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Article *in* Fungal Biology · February 2016

DOI: 10.1016/j.funbio.2016.01.014

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Molecular taxonomy of scopulariopsis-like fungi with description of new clinical and environmental species

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ARTICLE INFO

Article history:
Received 19 October 2015
Received in revised form
22 January 2016
Accepted 22 January 2016
Available online 3 February 2016
Corresponding Editor:
Marc Stadler

Keywords:
DNA barcoding
Fungal identification
Microascus
Phylogeny
Pithoascus
Pseudoscopulariopsis and *Scopulariopsis*

ABSTRACT

The taxonomy of scopulariopsis-like fungi, comprising numerous human opportunistic species, has recently been reassessed with delineation of the genera *Microascus*, *Pithoascus*, *Pseudoscopulariopsis*, and *Scopulariopsis*, using morphological data and multilocus sequence analysis based on four loci (ITS, LSU, *EF-1α*, and *TUB*). In this study, the same genetic markers were used to investigate a set of clinical and environmental isolates, morphologically identified as *Microascus* and *Scopulariopsis* spp. The ingroups of the concatenated phylogenetic tree resolved 41 species clades, with isolates distributed in four main lineages corresponding to the genera *Microascus*, *Pithoascus*, *Scopulariopsis*, and newly established genus *Fuscoannellis*, typified by *Scopulariopsis carbonaria*. The new species *Microascus chinensis*, *Microascus onychoides*, *Microascus pseudolongirostris*, *Pithoascus lunatus*, and *Scopulariopsis macurae* were described. *Microascus trigonosporus* var. *terreus* and *Scopulariopsis alboflavescens* were found different from *M. trigonosporus* and *Scopulariopsis brevicaulis*, respectively. All the species identified in the study, except *Fuscoannellis carbonaria* and *S. macurae*, originated from clinical samples, suggesting their potential role in human disease. The use of a four marker combination was demonstrated an efficient and reliable approach to infer phylogenetic relationships among the scopulariopsis-like fungi. Yet, the only genetic marker able to discriminate all species was *EF-1α*, therefore proposed as a secondary barcode for the identification of these fungi.

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http://dx.doi.org/10.1016/j.funbio.2016.01.014

Introduction

The genus *Scopulariopsis* contains both hyaline and somewhat pigmented filamentous fungi that normally live in soil as saprotrophs, deriving their nourishment from plant debris and other organic matter (Morton & Smith 1963, Domsch *et al.* 2007). Several species, however, are able to cause opportunistic infections in humans. Most of these infections involve skin and nails, but cases of subcutaneous, deep tissue and disseminated mycoses have also been described, predominantly in patients with a severely impaired immunological status (De Hoog *et al.* 2011; Sandoval-Denis *et al.* 2013). *Scopulariopsis* infections have recently gained more attention due to their increasing incidence and expanding geographical range.

Traditionally, the genus *Scopulariopsis* was established to include fungi that only showed asexual reproduction with long chains of dry conidia and annellidic conidiogeneous cells (annellides), and those species which produced an additional sexual state were commonly placed under the teleomorphic genus *Microascus* (Barron *et al.* 1961; von Arx 1975; Abbott *et al.* 2002; Guarro *et al.* 2012). In this context, several taxonomic studies on *Scopulariopsis* and *Microascus* have been published, the most relevant ones probably being those by Barron *et al.* (1961) and Morton & Smith (1963) which, using only morphological criteria, distinguished nearly 30 species in the two genera. The phenotypic scheme of classification proposed by these authors has been followed over decades and numerous new species have been added to this group of fungi. These genera belong to the *Microascaceae* family of the *Sordariomycetes* (von Arx *et al.* 1988; Guarro *et al.* 2012), a large and complex ascomycetous class whose phylogeny has not yet been well defined.

Considering the limitations related to morpho-taxonomy, a wide array of methods based on DNA sequencing technologies has been employed to classify fungi and to assess their genetic diversity. DNA barcoding has become a commonly used tool for species identification, the ribosomal internal transcribed spacer (ITS) in particular being the barcode marker generally accepted for fungal identification (Schoch *et al.* 2012). However, that locus may display insufficient variation to unequivocally recognize species so alternative markers are required. For instance, the large subunit ribosomal RNA gene (LSU rDNA), protein-encoding genes such as *EF-1α*, *TUB*, and *RPB2*, coding respectively for translation elongation factor 1-α, β-tubulin, and the second largest subunit of RNA polymerase II, have all been used to study phylogenetic relationships within *Sordariomycetes* (Zhang *et al.* 2006; Tang *et al.* 2007; Walker *et al.* 2012). Particularly in *Scopulariopsis* and *Microascus* species, the LSU and ITS regions are insufficient barcode markers to distinguish all species (Issakainen *et al.* 2003; Ropars *et al.* 2012; Jagielski *et al.* 2013; Sandoval-Denis *et al.* 2013; Lackner *et al.* 2014). With the aim of defining molecular targets for species identification in *Scopulariopsis* and other molds isolated from cheese, Ropars *et al.* (2012) compared phylogenies of partial LSU, *TUB*, and *EF-1α* genes. These authors suggested *EF1-α* as a marker for species discrimination in *Scopulariopsis*, although many ambiguities in the phylogenies of *Scopulariopsis* and *Microascus* species remained. In order to establish species boundaries, clarify the taxonomy of genera, and to comply with the new rules of nomenclature for fungi (Hawksworth 2011), a polyphasic approach was adopted by Sandoval-Denis *et al.* (2016). These authors, combining a multilocus sequence analysis of four loci (i.e., ITS and D1/D2 regions of the rDNA, *TUB*, and *EF-1α*) and morphological data, recognized *Microascus* and *Scopulariopsis* as two distinct genera, comprising respectively 20 and six species. These two genera exclusively include the species reported as human opportunistic pathogens. Additionally, the genus *Pithoascus* (Pi.) was reinstated with five species, and *Pseudoscopulariosis* (Ps.) was proposed as a new genus of Microascales with two species. Currently, the identification of scopulariopsis-like fungi becomes easier due to the availability of DNA sequences of type and authentic strains for comparison.

The purpose of the present study was to investigate a set of clinical and environmental isolates of *Scopulariopsis* and *Microascus*, in view of species circumscription through the DNA strategy used by Sandoval-Denis *et al.* (2016), and also to propose a DNA barcode marker for a rapid and reliable identification of the species of these genera.

Materials and methods

Strains and sequences

A total of 49 strains from clinical and environmental sources, morphologically identified as *Scopulariopsis* (n = 36) and *Microascus* (n = 13), were investigated in the present study (Table 1). The strains were obtained from the Peking University Health Science Center (Beijing, China) and from the Centraalbureau voor Schimmelcultures (CBS) culture collection (Utrecht, the Netherlands). In addition, a total of 172 sequences of the four selected loci (ITS, LSU, *TUB*, and *EF-1α*) of type or reference strains of the currently accepted species of *Scopulariopsis*, *Microascus*, *Pithoascus*, and *Pseudoscopulariopsis* (Sandoval-Denis *et al.* 2016) were included for comparative analyses (Table 1).

Morphological identification

The strains were cultured onto potato dextrose agar (PDA; LAB-AGAR, Biocorp, Poland), oatmeal agar (OA; 30 g boiled and filtered oat flakes, 15 g agar, 1 L distilled water), and potato carrot agar (PCA; 40 g of each boiled and filtered carrots and potatoes, 15 g agar, 1 L distilled water). They were incubated at 25, 30, 35, and 37 °C, with growth being monitored every 7 d for up to 4 weeks. Micromorphological features were examined in wet mount preparations, with lactophenol and lactophenol cotton blue. Subcultures of the strains corresponding to new species were deposited in the CBS culture collection. Dried cultures were deposited in the CBS and in the Research Center for Medical Mycology, Peking University (BMU), Beijing, China.

DNA isolation, amplification, and sequencing

All the strains were grown for 4–5 d on Sabouraud dextrose agar (LAB-AGAR, Biocorp) at 25 °C. To extract the total

Table 1 – Strains of *Scopulariopsis*, *Microascus* and related fungi included in the study.

