, Guangzhou, China; Shanghai Institute of Medical Mycology, Changzheng Hospital, Second Military Medical University, Shanghai, China; Basic Pathology Department, Federal University of Paraná State, Curitiba, Paraná, Brazil; King Abdulaziz University, Jeddah, Saudi Arabia.

**Table 1** Source, origin, and GenBank accession No. for the species included in this study. CBS (CBS-KNAW Fungal Biodiversity Centre), dH: (G.S. de Hoog working collection). Accession numbers in **bold** were retrieved from GenBank.

Name	Culture no.	Source	Origin	GenBank accessions					
				ITS	LSU	SSU	RPB2	TEF1	
<i>Biatrispora mackinnonii</i>	CBS 674.75 (T)	Mycetoma	Venezuela	KF015654	KF015612	<b>GQ387613</b>	KF015703	KF407986	
	CBS 110022	Mycetoma	Mexico	KF015653	KF015609	<b>GQ387552</b>	KF015704	KF407985	
<i>Falciformispora senegalensis</i>	CBS 196.79 (T)	Mycetoma	Senegal	KF015673	KF015631	KF015636	KF015717	KF015687	
	CBS 197.79	Human	Senegal	KF015677	KF015626	KF015633	KF015712	KF015690	
	CBS 198.79	Mycetoma	Senegal	KF015675	KF015627	KF015634	KF015713	KF015688	
	CBS 199.79	Human	Senegal	KF015672	KF015630	KF015638	KF015716	KF015689	
	CBS 132257	Mycetoma	Sudan	KF015676	KF015629	KF015635	KF015714	KF015692	
	CBS 132272	Mycetoma	Sudan	KF015674	KF015628	KF015637	KF015715	KF015691	
<i>Falciformispora tompkinsii</i>	CBS 200.79	Man	Senegal	KF015670	KF015625	KF015639	KF015719	KF015685	
	CBS 201.79	Man	Senegal	KF015671	KF015624	KF015640	KF015718	KF015686	
<i>Medicopsis romeroi</i>	CBS 252.60 (T)	Mycetoma	Venezuela	KF366446	<b>EU754207</b>	<b>EU754108</b>	KF015708	KF015678	
	CBS 132878	Mycetoma	India	KF015658	KF015622	KF015648	KF015709	KF015682	
	CBS 122784	Plant		KF366447	<b>EU754208</b>	<b>EU754109</b>	KF015707	KF015679	
	CBS 123975	Phaeohyphomycosis	India	KF015657	KF015623	KF015650	KF015710	KF015681	
	CBS 128765	Subcutaneous cyst	Kuwait	KF015659	KF015621	KF015649	KF015711	KF015680	
<i>Pseudochaetosphaeronema larense</i>	CBS 640.73 (T)	Mycetoma	Venezuela	KF015656	KF015611	KF015652	KF015706	KF015684	
	CBS 639.94	Man	Venezuela	KF015655	KF015610	KF015651	KF015705	KF015683	
<i>Rousoella</i> sp.	CBS 128203	Mycetoma	India	KF322117	KF366448	KF366450	KF366453	KF407988	
<i>Rousoella</i> sp.	CBS 868.95	Mycetoma	Aruba	KF322118	KF366449	KF366451	KF366452	KF407987	
<i>Trematosphaeria grisea</i>	CBS 332.50 (AUT)	Mycetoma	Chili	KF015662	KF015614	KF015641	KF015720	KF015698	
	CBS 246.66	Submandibular abscess	India	KF015661	KF015615	KF015646	KF366454	KF015697	
	CBS 120271	Tap water	The Netherlands	KF015660	KF015613	KF015647	KF015726	KF015699	
	CBS 135982	Pastry gel	The Netherlands	KF015663	KF015616	KF015645	KF015721	KF015693	
	CBS 135984	Water	The Netherlands	KF015666	KF015618	KF015632	KF015724	KF015694	
	CBS 136543	Water	The Netherlands	KF015665	KF015619	KF015642	KF015723	KF015696	
	CBS 135985	Water	The Netherlands	KF015667	KF015620	KF015643	KF015722	KF015700	
	CBS 135983	Water	The Netherlands	KF015664	KF015617	KF015644	KF015725	KF015695	
	CBS 136537	Water	The Netherlands	KF366444					
	V-0206-72	Biopsy, human	The Netherlands	KF366445					
	<i>Trematosphaeria pertusa</i>	CBS 122371	Plant	France	KF015669	FJ201992	FJ201993	GU371801	KF015702
		CBS 122368	Plant	France	KF015668	FJ201990	FJ201991	FJ795476	KF015701

**Table 2** Carbohydrate assimilation profile, and urease, laccase, and salt tolerance test results for the analysed species. – = Negative, + = positive, W = weak.

Carbohydrate/Test	<i>T. grisea</i>	<i>F. senegalensis</i>	<i>F. tompkinsii</i>	<i>Rousoella</i> sp.	<i>M. romeroi</i>	<i>B. mackinnonii</i>	<i>Pseud. larense</i>
GAL (D-galactose)	+	+	+	+	+	+	+
ACT (actidione)	+	+	+	+	+	–	–
SAC (D-saccharose)	+	+	+	+	+	+	+
NAG (N-acetyl-glucosamine)	+	+	+	+	+	+	+
LAT (DL-lactate)	–	–	–	–	–	–	–
ARA (L-arabinose)	–	+	–	+	–	+/-	–
CEL (D-cellobiose)	+	+	+	+	+	+	+
RAF (D-raffinose)	+	+	+	+	+	+	+
MAL (D-maltose)	+	+	+	+	+	+	+
TRE (D-trehalose)	+	+	+	+	+	+	+
2KG (potassium 2-keto-gluconate)	+	–	+	+	+	+	+
MDG (methyl-D-glucopyranoside)	+	+	+	+	–	+	+
MaN (D-mannitol)	+	+/-	+	+	+	+	+
LAC (D-lactose)	–	+	–	+/-	–	–	–
INO (inositol)	+	+/-	–	+	+	+	+
SOR (D-sorbitol)	+	+/-	–	+	+	+	+
XYL (D-xylose)	+	+	+	+	+	+	–
RIB (D-ribose)	–	–	–	–	–	+/-	-/+
GLY (glycerol)	–	–	–	–	–	–	+
RHA (L-rhamnose)	+	+	+	+	+	+	+
PLE (palatinose)	+	+	+	+	+	+	+
ERY (erythritol)	+/-	–	+	+	+	+	+
MEL (D-melibiose)	+	–	+/-	+	+/-	+	+
GRT (sodium glucuronate)	–	–	–	–	–	+/-	–
MLZ (D-melezitose)	+	+	+	+	+	+	+
GNT (potassium gluconate)	–	–	–	–	–	–	–
LVT (levulinate)	+/-	–	–	–	–	+	–
GLU (D-glucose)	+	+	+	+	+	+	+
SBE (L-sorbose)	+	+	W	+	+	–	–
GLN (glucosamine)	–	–	–	-/W	–	–	–
ESC (esculin)	+	+	+	–	W	+	+
Salt tolerance test (NACL)	20 %	20 %	20 %	20 %	20 %	20 %	10–20 %
Laccase test	+	+	-/W	+	+	+	+
Urease test	+	+	+	+	+	+	+

taxonomic position of *M. mycetomatis* within the *Sordariales* has been studied extensively (de Hoog et al. 2004, van de Sande 2012). However, the phylogeny and biology of *M. grisea* remains to be investigated.

Besides *Madurella* species, the order *Pleosporales* contains several other agents of black-grain mycetoma: *Leptosphaeria senegalensis*, *L. tompkinsii*, *Medicopsis romeroi* (syn. *Pyrenochaeta romeroi*), *Nigrograna mackinnonii* (syn. *Pyrenochaeta mackinnonii*), and *Pseudochaetosphaeronea larense*, as well as some undescribed species (Meis et al. 2000, de Hoog et al. 2004). The aim of the present study is to clarify the phylogeny and taxonomy of pleosporalean agents of mycetoma, with accent on *Madurella grisea*.

## MATERIALS AND METHODS

### Strains analysed

Fungal strains included in this study were obtained from the CBS-KNAW (Fungal Biodiversity Centre) reference collection ([www.cbs.knaw.nl](http://www.cbs.knaw.nl)), supplemented with fresh isolates recovered from patients with mycetoma. When available, type strains of species concerned were included in the analyses. Information on source and origin of the analysed strains is provided in Table 1.

### Morphology and physiology

Morphology and growth characteristics of the species were studied on malt extract agar (MEA, Oxoid, UK) and oatmeal agar (OA, home-made at CBS, The Netherlands, <http://www.cbs.knaw.nl/index.php/food-mycology/101-mycological-media-for-food-and-indoor-fungi>). Culture plates were incubated at 30 °C for at least 4 wk, with a maximum of 8 wk and colony colours were described using the colour chart of Kornerup & Wanscher (1967). Cultures were firstly examined under a Nikon C-DSD 230 dissecting microscope. Microscopic mounts were made in lactic acid and examined using a light microscope (Nikon Eclipse 80i). Pictures were taken with a digital camera (Nikon, digital sight, DS-5M) and adjusted in Adobe Photoshop CS3. Cardinal growth temperatures were determined by incubating MEA plates in the dark at temperatures ranging from 15 to 40 °C at 3 °C intervals and including 10 and 37 °C.

Carbohydrate assimilation profiles were determined using ID32 C (BioMérieux, Marcy l'Étoile, France). For a review of carbohydrates and abbreviations see Table 2. Inocula were homogenised by ultrasonic treatment of fungal material in API C medium at maximum power (Soniprep, Beun de Ronde, Abcoude, The Netherlands). Final inocula were adjusted to obtain a transmission of 68–72 % at 660 nm (Novaspec II, Pharmacia Biotech, Cambridge, UK) and used according to the manufacturer's instructions. Strips were incubated at 25 °C in a moist chamber and examined every 3 d for 9 d. Ability to produce urease was determined on 2 % urea agar plates (urea base, Oxoid, UK) incubated for one week. Laccase production was tested using ABTS agar medium containing 0.03 % ABTS (2, 2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt) (Kaluskar et al. 1999). ABTS plates were incubated at room temperature for 1 wk; *Cryptococcus neoformans* CBS 7229 was used as positive control. Salt tolerance was determined using nitrogen base medium (Difco) with sodium chloride at concentrations of 0, 2.5, 5, 10, 15, and 20 %.