Species		Strain accession no.	Origin	GenBank accession no.			
Current name	Original identification		Source/Country	ITS	LSU	*TUB*	*EF1-α*
Cephalotrichum stemonitis	*Cephalotrichum stemonitis*	CBS 103.19	Seed/Netherlands	LN850951	LN850952	LN850954	LN850953
Fuscoannellis carbonaria	*Scopulariopsis carbonaria*	CBS 205.61^{T}	Soil/Panama	LM652489	HG380462	LM652695	HG380385
gen. & comb. nov.	*Scopulariopsis brumptii*	**CBS 121662**	Black stromata of a pyrenomycete/USA	**LN850803**	**LN850852**	**LN850900**	**LN850948**
Microascus alveolaris	*Microascus trigonosporus*	**CBS 494.70**	Marine sediment/Norway	**LN850757**	**LN850806**	**LN850855**	**LN850903**
	Microascus alveolaris	CBS 139501^{T}	Bronchoalveolar lavage/USA	LM652385	HG380484	LM652601	HG380407
Microascus brunneosporus	*Microascus brunneosporus*	CBS 138276^{T}	Bronchoalveolar lavage/USA	LM652390	HG380497	HG380420	LM652605
	Scopulariopsis sp.	**BMU03919**	Nail/China	**LN850758**	**LN850807**	**LN850856**	**LN850904**
	Scopulariopsis sp.	**BMU06573**	Nail/China	**LN850759**	**LN850808**	**LN850857**	**LN850905**
Microascus campaniformis	*Microascus campaniformis*	CBS 138126^{T}	Bronchoalveolar lavage/USA	LM652391	HG380495	LM652606	HG380418
Microascus chartarus	*Microascus chartarus*	CBS 294.52^{T}	Mouldy wall paper/England	LM652393	HG380463	LM652607	HG380386
Microascus chinensis sp. nov.	*Scopulariopsis* sp.	**BMU01837 (CBS 139628)^{T}**	Nail/China	**LN850760**	**LN850809**	**LN850858**	**LN850906**
	Scopulariopsis sp.	**BMU01895**	Nail/China	**LN850761**	**LN850810**	**LN850859**	**LN850907**
Microascus cinereus	*Microascus cinereus* (isotype of *M. reniformis*)	**CBS 664.71**	Lung/USA	**LN850762**	**LN850811**	**LN850860**	**LN850908**
	Microascus cinereus	CBS 138709^{NT}	Bronchoalveolar lavage/USA	LM652397	HG380350	LM652611	HG380427
Microascus cirrosus	*Microascus cirrosus*	CBS 217.31^{T}	Leaf of *Prunus* sp./Italy	LM652400	HG380429	LM652614	HG380352
	Microascus cirrosus	**CBS 116405**	Antique tapestries/Poland	**LN850763**	**LN850812**	**LN850861**	**LN850909**
	Microascus cirrosus	**BMU04809**	Pulmonary tissue/China	**LN850764**	**LN850813**	–	**LN850910**
Microascus croci	*Scopulariopsis brumptii*	**BMU03912**	Nail/China	**LN850765**	**LN850814**	**LN850862**	**LN850911**
	Microascus croci	CBS 158.44^{T}	*Crocus* sp./Netherlands	LM652407	LM652508	LM652621	LM652560
	Microascus croci	CBS 296.61	Air/Brazil	LM652408	LM652509	LM652622	LM652561
Microascus expansus	*Microascus expansus*	CBS 138127^{T}	Sputum/USA	LM652410	HG380492	LM652624	HG380415
	Microascus expansus	FMR 12267	Pleural fluid/USA	LM652409	HG380491	LM652623	HG380414
Microascus gracilis	*Microascus gracilis*	CBS 369.70^{NT}	Food/Japan	LM652412	HG380467	LM652625	HG380390
	Microascus cinereus	**CBS 116059**	Polyethylene with starch/Poland	**LN850766**	**LN850815**	**LN850863**	**LN850912**
	Scopulariopsis gracilis	**BMU02787**	Nail/China	**LN850767**	**LN850816**	**LN850864**	**LN850913**
	Scopulariopsis gracilis	**BMU04786**	Bronchoalveolar lavage/China	**LN850768**	**LN850817**	**LN850865**	**LN850914**
Microascus hyalinus	*Microascus hyalinus*	CBS 766.70^{T}	Dung of cow/USA	LM652418	LM652513	LM652631	LM652564
Microascus intricatus	*Scopulariopsis* sp.	**BMU04915**	Nail/China	**LN850769**	**LN850818**	**LN850866**	**LN850915**
	Microascus intricatus	CBS 138128^{T}	Bronchoalveolar lavage/USA	LM652419	HG380496	LM652632	HG380419
Microascus longirostris	*Microascus longirostris*	CBS 196.61^{NT}	Wasp's nest/USA	LM652421	LM652515	LM652634	LM652566
	Microascus longirostris	CBS 415.64	Soil/Japan	LM652422	LM652516	LM652635	LM652567
Microascus macrosporus	*Microascus macrosporus*	CBS 662.71	Soil/USA	LM652423	LM652517	LM652636	LM652567
Microascus murinus	*Microascus murinus*	CBS 830.70^{T}	Composed municipal waste/Germany	LM652424	HG380481	HG380404	LM652637
	Scopulariopsis murina	**CBS 864.71**	Municipal waste/Germany	**LN850770**	**LN850819**	**LN850867**	**LN850916**
	Scopulariopsis murina	**CBS 621.70**	Composted municipal waste/Germany	**LN850771**	**LN850820**	**LN850868**	**LN850917**

Microascus onychoides	*Scopulariopsis* sp.	**BMU03909**	Nail/China	**LN850772**	**LN850821**	**LN850869**	**LN850918**
sp. nov.	*Scopulariopsis* sp.	**BMU03910**	Nail/China	**LN850773**	**LN850822**	**LN850870**	**LN850919**
	Scopulariopsis sp.	**BMU03911 (CBS 139629)^{T}**	Nail/China	**LN850774**	**LN850823**	**LN850871**	**LN850920**
Microascus paisii	*Scopulariopsis brumptii*	**CBS 116060**	Antique tapestries/Poland	**LN850775**	**LN850824**	**LN850872**	**LN850921**
	Microascus paisii	CBS 213.27^{T}	Man/Italy	LM652434	LM652518	LM652647	LM652569
	Scopulariopsis brumptii	**CBS 896.68**	Wheat-field soil/Germany	**LN850776**	**LN850825**	**LN850873**	**LN850922**
	Scopulariopsis brumptii	**CBS 345.58**	Skin and hair/Germany	**LN850777**	**LN850826**	**LN850874**	**LN850923**
	Scopulariopsis chartarum	**CBS 670.74**	Dead branches of *Picea excelsa*/ 2Czech Republic	**LN850778**	**LN850827**	**LN850875**	**LN850924**
Microascus pseudolongirostris sp. nov.	*Microascus cirrosus*	**CBS 462.97^{T}**	Nail/Netherlands	**LN850782**	**LN850831**	**LN850879**	**LN850927**
Microascus pyramidus	*Microascus pyramidus*	CBS 212.65^{T}	Desert soil/USA	LM652439	HG380435	LM652652	HG380358
	Microascus pyramidus	**CBS 668.71**	Mouse hair/USA	**LN850779**	**LN850828**	**LN850876**	**LN850925**
Microascus restrictus	*Scopulariopsis* sp.	**BMU07493**	Nail/China	**LN850780**	**LN850829**	**LN850877**	–
	Microascus restrictus	CBS 138277^{T}	Left hallux/USA	LM652440	HG380494	LM652653	HG380417
Microascus senegalensis	*Microascus senegalensis*	CBS 277.74^{T}	Mangrove soil/Senegal	LM652441	LM652523	LM652654	LM652574
	Microascus senegalensis	**CBS 594.78**	Skin/Algeria	**LN850781**	**LN850830**	**LN850878**	**LN850926**
Microascus terreus comb & stat. nov.	*Microascus trigonosporus* var. *terreus*	**CBS 601.67^{T}**	Soil/Ukraine	**LN850783**	**LN850832**	**LN850880**	**LN850928**
	Microascus alveolaris	FMR 12333	Lung tissue/USA	LM652388	HG380490	LM652604	HG380413
	Microascus alveolaris	FMR 12342	Sputum/USA	LM652384	HG380489	LM652600	HG380412
Microascus trigonosporus	*Microascus trigonosporus*	CBS 199.61	Milled rice/Japan	LM652444	HG380438	LM652656	HG380361
	Microascus trigonosporus	CBS 218.31^{T}	Unknown/USA	LM652443	HG380436	HG380359	LM652655
Microascus verrucosus	*Microascus verrucosus*	CBS 138278^{T}	Bronchoalveolar lavage/USA	LM652446	HG380493	LM652658	HG380416
Pithoascus ater	*Pithoascus ater*	CBS 400.34^{T}	Unknown/Unknown	LM652447	LM652526	LM652659	LM652576
Pithoascus exsertus	*Pithoascus exsertus*	CBS 819.70^{T}	*Megachile willoughbiella*/Denmark	LM652449	LM652528	LM652578	LM652661
Pithoascus intermedius	*Pithoascus intermedius*	CBS 217.32^{T}	Root of *Fragaria vesca*/USA	LM652450	LM652529	LM652662	LM652579
Pithoascus lunatus sp. nov.	*Microascus nidicola*	**CBS 103.85^{T}**	Skin (*Tinea plantaris*)/Germany	**LN850784**	**LN850833**	**LN850881**	**LN850929**
Pithoascus nidicola	*Pithoascus nidicola*	CBS 197.61^{T}	*Dipodomys merriami*/USA	LM652451	LM652530	LM652663	LM652580
Pithoascus stoveri	*Pithoascus stoveri*	CBS 176.71^{T}	Root of *Beta vulgaris*/USA	LM652453	LM652532	LM652664	LM652581
Pseudoscopulariopsis hibernica	*Pseudoscopulariopsis hibernica*	UAMH 2643	Soil/Ireland	LM652454	LM652533	LM652665	LM652582
Pseudoscopulariopsis schumacheri	*Pseudoscopulariopsis schumacheri*	CBS 435.86^{NT}	Soil/Spain	LM652455	LM652534	LM652666	LM652583
Scopulariopsis alboflavescens	*Scopulariopsis alboflavescens*	CBS 399.34^{T}	Skin/Austria	LM652466	LM652539	JQ434537	JQ434600
	Scopulariopsis brevicaulis	FMR 12211	Sputum/USA	LM652476	HG380448	LM652683	HG380371
	Scopulariopsis koningii	**CBS 152.22**	Unknown/France	**LN850785**	**LN850834**	**LN850882**	**LN850930**
	Scopulariopsis koningii	**CBS 208.61**	Elephant/unknown	**LN850786**	**LN850835**	**LN850883**	**LN850931**
Scopulariopsis asperula	*Scopulariopsis asperula*	**CBS 298.67**	*Triticum aestivum*/Turkey	**LN850789**	**LN850838**	**LN850886**	**LN850934**
	Scopulariopsis asperula	CBS 401.34	Carcass of rabbit/Austria	LM652463	HG380465	LM652670	HG380388
	Scopulariopsis asperula	CBS 853.68	Compost soil/Germany	LM652461	JQ434669	JQ434558	JQ434621
	Scopulariopsis fusca	**CBS 117767**	Wood/Germany	**LN850787**	**LN850836**	**LN850884**	**LN850932**
	Scopulariopsis fusca	**CBS 334.53**	Nail/Netherlands	**LN850788**	**LN850837**	**LN850885**	**LN850933**