### DNA extraction

Genomic DNA was isolated from fungal mass grown on the surface of MEA plates. About 10 mm<sup>2</sup> of material were added to a screw-capped tube containing 490 µL CTAB-buffer (2 % cetyltrimethyl ammonium bromide, 100 mM Tris-HCl, 20 mM

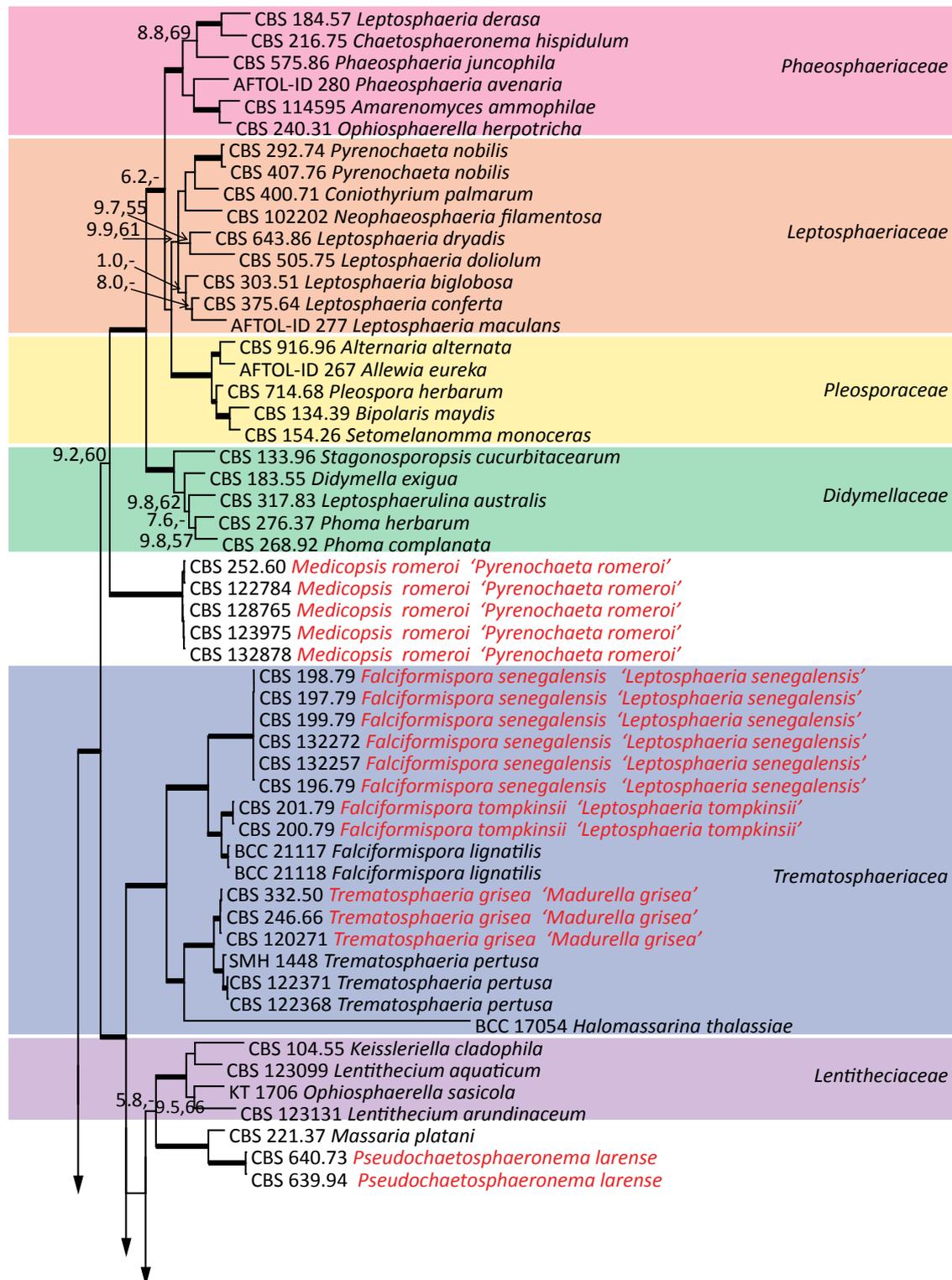
EDTA, 1.4 M NaCl) and 6–10 acid-washed glass beads. Ten microliters proteinase K were added to the mixture and vortexed with a MoBio vortex for a few min. Tubes were incubated at 60 °C for 60 min. After incubation the tubes were again vortexed and 500 µL of chloroform : isoamylalcohol (24 : 1) were added followed by shaking for 2 min. Tubes were spun at 14 000 r.p.m. in a microfuge for 10 min and the upper layer was collected in new sterile tubes with 0.55 volume ice-cold iso-propanol and spun again. Finally, the pellets were washed with 70 % ethanol, air-dried and re-suspended in 100 µL TE buffer.

### DNA amplification and sequencing

DNA amplification was performed for the Internal Transcribed Spacer region (ITS), small ribosomal subunit (SSU), and (partial) large ribosomal subunit (LSU), RNA polymerase second largest subunit (*RPB2*), and translation elongation factor 1- $\alpha$  (*TEF1*) loci. Primers used for amplification and partial sequencing of LSU, SSU, *RPB2*, and *TEF1* were LRoR and LR7 (Vilgalys & Hester 1990), NS1 and NS24 (Gargas & Taylor 1992, White et al. 1990), fRPB2-5F and fRPB2-7cR (Liu et al. 1999), and EF-983F and EF-2218R (<http://www.aftol.org/pdfs/EF1primer.pdf>), respectively. Additional primers that were used for sequencing SSU included NS2, NS3, NS6, and NS7, and rDNA ITS was amplified and sequenced using primers ITS1 and ITS4 (White et al. 1990). All PCR reactions were performed in a MyCycler™ Thermal Cycler (Bio-Rad, Munich, Germany) with 25 µL reaction mixture containing 10 ng of template DNA, 0.1 mM each dNTP, 1.0 U *Taq* polymerase, and 2.5 µL reaction buffer (0.1 M Tris-HCl, 0.5 M KCl, 15 mM MgCl<sub>2</sub>, 0.1 % gelatin, and 1 % Triton X-100). Amplification conditions for all fragments were taken from Feng et al. (in press) except for *RPB2*. Amplification conditions for *RPB2* included pre-denaturation at 95 °C for 3 min followed by 5 cycles of denaturation at 95 °C for 45 s and annealing at 58 °C for 45 s (reduced to 56 °C in another 5 cycles) and elongation at 72 °C for 2 min followed by 35 cycles of denaturation at 95 °C for 45 s, annealing at 52 °C for 45 s, and elongation at 72 °C for 2 min, with a final post-elongation step at 72 °C for 8 min. PCR products were used for sequencing using Big Dye terminator cycle sequencing RR mix protocol (ABI PRISM v. 3.1, Applied Biosystems) with cycling conditions of 95 °C for 1 min, 30 cycles of 95 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min. Final products were purified using Sephadex G-50 fine (GE Healthcare, Uppsala, Sweden) and analysed on an ABI 3730xl DNA Analyzer (Applied Biosystems).

### Alignment and phylogenetic analysis

Consensus sequences for each locus were obtained and edited using SeqMan in the Lasergene software (DNASTar, WI, USA). Sequences retrieved from GenBank for *Pleosporales* species were mainly from studies of Schoch et al. (2009), Zhang et al. (2009), and Lutzoni et al. (2004). Sequences derived from this study were deposited in GenBank; accession numbers are listed in Table 1. Sequences of ribosomal genes were aligned with MUSCLE using the EMBL-EBI web server (<http://www.ebi.ac.uk/Tools/msa/muscle/>). Introns were found in some sequences of SSU and they were removed from the alignment. Protein-coding gene sequences were aligned after removing of introns using RevTrans 1.4 Server (Wernersson & Pedersen 2003). Alignments were constructed for each gene separately and adjusted manually in BioEdit v. 7.1.3 software (Hall 1999). Missing data for partial or complete sequences for some taxa were coded as 'missing' but still can be used in the final matrix according to Wiens (2006) and Wiens & Moen (2008). A combined alignment was constructed for partial sequences of LSU, SSU, *TEF1*, and *RPB2*, alignments were concatenated using DataConvert 1.0 ([https://www.bigelow.org/research/srs/david\\_a\\_mcclellan/david\\_mcclellan\\_laboratory/dataconvert/](https://www.bigelow.org/research/srs/david_a_mcclellan/david_mcclellan_laboratory/dataconvert/)).

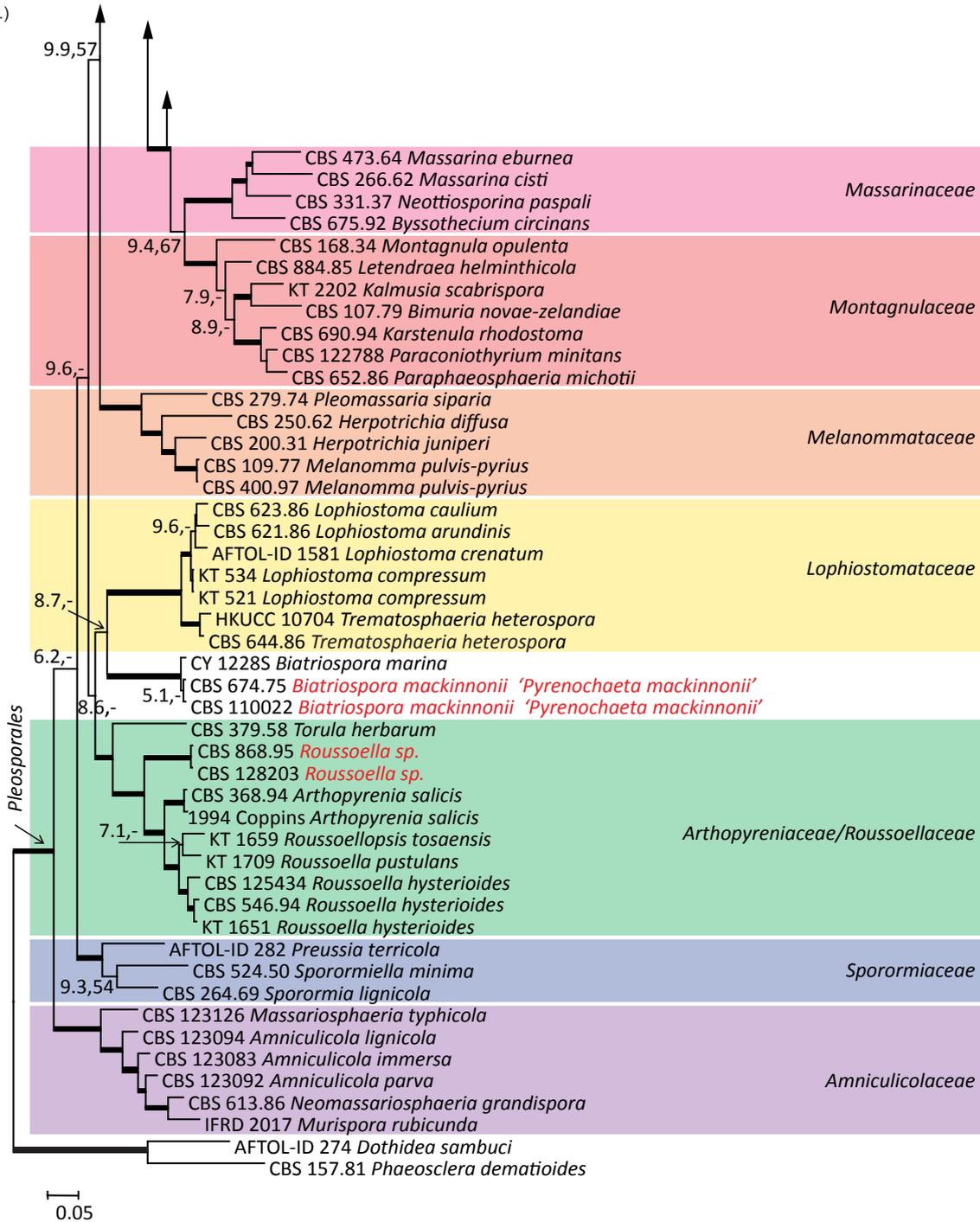


**Fig. 1** Phylogram of combined dataset (LSU, SSU, *RPB2*, *TEF1*) obtained by Bayesian analysis and maximum likelihood (values of  $\geq 0.70$  for Bayesian probability and  $\geq 70\%$  for maximum likelihood shown in **bold** branches). Species causing mycetoma shown in red, with obsolete names between inverted commas. *Dothidea sambuci* and *Phaeosclera dematioides* were used as outgroup.

Phylogenetic analyses were performed using Bayesian and maximum likelihood analyses. Bayesian command files were prepared using Mesquite v. 2.75 (Maddison & Maddison 2011). The combined dataset was partitioned per locus and the analysis was done in MrBayes v. 3.1.2 implemented in the Cipres web server (<http://www.phylo.org/>). Two parallel runs with four Markov chain Monte Carlo (MCMC) simulations for each run were set for 20 000 000 generations and the result was checked using Tracer v. 1.5 (Rambaut & Drummond 2007) for effective sample size (ESS). The run was then extended for another

10 000 000 generations with a sample frequency of 1 000 per each generation. Maximum likelihood analysis was done using RAXML v. 7.2.8 in the Cipres web server. All trees were constructed with outgroup method and edited in Mega v. 5.05 (Tamura et al. 2011). ITS sequences were aligned with MUSCLE and adjusted in BioEdit v. 7.1.3 (Hall 1999). Phylogenetic analysis for ITS was performed with Bayesian and maximum likelihood analysis as described before except with numbers of generations for MrBayes set at 10 000 000.

Fig. 1 (cont.)



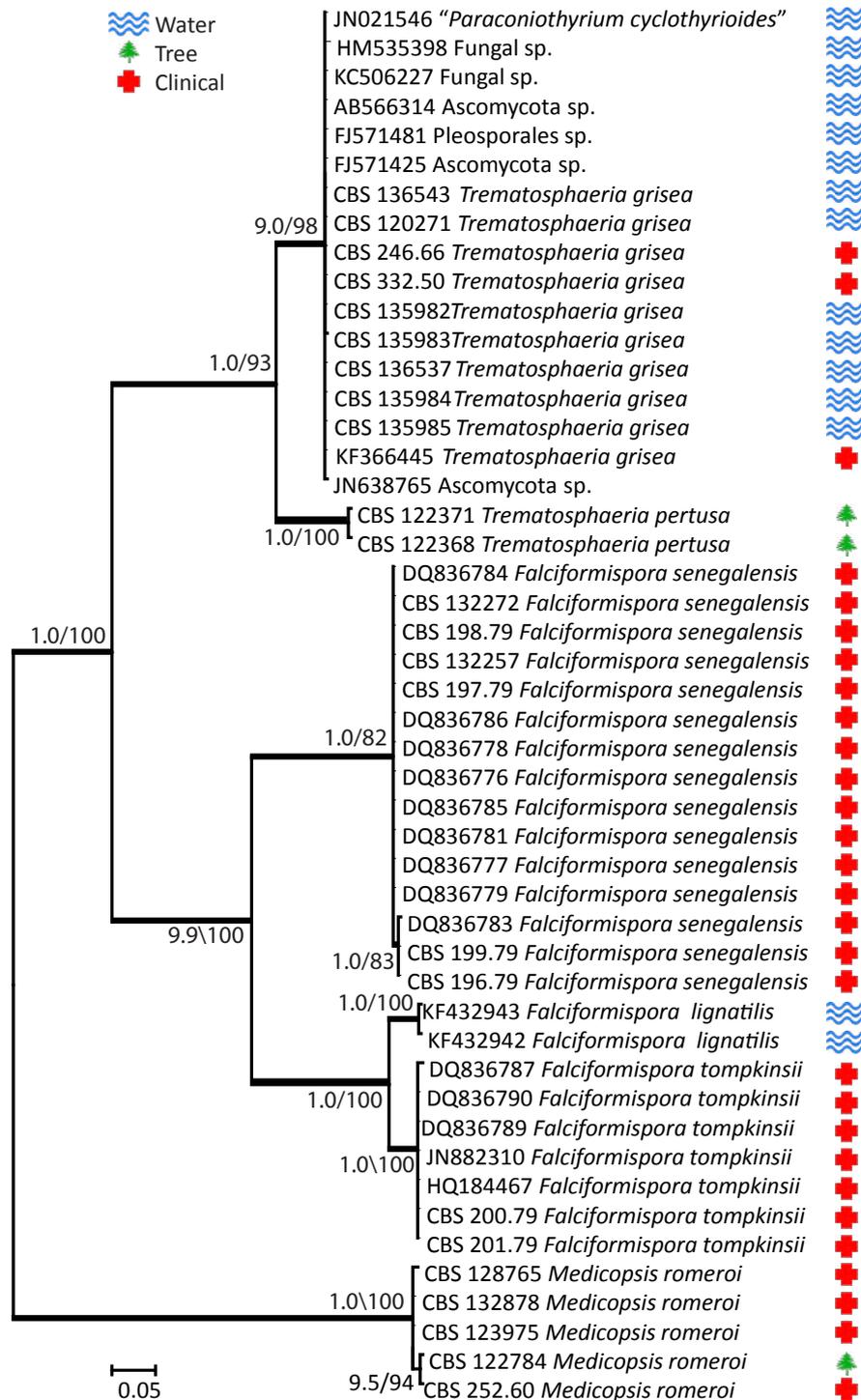
**RESULTS**

**Phylogenetic analysis**

The combined dataset of SSU, LSU, *RPB2*, and *TEF1* consisted of 101 taxa belonging to 13 families of *Pleosporales*. A total of 3 085 alignment characters were used of which 894 were derived from SSU, 706 from LSU, 711 from *RPB2*, and 774 from *TEF1*. Trees of the combined genes were rooted with *Dothidea sambuci* and *Phaeosclera dematioides*. The Bayesian and Maximum likelihood trees were congruent at both familial and intra-familial levels with few differences in the internal node support (Fig. 1). A 50 % majority consensus tree resulting from Bayesian analysis is presented in Fig. 1 in which the main clades represent the families of *Pleosporales* determined according to Zhang et al. (2009). An ITS alignment was constructed for 48 isolates belonging to 6 species. The total alignment length

was 531 characters of which 335 were constant. The ITS tree was rooted with *Medicopsis romeroi* as outgroup.

Based on the multi-gene phylogeny, pleosporalean mycetoma agents were found to belong to two families, viz. the *Trematosphaeriaceae* (*Madurella grisea*, *Leptosphaeria senegalensis*, and *L. tompkinsii*), and the *Arthopyreniaceae* / *Roussoellaceae*. Three additional agents of mycetoma were found to be unrelated to any known family. Within the *Trematosphaeriaceae*, *Madurella grisea* formed a well-supported clade with *Trematosphaeria pertusa* (Bayesian inference posterior probability (BII PP) / bootstrap values 1.00/100 %) (Fig. 1). This finding was confirmed with an ITS tree constructed for the family *Trematosphaeriaceae* (Fig. 2). In the ITS tree, additional ITS sequences were downloaded from GenBank and from strains in the CBS database which proved to be identical to the ITS sequence of *M. grisea*. These strains originally had been isolated from watery environments (Fig. 2, Table 1).



**Fig. 2** Phylogenetic tree resulting from Bayesian analysis and maximum likelihood for the ITS gene (values of  $\geq 0.8$  for Bayesian probability and  $\geq 80\%$  for maximum likelihood are shown with **bold** branches). *Medicopsis romeroi* was used as outgroup.