(*continued on next page*)

Table 1 – (*continued*)

Species		Strain accession no.	Origin	GenBank accession no.			
Current name	Original identification		Source/Country	ITS	LSU	*TUB*	*EF1-α*
Scopulariopsis brevicaulis	*Scopulariopsis flava*	**CBS 334.35**	Pupa/Czech Republic	**LN850790**	**LN850839**	**LN850887**	**LN850935**
	Scopulariopsis brevicaulis	**BMU0594**	Nail/China	**LN850791**	**LN850840**	**LN850888**	**LN850936**
	Scopulariopsis brevicaulis	**BMU3030**	Nail/China	**LN850792**	**LN850841**	**LN850889**	**LN850937**
	Scopulariopsis brevicaulis	**BMU3031**	Nail/China	**LN850793**	**LN850842**	**LN850890**	**LN850938**
	Scopulariopsis brevicaulis	**BMU3130**	Nail/China	**LN850794**	**LN850843**	**LN850891**	**LN850939**
	Scopulariopsis brevicaulis	**BMU3913**	Nail/China	**LN850795**	**LN850844**	**LN850892**	**LN850940**
	Scopulariopsis brevicaulis	**BMU3915**	Nail/China	**LN850796**	**LN850845**	**LN850893**	**LN850941**
	Scopulariopsis brevicaulis	**BMU3916**	Nail/China	**LN850797**	**LN850846**	**LN850894**	**LN850942**
	Scopulariopsis brevicaulis	**BMU3917**	Nail/China	**LN850798**	**LN850847**	**LN850895**	**LN850943**
	Scopulariopsis brevicaulis	**BMU4091**	Nail/China	**LN850799**	**LN850848**	**LN850896**	**LN850944**
	Scopulariopsis brevicaulis	FMR 12257	Toe nail/USA	LM652470	HG380443	HG380366	LM652677
	Scopulariopsis brevicaulis	MUCL 40726^{T}	Indoor air/Canada	LM652465	HG380440	LM652672	HG380363
Scopulariopsis candida	*Scopulariopsis flava*	**CBS 119.43**	Soil/Netherlands	**LN850800**	**LN850849**	**LN850897**	**LN850945**
	Microascus manginii	**CBS 132.78**	Human dentine/France	**LN850801**	**LN850850**	**LN850898**	**LN850946**
	Microascus manginii	**BMU3920**	Nail/China	**LN850802**	**LN850851**	**LN850899**	**LN850947**
	Scopulariopsis candida	MUCL 40743^{ET}	Indoor air/Canada	LM652484	HG380458	HG380381	LM652690
Scopulariopsis flava	*Scopulariopsis flava*	CBS 207.61^{NT}	Cheese/United Kingdom	LM652493	HG380464	LM652697	HG380387
	Scopulariopsis brevicaulis	**CBS 108960**	Cheese/Denmark	**LN850804**	**LN850853**	**LN850901**	**LN850949**
Scopulariopsis macurae sp. nov.	*Microascus manginii*	**CBS 506.66^{T}**	Chicken litter/Canada	**LN850805**	**LN850854**	**LN850902**	**LN850950**
Scopulariopsis soppii	*Scopulariopsis soppii*	UAMH 9169^{T}	Wood of *Populus tremuloides*/Canada	LM652495	LM652552	LM652698	LM652595
Wardomycopsis inopinata	*Wardomycopsis inopinata*	FMR 10306	Soil/Myanmar	LN850955	LN850956	LN850958	LN850957

CBS, Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands; BMU, Beijing Medical University (Peking University), Beijing, China; FMR, Facultat de Medicina i Ciències de la Salut, Reus, Spain; MUCL, Université Catholique de Louvain, Louvain-la-Neuve, Belgium; UAMH, University of Alberta Microfungus Collection and Herbarium, Canada. Strains characterized in this study and their newly generated sequences are highlighted in bold. Ex-epitype, -isotype, -type, and -neotype strains are indicated with ^{ET}, ^{IT}, ^{T}, and ^{NT}, respectively.

genomic DNA, *ca.* 0.2 g of mycelium was ground to a fine powder in a sterile pre-chilled mortar and pestle with liquid nitrogen. The obtained homogenate was further processed with the GeneMATRIX Plant & Fungi DNA Purification Kit (EURx, Poland), according to the manufacturer's protocol. The DNA concentration was measured with the NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, USA). Both the ITS and LSU (D1/D3 domains) loci were amplified in a single PCR run, with primers ITS5 and LR5 (White *et al.* 1990; Vilgalys & Hester 1990). Target regions within the *TUB* and *EF1-α* genes were PCR-amplified using primer pairs Bt2a/Bt2b (Glass & Donaldson 1995) and 983F/2218R (Rehner & Buckley 2005), respectively. All PCR reactions were performed using TopTaq PCR Master Mix kit (Qiagen, Germany) and run on a SensoQuest LabCycler (GmbH, Germany).

For sequencing, the PCR products were purified using the Clean-Up kit (A&A Biotechnology, Poland). Sequencing was done in both directions, with the same primer pairs as those used for amplification, except for the rDNA loci. Primers ITS4 and ITS5 (White *et al.* 1990) were used for sequencing of the ITS, whereas primers LR0R and LR5 were used for sequencing of partial LSU rDNA (Vilgalys & Hester 1990; Vilgalys & Sun 1994). Details of all primers used in this study are summarized in Table 2. Consensus sequences were obtained with ChromasPro v. 1.7.1 (Technelysium, Australia).

Alignment and phylogenetic analysis

Multiple sequence alignments were made for each individual locus using Mega version 6.06 (Tamura *et al.* 2013), with the ClustalW function (Thompson *et al.* 1994), checked visually, and refined using Muscle (Edgar 2004). The best nucleotide substitution model for each data set (GTR + I + G) was estimated using MrModeltest version 2.3 (Nylander 2004). Phylogenetic analyses using maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI) were carried out for each loci and the combined dataset under PAUP* version 4.0b10 (Swofford 2002), Mega and MrBayes v. 3.1.2 (Huelsenbeck & Ronquist 2001), respectively. In order to assess topological incongruences between the different genes, the resulting trees of the individual phylogenies were compared visually using a 70 % bootstrap cutoff and complemented with the partition homogeneity test, carried out as implemented in PAUP*. Since no incongruence was found ($P = 0.049$), the four loci were combined into a single data set. For MP analyses, 1000 replicates of random sequence addition were performed, with tree bisection-reconnection swapping algorithm. Gaps were treated as a fifth character and all characters were unordered and weighted equally. For ML analyses, nearest-neighbour interchange (NNI) was used as the heuristic method for tree inference. For MP and ML analyses, support for the internal branches was assessed by a search of 1000 bootstrapped sets of data. A bootstrap support (bs) value of ≥70 % was considered significant. For BI analysis, two simultaneous runs of 3 000 000 generations were performed, and samples were stored every 1000 generations. The 50 % majority-rule consensus tree and posterior probability (pp) values were calculated after the first 25 % of the samples were discarded. A pp value of ≥0.95 was considered significant. All sequences generated in this study and the alignments were deposited respectively in GenBank (Table 1) and TreeBASE (www.treebase.org).

Results

Species currently accepted in the genera *Microascus*, *Pithoascus*, *Pseudoscopulariopsis*, and *Scopulariopsis* were clearly differentiated in the combined phylogenetic analysis using the loci selected (Fig 1). A total of 41 supported terminal clades were configured, distributed into five main lineages, which corresponded to the afore-mentioned four genera and a fifth monotypic lineage representing the new genus *Fuscoannellis* described below. Final identification of all isolates investigated here is summarized in Table 1.

The *Microascus* lineage encompassed 24 well-supported terminal clades (M1–M24) that represented the species previously delineated by Sandoval-Denis *et al.* (2016) and four new species clades (i.e., M1, M20, M22, and M23). Clade M1 included two clinical strains previously identified as *Microascus alveolaris* (Sandoval-Denis *et al.* 2016) and the ex-type strain of

Table 2 – Primers used for PCR amplification and sequencing.

Gene	Primer		Product size [bp]	T_a [°C][a]	Reference
	Designation	Nucleotide sequence [5′ → 3′]			
ITS-LSU[b]	ITS5	GGAAGTAAAAGTCGTAACAAGG	*ca.* 1500	53	White *et al.* (1990)
	LR5	TCCTGAGGGAAACTTCG			Vilgalys & Hester (1990)
	ITS4*	TCCTCCGCTTATTGATATGC			
	LR0R*	ACCCGCTGAACTTAAGC			
TUB	Bt2a	GGTAACCAAATCGGTGCTGCTTTC	*ca.* 550	55	Glass & Donaldson (1995)
	Bt2b	ACCCTCAGTGTAGTGACCCTTGGC			
EF1-α	983F	GCyCCyGGhCAyCGTGAyTTyAT	*ca.* 1000	65	Rehner & Buckley (2005)
	2218R	ATGACACCrACrGCrACrGTyTG			

Degenerate nucleotides: y, C or T; h, A, C, or T; r, A or G.
An asterisk (*) indicates starters used for sequencing only.
[a] T_a, annealing temperature.
[b] ITS-LSU represents a fragment of the rDNA operon, encompassing 3′-end of the 18S rRNA gene, ITS1, the 5.8S rRNA gene, ITS2, and D1/D3 domains of the 28S rRNA gene.

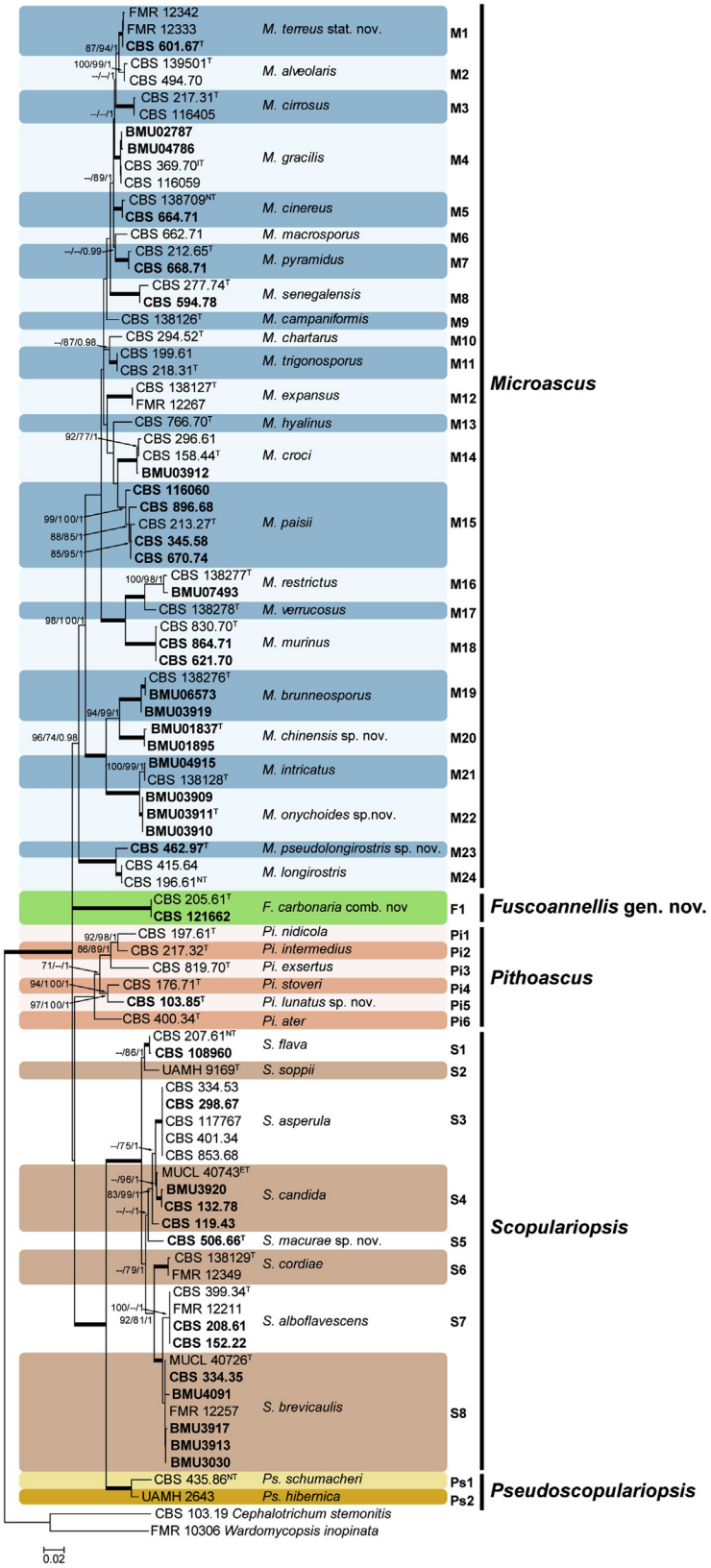

Fig 1 – Maximum likelihood (ML) tree obtained from the combined LSU, ITS, EF-1α, and *TUB* sequences of 90 strains of 41 species from the genera *Fuscoannellis* (F 1), *Microascus* (M 1–24), *Pithoascus* (Pi 1–6), *Pseudoscopulariopsis* (Ps 1, 2), and *Scopulariopsis* (S 1–8). Strains characterized in this study are highlighted in bold. Numbers on the nodes are MP and ML bootstrap values above 70 % and BI posterior probabilities above 0.95. Branches with full-statistical support are indicated in bold. Branch lengths are proportional to distance. Ex-epitype, -isotype, -type, and -neotype strains are indicated with ^{ET}, ^{IT}, ^{T}, and ^{NT}, respectively. The tree was rooted on *Cephalotrichum stemonitis* (CBS 103.19) and *Wardomycopsis inopinata* (FMR 10306).

Microascus trigonosporus var. *terreus*. Since this clade was distant from the ex-type strain of *M. trigonosporus* and differed morphologically from its sister species *M. alveolaris* in having shorter annellides (7–11.5 μm long *vs*. 6–17 μm in *M. alveolaris*) and subglobose to somewhat lemon-shaped conidia, it was regarded as a different species named *Microascus terreus*. Clades M20 and M22, comprising exclusively Chinese clinical isolates, were phylogenetically and morphologically distinct from the closely related species *Microascus brunneosporus* (M19) and *Microascus intricatus* (M21), respectively. Therefore, we considered them to represent undescribed species named here *Microascus chinensis* (M20) and *Microascus onychoides* (M22). *Microascus chinensis* was morphologically characterized by ellipsoidal dark brown (color in the web version) conidia, measuring 2–4 × 2–3.5 μm, whereas the conidia of *M. onychoides* were ovate, olive brown (color in the web version) and measured 2.8–3.8 × 2.5–3 μm. Clade M23, formed by a single strain CBS 462.97, was closely related to the type species of the genus, *Microascus longirostris*, but phylogenetically distant and morphologically different by having paler colonies and lacking the asexual state. This new species is described below as *Microascus pseudolongirostris*.