Based on the multi-gene phylogeny, *Leptosphaeria senegalensis* and *L. tompkinsii* were found to cluster within the *Trematosphaeriaceae*, with *Falciformispora lignatilis* as nearest sibling species (Fig. 1). *Leptosphaeria tompkinsii* was closer to *F. lignatilis* than to its sister species *L. senegalensis*. This was noticed not only in the combined gene tree but also in the pairwise similarities between the three species at the individual loci (Fig. 2). Two sequences of ITS derived from *Falciformispora lignatilis* were included in the ITS tree (Fig. 2). The phylogeny of *Falciformispora* and the two *Leptosphaeria* species in the tree was congruent with the multi-gene tree.

*Pseudochaetosphaerionema larense* was found to be close to *Massaria platani* as a basal lineage to the *Lentitheciaceae*;

however, this position was not well supported (Fig. 1). The relation to the genus *Massaria* also remained unclear since the genus is polyphyletic and *Massaria platani* is not the generic type species.

*Nigrograna mackinnonii* has recently been proposed as a new name for *Pyrenochaeta mackinnonii*, because it was found to be remote from the generic type species *Pyrenochaeta nobilis* (de Gruyter et al. 2010, 2013). In our multilocus analysis this species was found to be closely related to the monotypic genus *Biatrispora*, with *B. marina* as type species (Fig. 1). The two species could not unambiguously be separated using the five genes analysed.

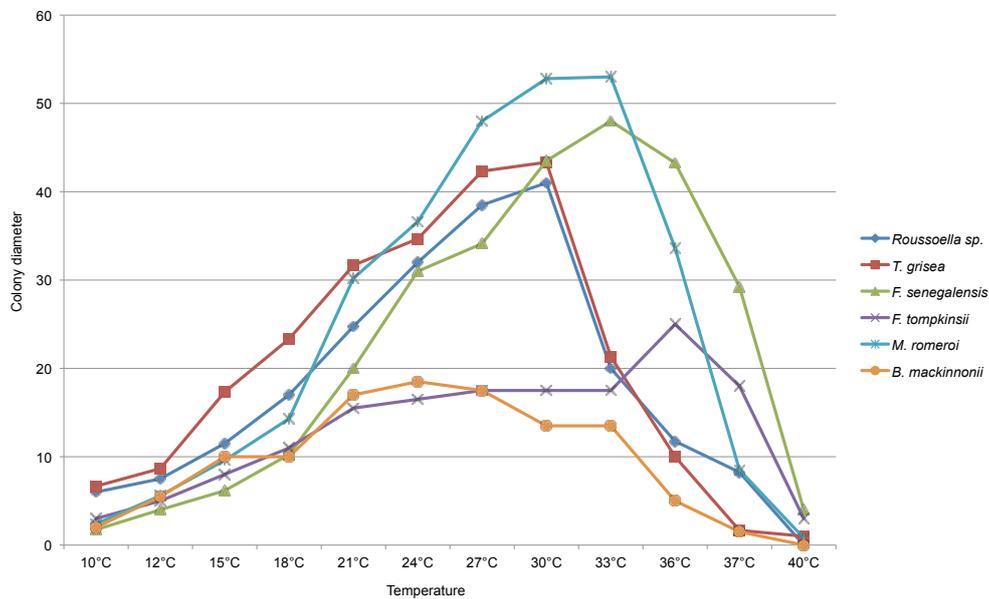
*Medicopsis romeroi* (formerly *Pyrenochaeta romeroi*) was found in a basal lineage of *Pleosporinae* with no close relation to any pleosporalean family. The monotypic genus *Medicopsis* was recently erected to accommodate *Pyrenochaeta romeroi* (de Gruyter et al. 2013).

A non-sporulating eumycetoma agent represented by two strains; one strain morphologically identified by Meis et al. (2000) as *Madurella mycetomatis*, and a second isolate recovered from subcutaneous mycoses clustered in the pleosporalean family *Arthopyreniaceae* / *Roussoellaceae* (Fig. 1). This new species with its clinical data will be published at a later date.

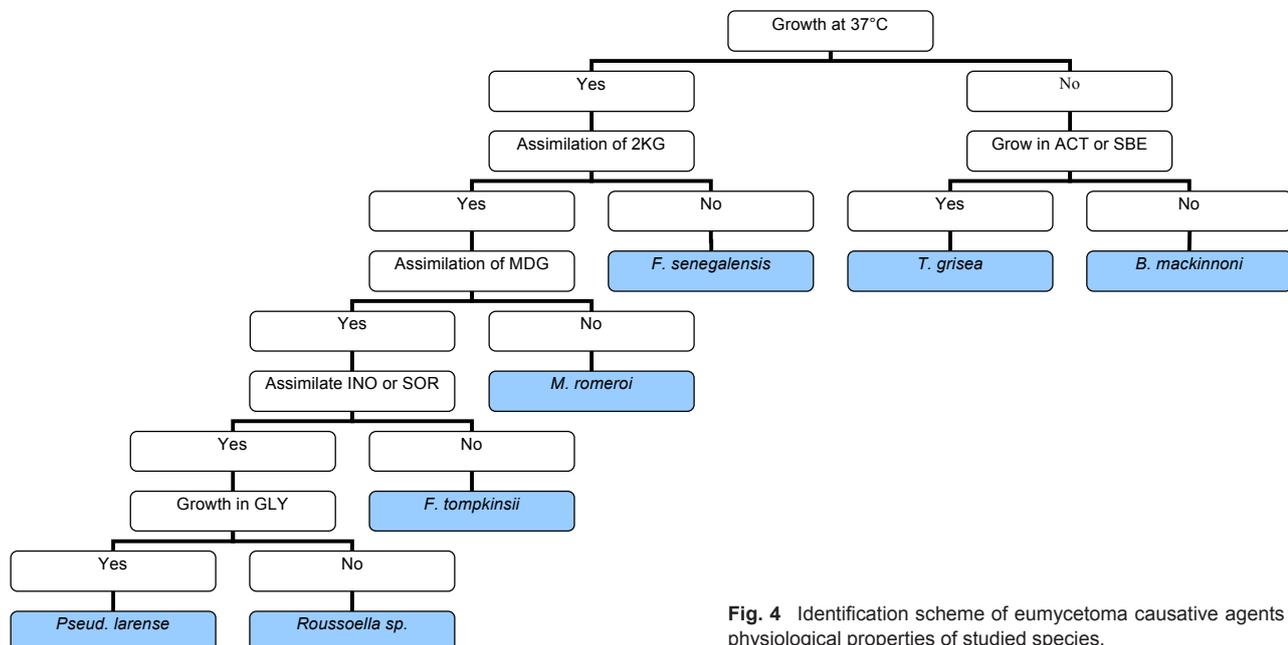
**Physiology**

To determine possible diagnostic phenotypic or virulence markers, the black-grain eumycetoma species of the order of the *Pleosporales* were analysed for their physiological properties, i.e. cardinal growth temperatures and carbohydrate assimilation profiles. Growth profiles at different temperatures for the tested strains are shown in Fig. 3. Most of the species showed optimal growth at temperatures ranging from 27 to 36 °C, with

the exception of *N. mackinnonii*, which had a lower optimum at 24 °C. The maximum growth temperature of all species tested was below 40 °C, but some individual strains of *L. tompkinsii* and *L. senegalensis* still were able to grow at this high temperature. Most strains were able to grow at 37 °C, with the exception of the clinical isolates of *M. grisea* (Fig. 3). However, strains of *M. grisea* still survived at 37 °C, because they could grow when re-incubated at 30 °C. Results of carbohydrate assimilation, urease, laccase, and salt tolerance tests are listed in Table 2. *Madurella grisea*, *Leptosphaeria senegalensis*, *L. tompkinsii*, *Medicopsis romeroi*, *Nigrograna mackinnonii*, *Pseudochaetosphaeronema larense*, and *Roussoella* sp. all were able to assimilate GAL, SAC, NAG, CEL, RAF, MAL, TRE, MAN, RHA, MLZ, PLE, and GLU, but not LAT, GNT, GRT, and GLN; no diagnostic difference was found between these carbohydrates. *Leptosphaeria senegalensis* was unique among the species tested in that it did not assimilate 2KG, ERY, and MEL, whereas the remaining species did. Furthermore, LAC was assimilated by this species, whereas the majority of the species was unable to use this compound. *Leptosphaeria*



**Fig. 3** Average of colony diameters of studied species after 2 wk of incubation at various temperatures ranging from 15 to 36 °C, with 3 °C intervals, including 10 °C, 37 °C, and 40 °C.



**Fig. 4** Identification scheme of eumycetoma causative agents based on physiological properties of studied species.

*tompkinsii* was the only species that did not assimilate INO and SOR. *Medicopsis romeroi* was the only species not assimilating MDG, and *Nigrograna mackinnonii* was unique by its sensitivity to cycloheximide and ability to use XYL, unlike *Pseudochaetosphaeronema larense*, which did not assimilate XYL. Based on these physiological properties, an identification scheme was developed, which is presented in Fig. 4. It appeared that all species were able to produce laccase indicated by a green halo around the colony on ABTS medium (Fig. 8), except for one strain of *L. tompkinsii*. All species were found to be osmotolerant and were able to survive in concentrations of salt up to 20 %.

### Taxonomy

***Biatriospora mackinnonii*** (Borelli) S.A. Ahmed, W.W.J. van de Sande, A. Fahal, de Hoog, *comb. nov.* — MycoBank MB805083

*Basionym.* *Pyrenochaeta mackinnonii* Borelli, *Castellania* 4: 230. 1976. MB322132.

≡ *Nigrograna mackinnonii* (Borelli) Gruyter, Verkley & Crous, *Stud. Mycol.* 75: 31. 2012. MB564795.

*Colonies* on MEA restricted, grey, velvety, becoming dark grey to black with age. Colonies on OA felty, greyish green to olivaceous. Description taken from de Gruyter et al. (2013): “*Pycnidia* solitary on the surface or submerged in agar, globose to subglobose or pyriform, with papillate ostioles. *Conidiogenous*

*cells* hyaline, phialidic, discrete. *Conidia* subhyaline, brown in mass, aseptate, ellipsoidal”. Sexual morph unknown.