The *Pithoascus* lineage included 6 species clades (Pi1–Pi6), and is the branch of the clinical strain CBS 103.85 (Pi6), previously identified as *Microascus nidicola*, representatives of an undescribed species. This strain differed from other species of the genus by its small broadly lunate ascospores and the absence of an asexual morph.

The *Scopulariopsis* lineage had 8 terminal clades (S1–S8), two of which (S5 and S7) corresponded to two species not delineated by Sandoval-Denis *et al.* (2016). Clade S5 was represented by a single strain, previously identified as *Microascus manginii* (CBS 506.66) and described below as a new species, *Scopulariopsis macurae*. Clade S7 included both clinical and environmental isolates formerly considered to belong to *Scopulariopsis brevicaulis* (Sandoval-Denis *et al.* 2016). However, genetic differences observed between S7 and S8 indicate that they represent two distinct phylogenetic entities, which can also be distinguished morphologically. Clade S8 included the ex-type strain of *S. brevicaulis* and clinical strains showing typical features of that species (i.e., tan colonies and pale brown (color in the web version) verrucose conidia). In contrast, strains of clade S7 showed white to cream or pale yellowish colonies, producing subhyaline and mostly smooth-walled conidia. Since this latter clade included the ex-type strain of *Scopulariopsis alboflavescens* (CBS 399.34), we used its name to define the whole clade.

The particular phylogenetic analysis of each of the four loci used showed different degrees of successful species identification. LSU resolved less than 10 % (4/41) of the species investigated and only *Microascus croci*, *Microascus expansus*, *Microascus murinus*, and *Microascus restrictus* could be identified with confidence. The alignment included 788 positions, with 702 were conserved, 84 variable and 66 parsimony informative. ITS resolved 63 % (26/41) of the species; it was unable to separate *M. alveolaris* and *M. terreus*, *Microascus campaniformis* and *Microascus gracilis*, *M. onychoides* and *M. restrictus*, as well as none of the species of *Pithoascus* nor *Scopulariopsis asperula*, *Scopulariopsis candida*, and *S. macurae*. The alignment contained 364 positions with 238 conserved, 120 variable, and 94 parsimony informative positions. A higher number (95 % or 39/41) species resolution was achieved with the *TUB* dataset. The only species that could not be separated with such marker were *M. restrictus* and *Microascus verrucosus*. The alignment contained 475 positions, of which 245 were conserved, 216 variable, and 178 parsimony informative. Finally, *EF-1α* was the only locus able to resolve all species studied. The alignment comprised 819 positions, with 572, 241, and 191 being conserved, variable, and parsimony informative, respectively.

The interspecific distances among the 41 species ranged from 0 to 4.9 % for the LSU region, 0–9.8 % for the ITS region, 0.9–12.2 % for the *EF-1α* gene, 0–17 % for the *TUB* gene, and 1–10.8 % for the whole concatenated loci data set. Whereas, the intraspecific variability ranges were 0–0.4 % for LSU, 0–2.4 % for ITS, 0–1 % for*EF-1α*, 0–1.8 % for *TUB*, and 0–0.9 % for the combined data set. Overall, *Microascus paisii* was the most genetically variable species at all loci, except for the ITS region, for which *Microascus senegalensis* and *M. croci* showed the highest variability.

Taxonomy

Fuscoannellis Sandoval-Denis, Jagielski, Jin Yu & Gené, **gen. nov.** – MycoBank No.: MB814494.

Etymology – name refers to the dark pigmented sporogenous structures of the type species.

Type species – *Fuscoannellis carbonaria* (F.J. Morton & G. Sm.) Sandoval-Denis, Jagielski, Jin Yu & Gené.

Colonies spreading moderately, velvety to funiculose, greenish grey, dark grey to black. *Conidiophores* unbranched and bearing terminally a compact group of 2–10 conidiogenous cells, or more frequently branched, with several stages of branching, each branch terminally swelling and bearing a compact group of conidiogenous cells. *Conidiogenous cells* annellidic, mostly ampulliform, with a swollen base followed by an annellated zone never greatly elongating, pale brown to brown (color in the web version), smooth-walled. *Conidia* 1-celled, ovate, with a rounded or slightly pointed apex and a wide truncate base, smooth-walled, brown or greyish brown (color in the web version), near black in mass, arranged in long basipetal dry chains often adhering in fairly dense columns. *Sexual morph* absent.

Fuscoannellis carbonaria (F.J. Morton & G. Sm.) Sandoval-Denis, Jagielski, Jin Yu & Gené, **comb. nov.** – MycoBank No.: MB814495; Fig 2.

Basionym – *Scopulariopsis carbonaria* F.J. Morton & G. Sm, Mycol. Pap. 86:59 (1963).

Specimens examined: **Panama**: soil, *R. Coghill* (CBS 205.61 – culture ex-type). **USA**: Hawaii, on black stromata of an unidentified pyrenomycete on a dead hardwood branch, *Eucalyptus* forest planting, Nov. 2002, *D.T. Wicklow* (as *Scopulariopsis brumptii* CBS 121662).

Notes – Issakainen *et al.* (2003) and Ropars *et al.* (2012), although they did not propose any taxonomic change, had already demonstrated that the ex-type strain of *S. carbonaria* (CBS 205.61) constituted a monophyletic branch that might represent a genus distinct from *Scopulariopsis* and *Microascus*. That was recently confirmed by Sandoval-Denis *et al.* (2016) through multilocus analysis. Since these authors were not

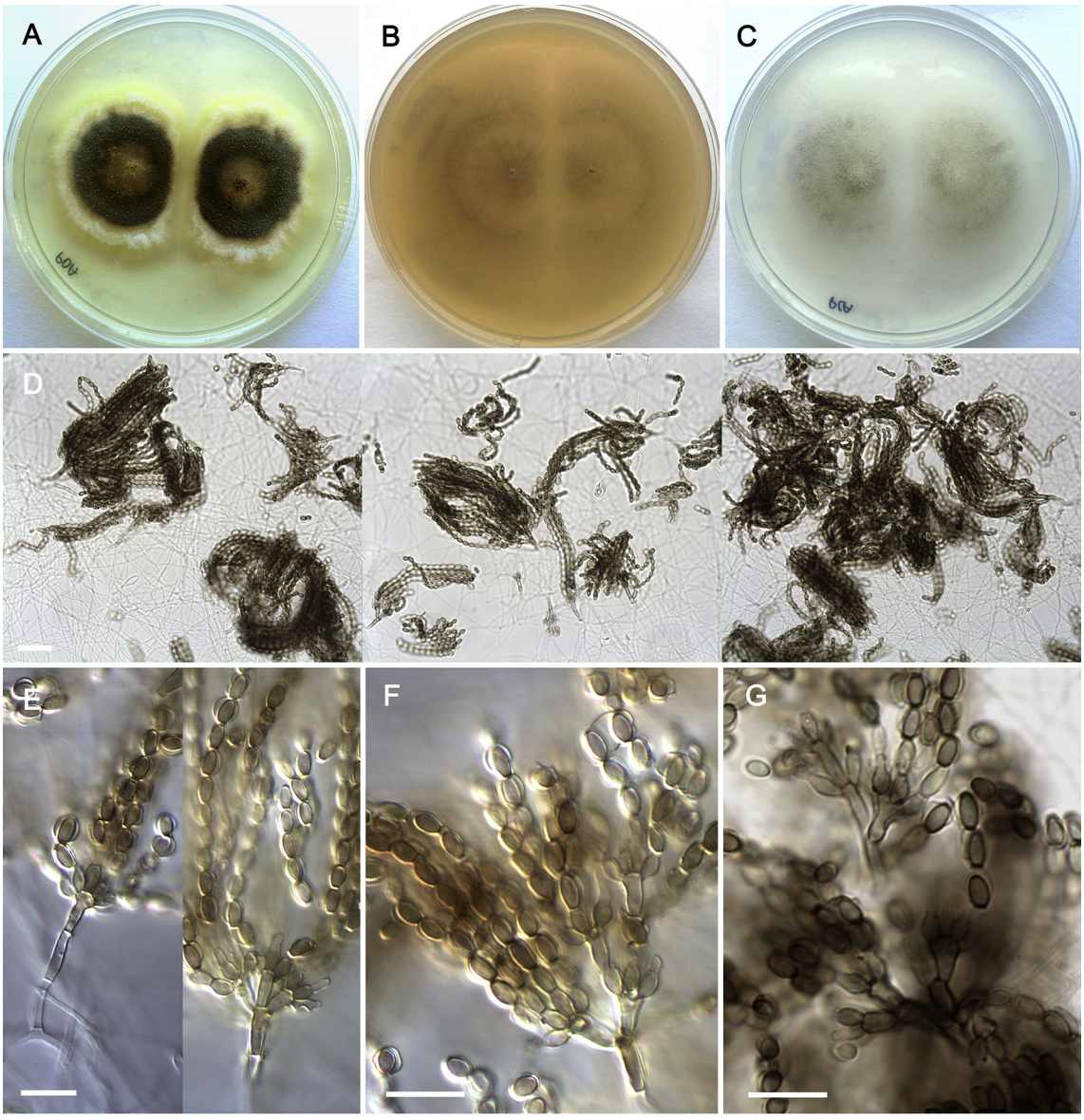

Fig 2 – *Fuscoannellis carbonaria* (CBS 205.61). Colony morphology after 21 d at 25 °C on PDA (A), OA (B), and PCA (C). Conidia forming fairly solid columns (D). Simple and branched conidiophores and conidia (E–G). Scale bars: D = 20 μm; E–G = 10 μm.

able to get sporulated cultures of that strain, it was treated as a doubtful species. In the present study, well-sporulated cultures of *S. carbonaria* could be examined, including the ex-type strain and an additional isolate received as *S. brumptii* (CBS 121662). Both strains showed a combination of features (i.e., brown (color in the web version) and branched conidiophores, very short annellides and conidia arranged in long chains adhering in more or less compact columns) not observed in the currently accepted *Microascus* and *Scopulariopsis* species (Sandoval-Denis *et al.* 2016) and considered of generic value. Our observations agree with those of Morton & Smith (1963) who provided a detailed description of *S. carbonaria*. The main morphological features of *F. carbonaria* can be outlined briefly in its dark grey almost black colonies on PDA at 25 °C, branched conidiophores up to 40 μm long, ampulliform 4–7 × 2–3 μm annellides, producing ovate, greyish brown (color in the web version), 3.5–5 × 2.5–4 μm conidia and absence of growth at 37 °C. Because of the colony colour and conidiophore pattern, this fungus resembles *Microascus gracilis*, but the latter can be easily differentiated by its growth at 40 °C. Another dark pigmented similar species is *Microascus paisii*. However, the latter shows simpler conidiophores and can grow and sporulate well at 37 °C. *Microascus gracilis* and *M. paisii* are two species often isolated from clinical specimens (Sandoval-Denis *et al.* 2013), while *F. carbonaria* has only been isolated from environmental sources such as soil, dung or plants (Morton & Smith 1963; Matsushima 1971).

Microascus chinensis Jin Yu, Sandoval-Denis & Gené, **sp. nov.** – MycoBank No.: MB814497; Fig 3.

Etymology – name refers to the geographical origin of the isolates.

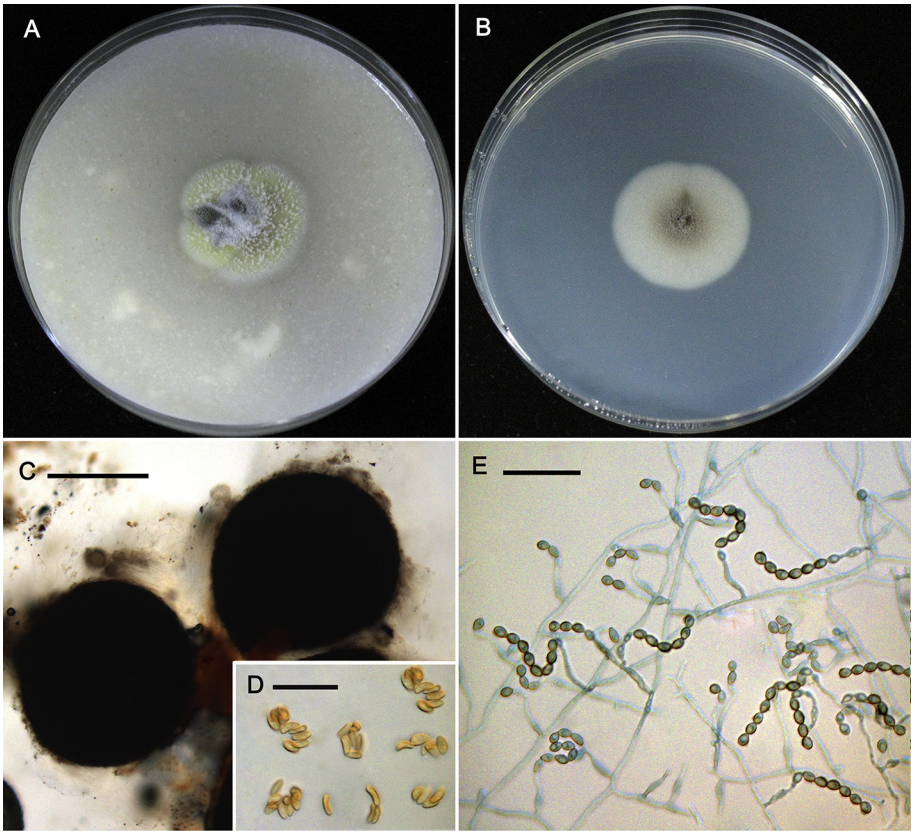

Fig 3 – *Microascus chinensis* (CBS 139628). Colony morphology after 14 d at 25 °C on OA (A) and PCA (B). Ascomata (C). Ascospores (D). Annellides and conidia (E). Scale bars: C = 100 μm; D, E = 20 μm.

Colonies on OA and PCA attaining a diameter of 9 mm in 7 d (25–28 mm after 14 d) at 25 °C, greyish olivaceous at the centre, white grey towards the periphery, flat, velvety or somewhat floccose; reverse olivaceous to light grey. No production of diffusible pigment. *Mycelium* composed of hyaline, branched, septate, smooth-walled, hyphae of 1–1.8 μm wide. *Ascomata* globose to subglobose, 120–200 μm, usually with a short ostiolar neck, black, glabrous; peridium with a *textura angularis*. *Asci* more or less ellipsoidal, 8.2–12 × 6.5–9 μm. *Ascospores* mostly allantoid, some clavate or ellipsoidal, 3.5–7 × 1.5–3 μm, yellowish brown (color in the web version), with a single and inconspicuous germ pore. *Annellides* mostly sessile and single, arising laterally on vegetative hyphae, flask-shape with a slightly swollen base and a narrow cylindrical annellated zone of variable length, 1.5–2.8 μm. *Conidia* ellipsoidal, 2–4 × 2–3.5 μm, with a truncate base, dark brown (color in the web version), smooth-walled.

Temperature for growth – optimum 25 °C, maximum 30 °C.

Specimens examined: **China**: Beijing, Peking University First Hospital, human nail, Nov. 2000, *Jin Yu* (BMU01837 – holotype, a dried culture on PDA; CBS 139628 – culture ex-type). **China**: Liaoning, Dalian, The First Affiliated Hospital of Dalian Medical University, unknown clinical specimen, Dec. 2000, *Jin Yu* (BMU01895).

Notes – *Microascus chinensis* together with *Microascus onychoides*, also described in the present study, and another two species *Microascus brunneosporus* and *Microascus intricatus*, recently proposed by Sandoval-Denis *et al.* (2016), conformed a lineage of species with ellipsoidal, fusiform or allantoidal ascospores, features that clearly distinguish them from all the other members of *Microascus*. *Microascus chinensis* can be differentiated from the phylogenetically close species mainly by the morphology of its conidia, which are ellipsoidal and measure 2–4 × 2–3.5 μm, while in *M. brunneosporus* they are subglobose to navicular and 4–5 × 2.5–5 μm, in *M. intricatus* they are globose to broadly ellipsoidal and 4–5 × 3–3.5 μm, and in *M. onychoides* they are ovate and measure 2.8–3.8 × 2.5–3 μm.

Microascus onychoides Jin Yu, Sandoval-Denis & Gené, **sp. nov.** – MycoBank No.: MB814496; Fig 4.

Etymology – name refers to the clinical specimen where the species was found.