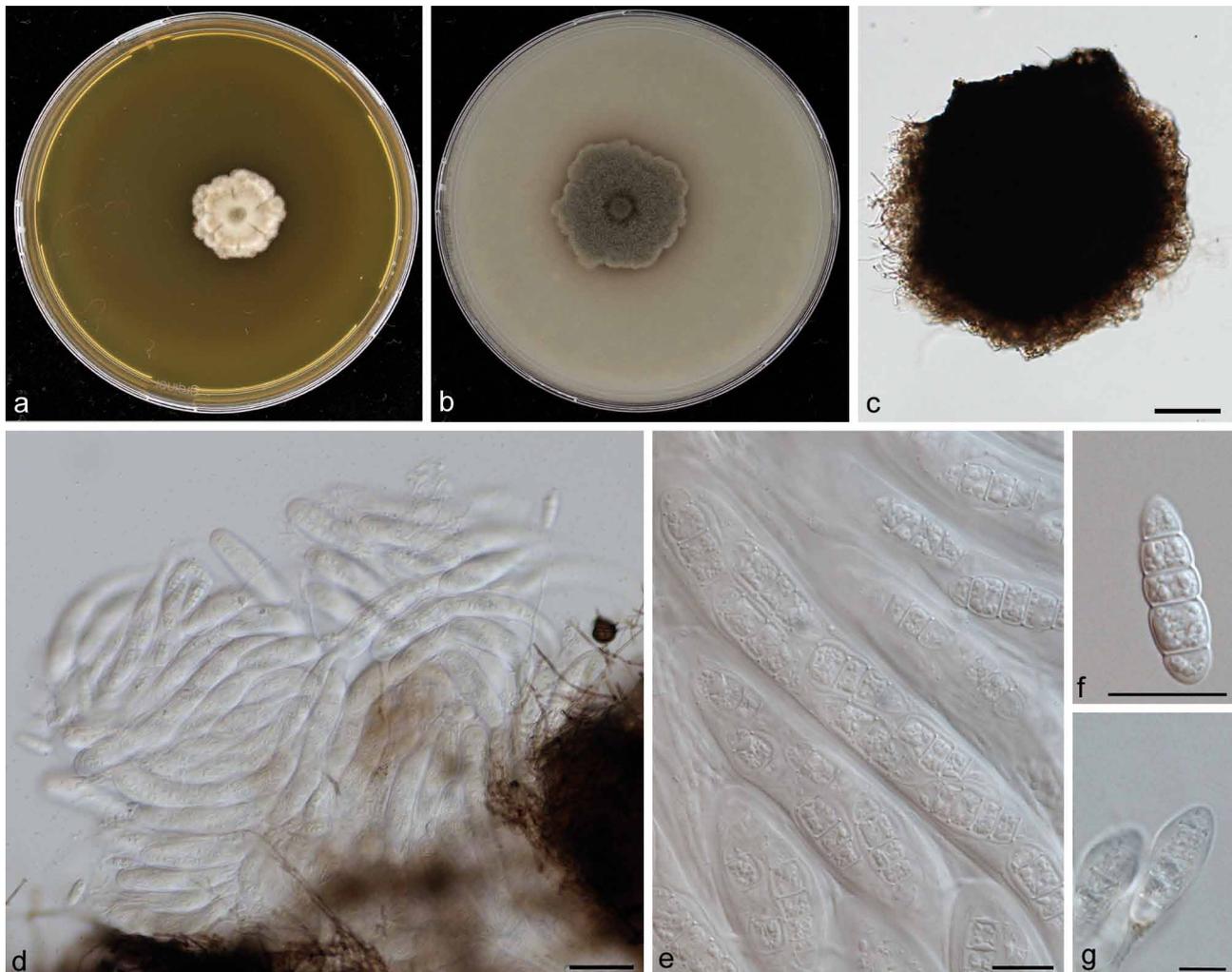
Cardinal temperatures — Min. below 10 °C, opt. 24 °C, max. 37 °C (Fig. 3).

*Specimens examined.* VENEZUELA, human mycetoma, CBS H-21354 holotype, culture ex-type CBS 674.75. Additional strain listed in Table 1.

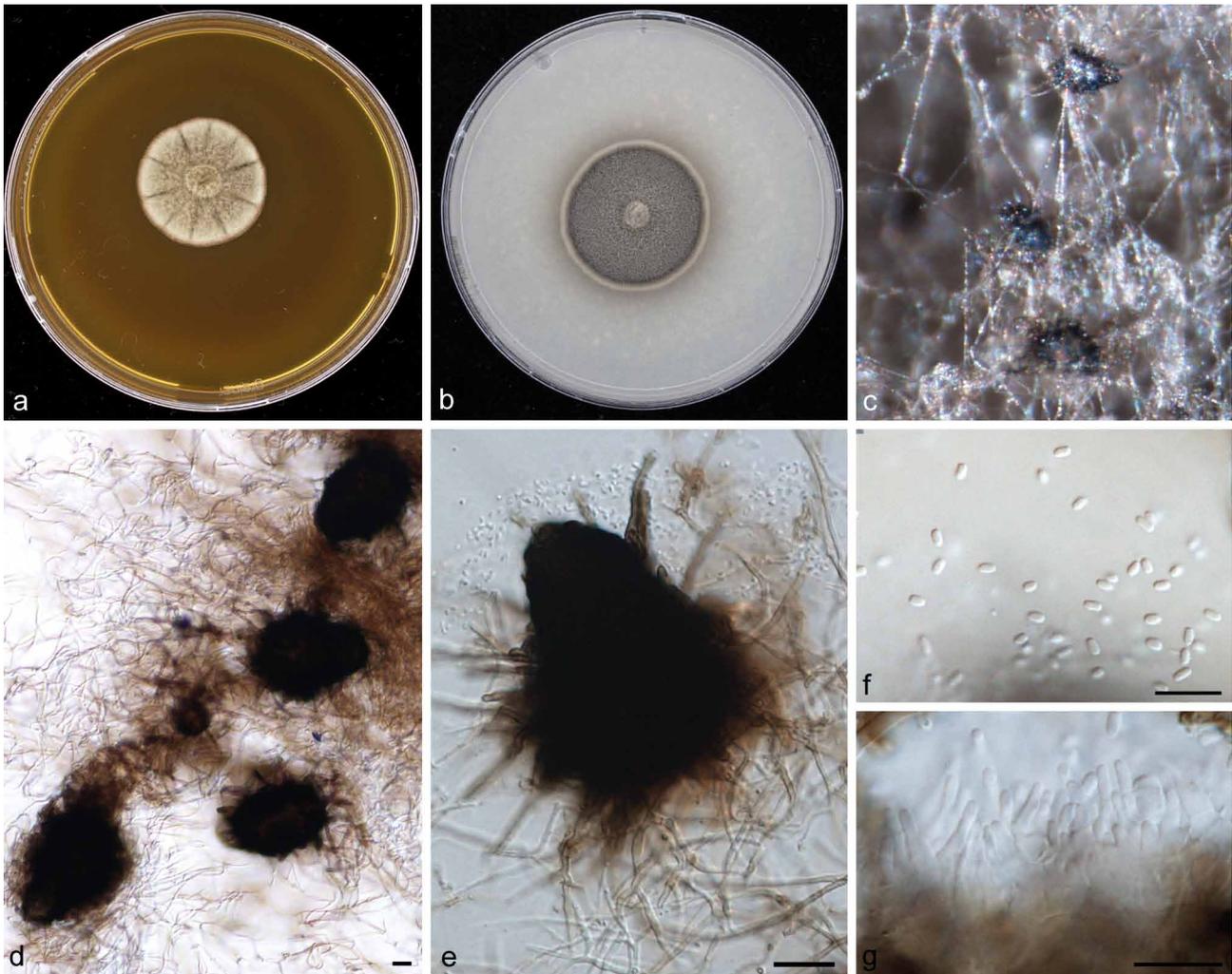
***Falciformispora senegalensis*** (Segretain, Baylet, Darasse & Camain) S.A. Ahmed, W.W.J. van de Sande, A. Fahal & de Hoog, *comb. nov.* — MycoBank MB804853; Fig. 5

*Basionym.* *Leptosphaeria senegalensis* Segretain, Baylet, Darasse & Camain, *Compt. Rend. Acad. Sci.* 248: 3732. 1959. MB299640.

*Colonies* on MEA radially folded, marble white, pale greyish green at some parts, velvety, with brown exudate produced on the colony surface; reverse dark brown to black, with dark brown pigment diffused into the agar. Colonies on OA dark greyish green, felty, radially folded with reddish brown pigment exuded below and around the colony. *Asexual morph* sporulation absent. *Mature ascomata* observed after 8 wk incubation as olivaceous, greyish green dots at the surface of the agar medium, 100–116 µm diam, black, solitary, spherical to subspherical or ovoidal, thick-walled. *Pseudoparaphyses* hyaline, septate. *Asci* clavate, rounded at the apex, bitunicate, containing 8 ascospores. *Ascospores* 30–32 × 9–10 µm ellipsoidal, 4-septate with constrictions at the septa, second cell from the top is the largest, leading to widening of the thin



**Fig. 5** *Falciformispora senegalensis* (CBS 196.79). Colonies after 2 wk of incubation on: a. MEA; b. OA; c. cleistothecia; d, e. asci; f. ascospore; g. ascospore surrounded by thin sheath. — Scale bars = 10 µm.



**Fig. 6** *Medicopsis romeroi* (CBS 252.60). Colonies after 2 wk of incubation on: a. MEA; b. OA; c–e. pycnidia; f. conidia; g. conidiophores. — Scale bars = 10  $\mu$ m.

sheath surrounding the ascospore (Fig. 5). The species did not assimilate potassium 2-keto-gluconate (2KG), erythritol (ERY), and D-melibiose (MEL).