Colonies on OA and PCA attaining a diameter of 15–17 mm in 7 d (28–35 mm after 14 d) at 25 °C, olivaceous grey, white or paler grey at the periphery, flat, velvety or slightly floccose; reverse light grey. No production of diffusible pigment. *Mycelium* composed of hyaline, branched, septate, smooth-walled, hyphae of 1–2 μm wide. *Ascomata* globose to subglobose, (130–) 170–200 (–250) μm diam, usually with a short ostiolar neck, black, glabrous; peridium with a *textura angularis*. *Asci* broadly ellipsoidal to subglobose, 7.5–11 × 8.5–10 μm. *Ascospores* ellipsoidal, some slightly allantoid, 4.5–6 × 2–2.5 μm, yellowish brown (color in the web version), with a single and inconspicuous germ pore. *Annellides* mostly borne terminally on short unbranched conidiophores arising laterally on vegetative hyphae, flask-shaped with a slightly swollen base and a narrow

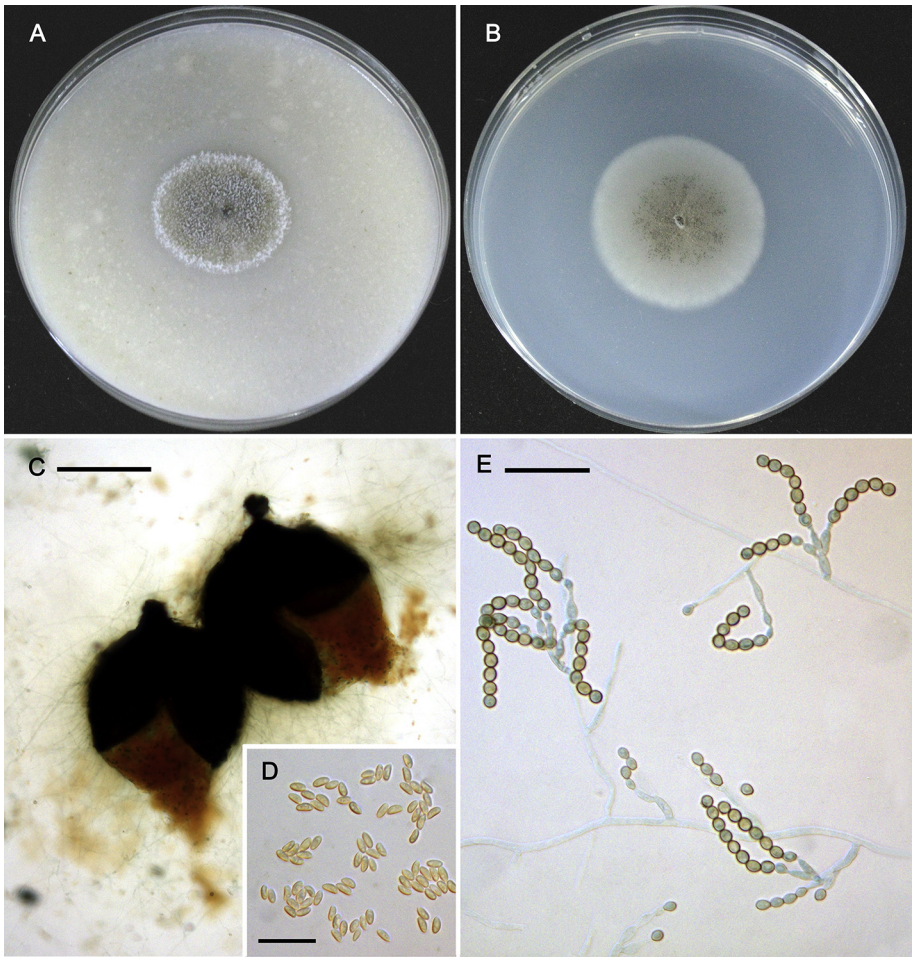

Fig 4 – *Microascus onychoides* (CBS 139629). Colony morphology after 14 d at 25 °C on OA (A) and PCA (B). Ascomata, ascospores, and some conidia (C, D). Annellides and conidia (E). Scale bars: C = 100 μm; D, E = 20 μm.

cylindrical annellated zone of variable length, 1.5–2.6 μm. *Conidia* ovate, 2.8–3.8 × 2.5–3 μm, with a slightly truncate base, olivaceous brown (color in the web version), dark brown (color in the web version) in mass, smooth-walled, arranged in long dry chains.

Temperature for growth – optimum 25 °C, maximum 30 °C.

Specimens examined: **China**: Beijing, Peking University First Hospital, Human nail, Feb. 2006, *Jin Yu* (BMU03911 – holotype, a dried culture on PDA; CBS 139629 – culture ex-type). **China**: Beijing, Peking University First Hospital, human nail, Feb. 2006, *Jin Yu* (BMU03909). **China**: Beijing, Peking University First Hospital, human nail, Feb. 2006, *Jin Yu* (BMU03910).

Notes – *Microascus intricatus*, recently described by Sandoval-Denis *et al.* (2016), is the phylogenetically closest species to *M. onychoides*, from which it can be morphologically differentiated by its perithecia with a peridium of *texture intricata* (*texture angularis* in *M. onychoides*) and by its fusiform ascospores of 5–6 × 2.5–3.5 μm (ellipsoidal or slightly allantoid, 4.5–6 × 2–2.5 μm, in *M. onychoides*).

Microascus pseudolongirostris Jagielski, Sandoval-Denis, Krzyściak & Gené, **sp. nov.** – MycoBank No.: MB814502; Fig 5.

Etymology – name refers to the morphological resemblance and phylogenetic closeness to *Microascus longirostris*.

Colonies on OA attaining a diameter of 14–19 mm after 14 d at 25 °C, flat, white to cream-coloured, beige and with black ascomata at the centre, margin regular; reverse light grey at the centre and cream-coloured at the periphery. On PCA at 25 °C attaining 15–20 mm diam in 14 d, slightly convex, glabrous, cream-coloured, granular and black at the centre due to the presence of ascomata, with a slightly lobulate margin; reverse dark grey at the centre, light beige at the periphery. *Mycelium* composed of septate, hyaline, smooth-walled hyphae of 1–1.5 μm wide. *Ascomata* globose, 269–329 μm diam, with a cylindrical neck, 64.5–85 × 35–37 μm, black, glabrous or covered with a small amount of hairs; peridium with a *textura angularis* to *textura epidermoidea*. *Asci* globose to broadly ellipsoidal, 7.5–9.5 × 6.5–9 μm. *Ascospores* reniform, 3.5–4 × 2–3 μm, straw coloured, yellowish brown (color in the web version) in mass. *Asexual morph* not observed.

Temperature for growth – optimum 20–25 °C, maximum 30 °C.

Specimen examined: **the Netherlands**: Harderwijk, onychomycosis, Nov. 1996, (CBS-H 22296 – holotype, a dried culture on PDA; CBS 462.97 – culture ex-type).

Notes – This species differs from its sibling species, *M. longirostris*, solely by having paler colonies and the absence

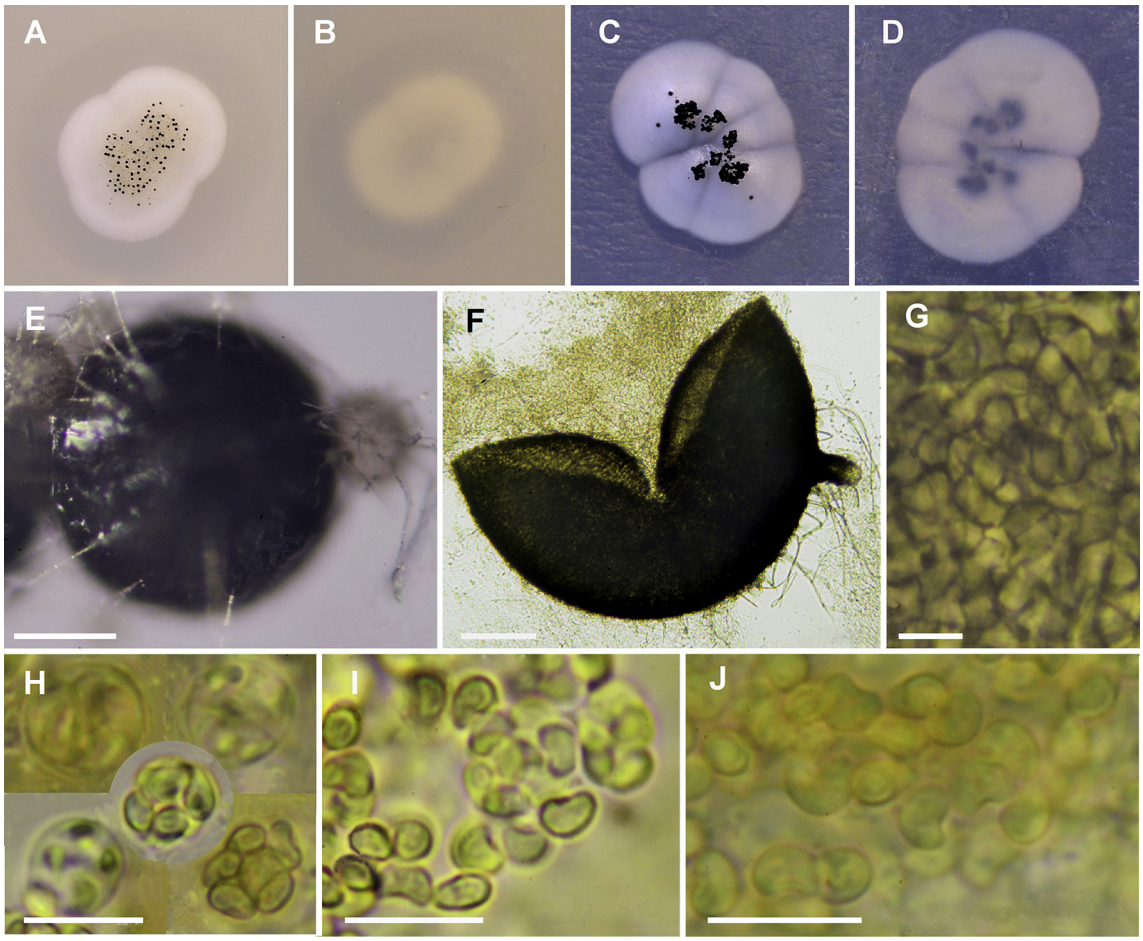

Fig 5 – *Microascus pseudolongirostris* (CBS 462.97). Colony morphology after 14 d at 25 °C on OA (A, B – reverse) and PCA (C, D – reverse). Ascomata (E, F). Detail of the peridium (G). Asci and ascospores (H, I). Scale bars: E, F = 100 μm; G–J = 10 μm.