Cardinal temperatures — Min. below 10 °C, opt. 33 °C, max. above 40 °C (Fig. 3).

*Specimens examined.* SENEGAL, Dakar, C. de Bièvre, human mycetoma with black grains, CBS H-21349 holotype, culture ex-type CBS 196.79 = IHM 3278 = IHM 3814 = IP 614.60. Additional strains listed in Table 1.

***Falciformispora tompkinsii*** (El-Ani) S.A. Ahmed, W.W.J. van de Sande, A. Fahal & de Hoog, *comb. nov.* — MycoBank MB804855

*Basionym.* *Leptosphaeria tompkinsii* El-Ani, *Mycologia* 58: 409. 1966. MB333276.

*Colonies* on MEA restricted, flat, initially marble white, becoming greyish green with age. Reverse dark brown to black at the centre and brown pigment seen on agar. Colonies on OA grey, with a halo of a pale red pigment diffusing into agar. Asexual morph sporulation absent. Sexual morph description according to El-Ani (1966): “*Perithecia* are non-ostiolate, scattered, innate or superficial, globose or subglobose, black, covered with brown flexuous hyphae, frequently flattened at the base measuring 214–535  $\mu$ m. *Asci* are octosporic, clavate to cylindrical, bitunicate, rounded at the tip and tapered at the base 80–115  $\times$  20–25  $\mu$ m. The *ascospores* are hyaline, fusoid, straight or curved, 4–8-septate, predominantly 6- and 7-septate, constricted at the septa, 32–45  $\times$  8.8–11  $\mu$ m”. Unable to assimilate inositol (INO) and D-sorbitol (SOR).

Cardinal temperatures — Min. below 10 °C, opt. 36 °C, max. above 40 °C (Fig. 3).

*Specimens examined.* SENEGAL, Dakar, C. de Bièvre, human, CBS H-21350 holotype, culture ex-type CBS 200.79. Additional strain listed in Table 1.

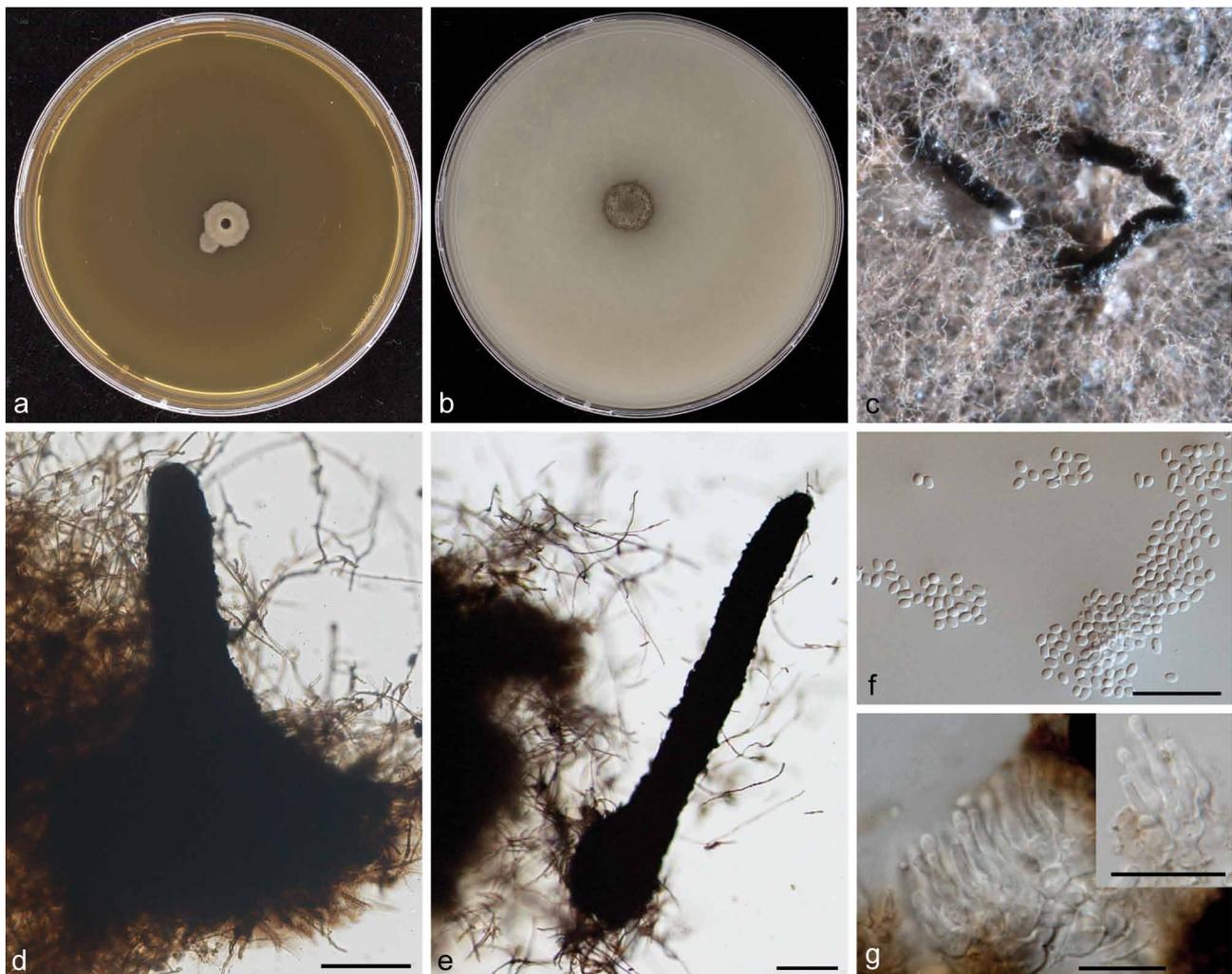
***Medicopsis romeroi*** (Borelli) Gruyter, Verkley & Crous, *Stud. Mycol.* 75: 28. 2012. — MycoBank MB564792; Fig. 6

$\equiv$  *Pyrenochaeta romeroi* Borelli, *Dermatol. Venez.* 1: 326. 1959. MB338075.

*Colonies* on MEA greyish green, velvety, becoming dark grey and floccose with age; reverse dark grey to black. Colonies on OA flat, dark grey to olivaceous or black. *Hyphae* broad, septate, branched, dark brown, verruculose. *Pycnidia* observed after 4 wk incubation as small grey black dots on the surface of agar plates, 105–127  $\times$  70–96  $\mu$ m, black, ostiolate, pyriform, solitary with short dark brown to black setae. *Conidia* 2.0–2.8  $\times$  1.2–1.5  $\mu$ m, hyaline, unicellular, cylindrical to ellipsoidal. *Conidiophores* seen after crushing the pycnidia, phialidic, smooth-walled, hyaline, ampulliform (Fig. 6). *Sexual morph* unknown. Differential diagnosis from other pleosporalean mycetoma agents: inability to assimilate methyl- $\alpha$ -D-glucopyranoside (MDG).

Cardinal temperatures — Min. below 10 °C, opt. 30–33 °C, max. 40 °C (Fig. 3).

*Specimens examined.* VENEZUELA, from human mycetoma, D. Borelli, culture ex-type CBS 252.60 = ATCC 13735 = FMC 151 = UAMH 10841. Additional strains listed in Table 1.



**Fig. 7** *Pseudochaetosphaeronema larense* (CBS 640.73). Colonies after 2 wk of incubation on: a. MEA; b. OA; c–e. pycnidia; f. conidia; g. conidiophores. — Scale bars = 10  $\mu$ m.

***Pseudochaetosphaeronema larense*** (Borelli & R. Zamora) Punith., Nova Hedwigia 31: 127. 1979. — MycoBank MB321757; Fig. 7

**Colonies** on MEA restricted, velvety, grey green; reverse dark grey to black. Colonies on OA woolly, dark grey to olivaceous. **Pycnidia** observed after 8 wk of incubation on OA, 245–248  $\times$  177–231  $\mu$ m black, solitary, obpyriform with long neck reaching 140  $\mu$ m in length. **Conidia** 2.7–3.6  $\times$  2.0–2.5  $\mu$ m, hyaline, unicellular, subspherical to ellipsoidal. **Conidiophores** ampulliform hyaline, phialidic. No assimilation of xylose; sensitive to cyclohexamide. **Sexual morph** unknown.

**Cardinal temperatures** — Min. below 10  $^{\circ}$ C, opt. 24–30  $^{\circ}$ C, max. 40  $^{\circ}$ C (Fig. 3).

**Specimens examined.** VENEZUELA, human mycetoma, *D. Borelli*, culture ex-type CBS 640.73 = FMC 250. Additional strain listed in Table 1.

***Trematosphaeria grisea*** (J.E. Mackinnon, Ferrada & Montemart) S.A. Ahmed, W.W.J. van de Sande, A. Fahal & de Hoog, *comb. nov.* — MycoBank MB804854; Fig. 8

**Basionym.** *Madurella grisea* J.E. Mackinnon, Ferrada & Montemart, Mycopathologia 4: 389. 1949. MB287885.

**Colonies** on MEA restricted, felty to floccose, greenish grey at the centre, becoming faint towards the margin; reverse dark brown to black. Colonies on OA flat, olivaceous to black (Fig. 8). Environmental isolates of *T. grisea* rapidly growing, with expanding, grey colonies. **Hyphae** branched, septate, hyaline or brown, thick-walled, verruculose. **Pycnidia** observed in envi-

ronmental strains after 8 wk incubation as small black dots with white droplets on top, 128–190  $\times$  96–115  $\mu$ m, black, solitary or aggregated, subspherical to pyriform, ostiolate, setose. **Conidia** 4.0–5.4  $\times$  2.0–2.4  $\mu$ m, hyaline to pale brown, unicellular, clavate to ellipsoidal. **Conidiophores** hyaline, rostrate with very short, hardly detectable collarette. **Sexual morph** unknown.

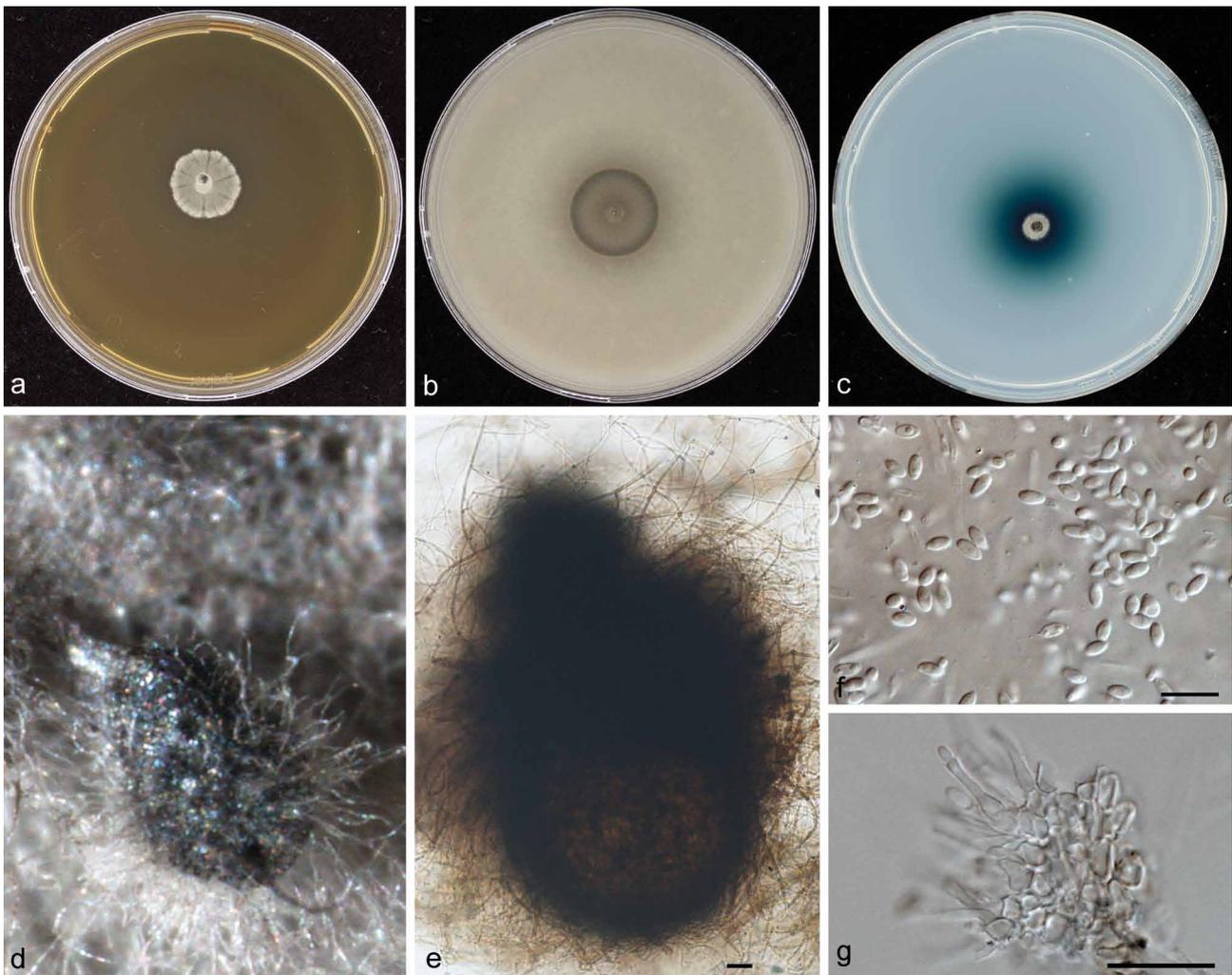
**Cardinal temperatures** — Min. below 10  $^{\circ}$ C, opt. 27–30  $^{\circ}$ C, max. 40  $^{\circ}$ C (Fig. 3).

**Specimens examined.** CHILI, Mycetoma, *J.E. MacKinnon*, CBS-H 21348 holotype, culture ex-type CBS 332.50 = ATCC 10794 = IHM 927. Strain CBS 120271 isolated from tap water, The Netherlands, was used for morphological description of pycnidia and conidia, additional strains listed in Table 1.

***Trematosphaeria pertusa*** (Pers.) Fuckel, Jahrb. Nassauischen Vereins Naturk. 23–24: 161. 1870. — MycoBank MB197662; Fig. 9

**Basionym.** *Sphaeria pertusa* Pers., Syn. Meth. Fung.: 83. 1801. MB405380.

**Colonies** on MEA velvety, dark greyish green with pale grey margin; reverse dark grey to black. Colonies on OA floccose, dark greyish green. **Pycnidia** 70–100  $\times$  38–63  $\mu$ m, black, solitary or in aggregates, ovoidal, ostiolate, with dark brown to black setae. **Conidia** 2.0–3.6  $\times$  2.0–2.3  $\mu$ m, hyaline to pale brown, unicellular, ellipsoidal. **Conidiophores** short, hyaline, obpyriform. **Sexual morph:** description according to Zhang et al. (2008): “*Ascomata* were 350–550  $\times$  320–480  $\mu$ m, solitary or in groups and subglobose. *Asci* were 100–145  $\times$  15–17  $\mu$ m, contain 8 ascospores 27.5–32.5  $\times$  7.5–8.5  $\mu$ m, verruculose



**Fig. 8** *Trematosphaeria grisea* (a–c. CBS 332.50, d–g. CBS 120271). Colonies after 2 wk of incubation on: a. MEA; b. OA c. ABTS media; d, e. pycnidia; f. conidia; g. conidiophores. — Scale bars = 10  $\mu$ m.

with 1–3 septa, deeply constricted at the median septum, the upper cell often shorter and broader than the lower one”.

Cardinal temperatures — Min. below 10 °C, opt. 27 °C, max. 37 °C.

*Specimens examined.* FRANCE, 25 Apr. 2004, from *Fraxinus excelsior*, J. Fournier, Herbarium IFRD 2002, culture ex-type CBS 122368. Additional strain listed in Table 1.

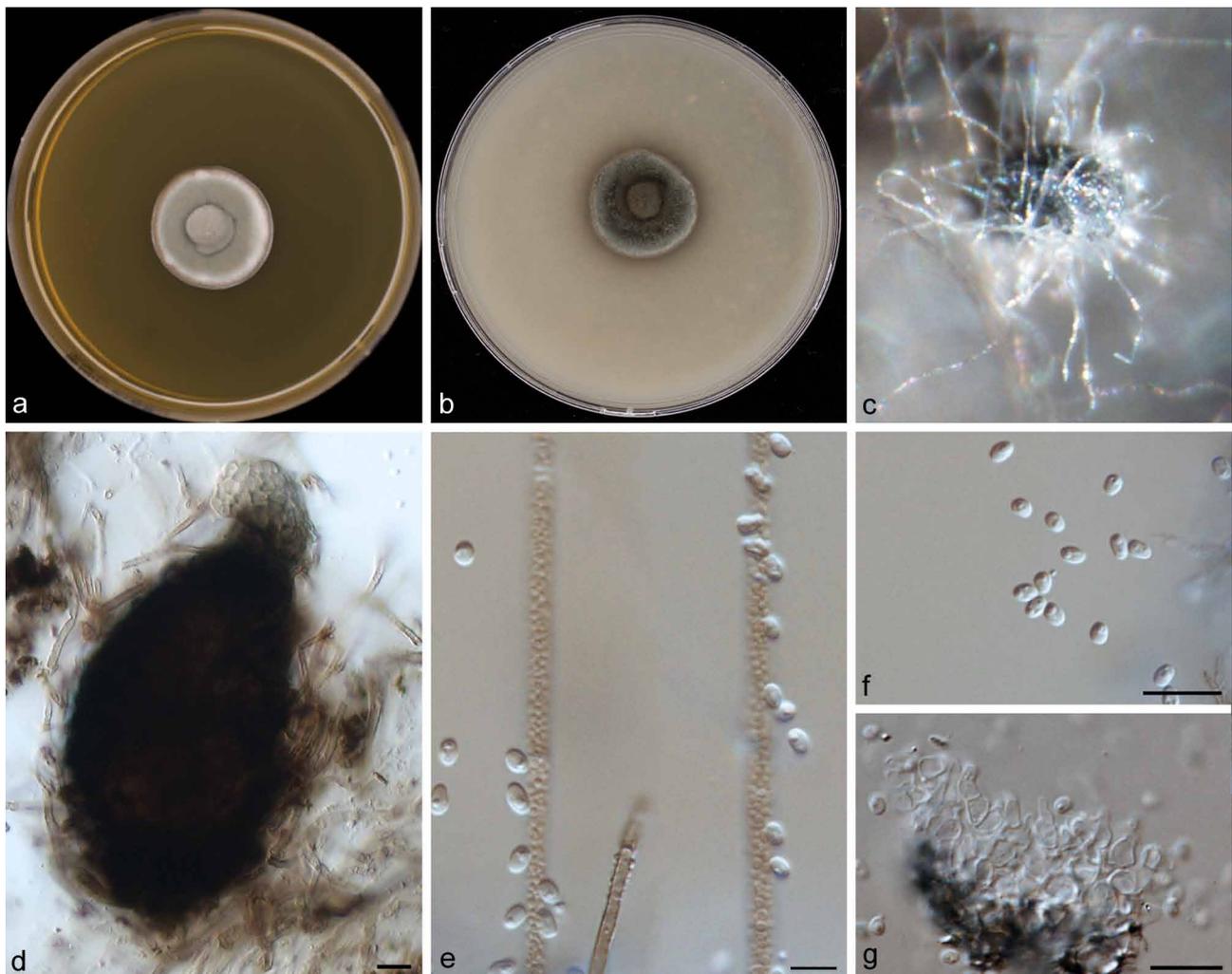
## DISCUSSION

A wide range of fungal species has been reported as causative agents of human eumycetoma. To date more than 30 species are listed, belonging to different genera throughout the *Ascomycota*, predominantly in the orders *Sordariales* and *Pleosporales* (de Hoog et al. 2004). In the present study, we analysed the molecular taxonomy of pleosporalean agents of mycetoma using five loci, to establish their higher phylogenetic affiliations as well as their specific taxonomy. Some of the species have exclusively been recovered from cases of human mycetoma and are not known from the environment. They are nevertheless judged to be environmental opportunists causing a general foreign body response in the host (de Hoog et al. 2000). A habitat involving living plants or plant debris is known for numerous species of genera such as *Leptosphaeria* and *Pyrenochaeta*, but for the clinical members of these genera the natural niche has yet to be discovered.

The present study shows that agents of mycetoma are phylogenetically remote from the genera where they were originally classified, and thus entirely different habitats may be

expected. A striking example is *Nigrograna mackinnonii* (= *Pyrenochaeta mackinnonii*), which we found to be molecularly indistinguishable from *Biatrispora marina*, a marine ascomycete consistently inhabiting tropical mangrove wood (Hyde & Borse 1986, Jones et al. 2006). *Nigrograna* was proposed to accommodate *Pyrenochaeta mackinnonii*. However, the high degree of sequence similarity demonstrated the single identity of the two species, *N. mackinnonii* and *B. marina*. Therefore, the genus *Nigrograna* proved to be redundant and *Nigrograna mackinnonii* might be the asexual morph of the ascospore-producing species *B. marina*. Mangrove wood may be the natural habitat of the fungus; cases of mycetoma are extremely rare, so that human infection is unlikely to be the natural behaviour of the fungus. An unidentified *Biatrispora* species was isolated from a marine sponge in Brazil by Passarini et al. (2013). In addition to mycetomata, *N. mackinnonii* was also reported from phaeohyphomycosis in a renal transplant patient in Brazil (Santos et al. 2013). No morphological similarity was found between *B. marina* and *N. mackinnonii* and the two strains of *N. mackinnonii* (*B. marina*) included in our analysis, originating from Mexico and Venezuela, did not show any sporulation even after 3 mo of incubation. De Gruyter et al. (2013) reported the formation of pycnidia and conidia by the ex-type strain of *N. mackinnonii*, CBS 674.75.

Most clinical isolates from mycetoma are non-sporulating or produce conidia or ascospores only after a very long incubation time. Applying phenotypic, morphological diagnostics has thus led to many misidentifications and also to misclassifications of the fungus within the fungal kingdom. Previous generic concepts



**Fig. 9** *Trematosphaeria pertusa* (CBS 122368). Colonies after 2 wk of incubation on: a. MEA; b. OA. c, d. pycnidia; e. verrucose hyphae and conidia; f. conidia; g. conidiophores. — Scale bars = 10  $\mu$ m.

based on sterile mycelia led to assignment of *Madurella mycetomatis* and *M. grisea* as sister and only species in the genus, whereas we now know that the DNA sequence differences are great enough that they are placed in different orders (de Hoog et al. 2004). More precisely, whereas *Madurella mycetomatis*, the most frequent agent of eumycetoma (McGinnis 1996) appeared to be closely related to dung-associated species of *Chaetomium* in the *Sordariales* (van de Sande 2012, de Hoog et al. 2013), we found *M. grisea* to be related to marine wood-inhabiting species of *Trematosphaeria* in the *Pleosporales*.

*Madurella grisea*, *Leptosphaeria senegalensis*, and *L. tompkinsii* were found to belong to the *Trematosphaeriaceae*, a small family of fungi in marine environments affiliated to the sub-order *Massarineae* (Zhang et al. 2012). This adds to *N. mackinnonii* above, where also a marine natural habitat was suggested. De Hoog et al. (2005) noted that mild halotolerance tends to be associated with opportunism and may thus be regarded as a potential virulence factor. The ecological similarity with *Trematosphaeria* is striking; possibly also here mild osmotolerance can be regarded as an ancestral virulence factor in these fungi.

The family *Trematosphaeriaceae* consists of three genera, *Trematosphaeria*, *Halomassarina*, and *Falciformispora*. These genera mainly comprise fungi inhabiting marine environments, e.g. mangrove wood (Suetrong et al. 2011). *Madurella grisea* was found closely related to *Trematosphaeria pertusa*, the type species of the genus *Trematosphaeria*. Both species produce a coelomycetous asexual morph forming well-characterised conidiomata (Fig. 8, 9), but *T. pertusa*, described from its

natural habitat, produces a sexual morph with ascomata and ascospores (Zhang et al. 2008). In our study, we analysed two strains of *T. pertusa*, including the ex-type strain (CBS 122368). Both strains only displayed asexual sporulation, which was not described before (Fig. 9). Zhang et al. (2008) reported formation of hyphopodia-like structures in *T. pertusa* after 6 mo of incubation in water. To compare the morphology with other species of the genus *Trematosphaeria*, strains of *T. heterospora*, *T. hydrela*, and *T. crassiseptata* have been analysed, but they were found to be phylogenetically distant from *T. pertusa* and could not be included in the tree (data not shown) (Wang et al. 2007). Because of similarities in both phylogenetic position and morphology with *T. pertusa*, *M. grisea* is transferred to the genus *Trematosphaeria* and renamed *Trematosphaeria grisea*. Physiological properties established for *T. grisea*, i.e. assimilation of sucrose and galactose but not lactose are consistent with the original description of the species given by Mackinnon et al. (1949). The GenBank database contained several unnamed depositions of identical ITS sequences, which are now re-identified as *T. grisea* (Fig. 2). Interestingly, all sequences for which information on origins was available were derived from marine and marine-related environments (Fig. 2). In addition, several strains from the CBS were added which were mainly obtained from water or other environmental sources in The Netherlands (Table 1). In the present study, only 3 out of 10 strains genotypically identified as *T. grisea* and included in the ITS tree (Table 1, Fig. 2) originated from a human infection (mycetoma, abscess, biopsy specimen). Thus *T. grisea* is an occasional opportunist. Six of the strains were recovered from

water, and one from pastry gel. This suggests that *T. grisea* is likely to be an oligotrophic saprobe. Reported cases of mycetoma caused by *T. grisea* originated from South America and India. Unconfirmed reports were published from North and Central America, Africa, and Asia (Green & Adams 1964, Butz & Ajello 1970, Mahgoub 1973, Venugopal et al. 1990, Vilela et al. 2004). In most of these publications, identification was based on morphological or physiological properties and may have been misidentifications (Desnos-Ollivier et al. 2006). One of the strains of *T. grisea* examined by Mackinnon et al. (1949) in the original description of the species was re-identified with ITS sequences as *Madurella tropicana* (*Sordariales*, *Chaetomiaceae*). Recently, a case of mycetoma in an Indian patient in the UK was ascribed as *T. grisea* by Gulati et al. (2012). However, sequence comparison revealed that the strain was phylogenetically remote from *T. grisea* (data not shown).

Two *Leptosphaeria* agents of mycetoma appear in the literature, viz. *L. senegalensis* and *L. tompkinsii*. Both species were found to be phylogenetically remote from the *Leptosphaeriaceae* but instead clustered in the *Trematosphaeriaceae* close to *Falciformispora*, another monotypic genus in which *F. lignatilis* is associated with the mangrove environment (Hyde 1992). The position of *L. tompkinsii* and *F. lignatilis* is concordant with the morphology, both forming ascospores with 6–8 septa, whereas in *L. senegalensis* ascospores have 4 septa (El-Ani 1966, Hyde 1992). With *F. lignatilis* as the type species of the genus *Falciformispora*, both *L. senegalensis* and *L. tompkinsii* are to be transferred to this genus. All strains of *F. senegalensis* and *F. tompkinsii* included in this study originated from human infections, mostly mycetoma or other subcutaneous infections, and were geographically distributed in tropical areas of Sudan and Senegal. Segretain & Mariat (1968) reported the isolation of *F. senegalensis* from *Acacia* thorns in West and Central Sub-Saharan Africa. *Falciformispora lignatilis* has thus far only been recovered from mangrove wood and fresh water in North America and Thailand (Hyde 1992, Suetrong et al. 2009).

Of the five strains of *Pyrenochaeta romeroi* included in the present study, two were derived from mycetoma, two from phaeohyphomycosis or subcutaneous cysts, and one was recovered from plant material (Table 1). De Gruyter et al. (2010) excluded *P. romeroi* from the genus *Pyrenochaeta*, because it was proven to be phylogenetically distant from its type species *P. nobilis*. In another study, de Gruyter et al. (2013) proposed a new genus *Medicopsis* to accommodate *P. romeroi*. In our multi-gene phylogeny the as yet monotypic genus *Medicopsis* was found as a basal lineage to the *Pleosporinae* rather than as a member of *Trematosphaeriaceae* as reported by de Gruyter et al. (2013) based on rDNA LSU analyses. This might be due to the larger number of loci analysed, a more extended taxon sampling of species of *Trematosphaeriaceae*, and more strains of *M. romeroi* included.

Very few cases of eumycetoma have been reported with *Pseudochaetosphaerionema larense* as causative agent. Two strains isolated from Venezuela were analysed in the present study. The species formed a well-supported clade with strains identified as *Massaria platani*, and with *Lentitheciaceae* as the closest family, but at a significant phylogenetic distance. This was in accordance with the phylogenetic tree published by Schoch et al. (2009), while another tree published by Suetrong et al. (2009) showed that *M. platani* strains were basal lineage of *Trematosphaeriaceae*. In general, the phylogeny of many genera of *Pleosporales* is poorly resolved at the familial level mainly because this order is still under-sampled (Schoch et al. 2009).

Two sterile strains of a madurella-like species from human mycetoma were studied, one originating from the Caribbean and the other from India. Both strains appeared to represent

a new pathogenic species in the family *Arthopyreniaceae* / *Roussioellaceae* (Ahmed et al. 2014).

The different species of mycetoma analysed in the present study share some physiological and virulence factors like growth at human body temperature, laccase production, and melanisation, in addition to halotolerance. The ecology and epidemiology of these species need to be further determined by analysing more strains from different regions.

**Acknowledgements** We thank Satinee Na and E. Gareth Jones for providing sequences of *Falciformispora* strains. Katrien Lagrou is thanked for providing information on some clinical strains.

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