of asexual morph. The ex-type strain of *M. pseudolongirostris* was originally identified as *Microascus cirrosus* (Table 1), but both species are phylogenetically very distant and morphologically the latter mainly differs by its larger ascospores (5–6 × 3–4 μm), the presence of asexual morph and by its ability to grow at 40 °C (Sandoval-Denis *et al.* 2016).

Microascus terreus (Kamyschko) Jagielski, Sandoval-Denis & Gené, **comb. & stat. nov.** – MycoBank No.: MB814498; Fig 6.

Basionym – *Microascus trigonosporus* C.W. Emmons & B.O. Dodge var. *terreus* Kamyschko, Novosti Sistematiki Nizshikh Rastenii 3: 175 (1966).

Colonies on OA and PCA attaining a diameter of 24–26 mm after 14 d at 25 °C, flat, somewhat floccose, white or greyish beige, granular at the centre due to the presence of black ascomata, with a white immersed and regular margin; reverse brownish grey (color in the web version) or greyish at the centre, colourless towards the periphery. *Mycelium* composed of septate, hyaline, smooth-walled hyphae of 1–2.5 μm wide. *Ascomata* globose or subglobose, 176.5–271.5 μm diam, usually with a short ostiolar neck, 42–44.5 × 30.5–33.5 μm, black, glabrous; peridium with a *textura angularis*. *Asci* globose to ellipsoidal, 9–11 × 7–9.5 μm. *Ascospores* triangular with concave sides and rounded apices, 5–6 × 3.5–4 μm, subhyaline or straw coloured, yellowish brown (color in the web version) in mass. *Annellides* single, lateral and sessile on vegetative hyphae, mostly lageniform, 7–11.5 × 2.5–3 μm, tapering to a cylindrical annellated zone 1–1.5 μm wide. *Conidia* subglobose or somewhat lemon-shaped, 3.5–4.5 × 3–3.5 μm, with a truncate base, 0.5–1 μm wide, subhyaline, smooth-walled, arranged in long chains.

Temperature for growth – optimum 25–30 °C, maximum 40 °C.

Specimen examined: **Ukraine**: soil, Nov. 1967, O.P. *Kamyschko* (*M. trigonosporus* var. *terreus* CBS 601.67, ATCC 22360, NRRL A-18283 and VKM F-1144 – cultures ex-type).

Notes – Traditionally, *M. trigonosporus* comprised four varieties (i.e., *trigonosporus*, *macroperithecia*, *macrosporus*, and *terreus*). While the variety *macroperithecia* was considered a *nom inval.* In *Index Fungorum* (ICBN Art. 40.5), the variety *macrosporus* has been recently recognized as a species different from *M. trigonosporus* by Sandoval-Denis *et al.* (2016). The ex-type strain of the variety *terreus* was not studied by the latter authors and according to the present phylogenetic analysis it also represents a species distinct from *M. trigonosporus*. To distinguish morphologically *M. terreus* from *M. trigonosporus* is quite challenging. The variety *terreus* was introduced by Kamyschko (1966) and differentiated from *M. trigonosporus* var. *trigonosporus* mainly by its larger ascospores (5–6 μm

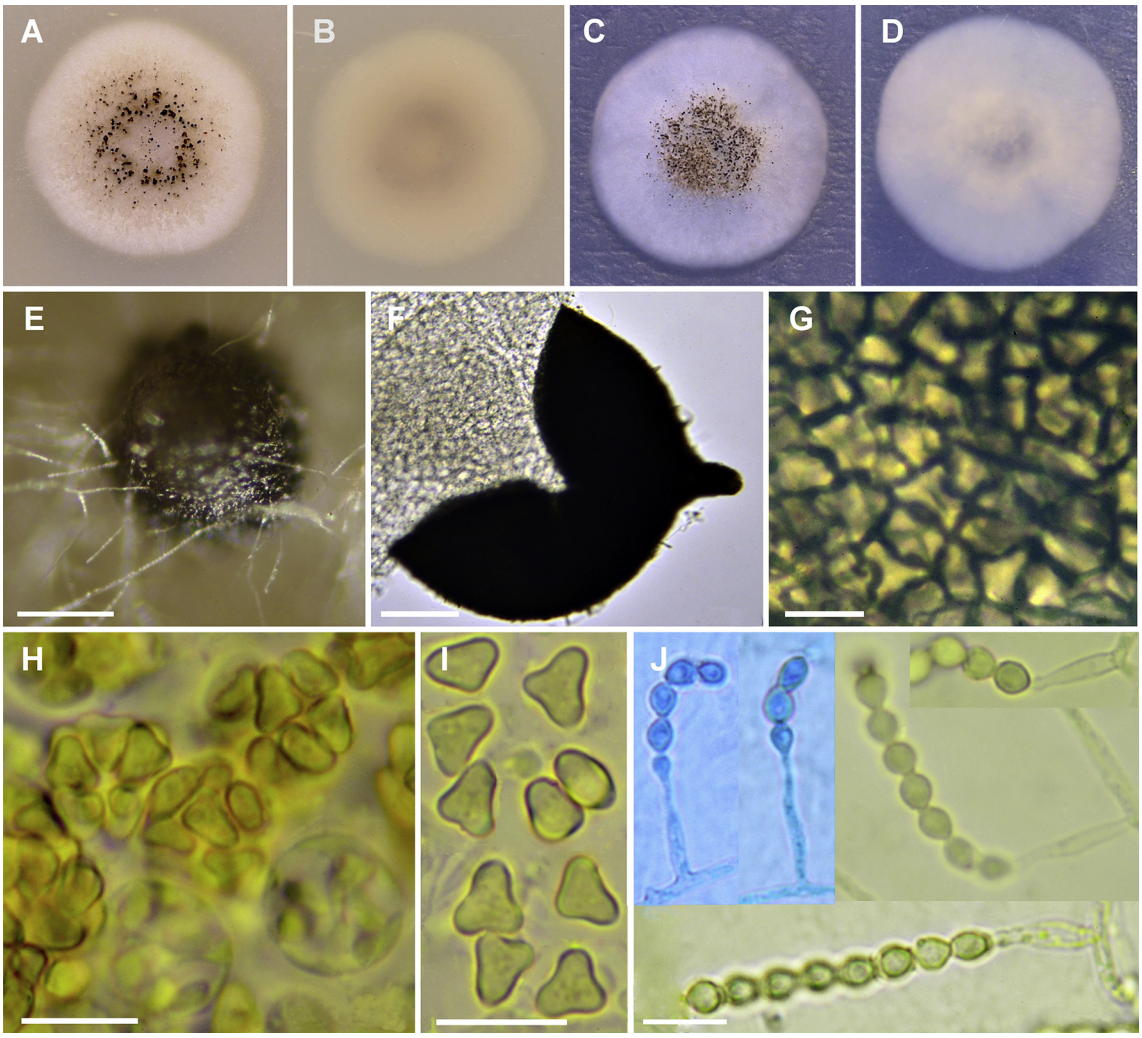

Fig 6 – *Microascus terreus* (CBS 601.67). Colony morphology after 14 d at 25 °C on OA (A, B – reverse) and PCA (C, D – reverse). Ascomata (E, F). Detail of the peridium (G). Asci (H). Ascospores (I). Annellides and conidia (J). Scale bars: E, F = 100 μm; G – J = 10 μm.

long), a feature confirmed by our observations. Both taxa can also be differentiated by the organization of the conidiogenous apparatus, which is less complex in *M. terreus* and consists of mostly sessile annellides arising directly on the vegetative hyphae, while in *M. trigonosporus* branched conidiophores are more common.

Pithoascus lunatus Jagielski, Sandoval-Denis, Skóra & Gené, **sp. nov.** – MycoBank No.: MB814503; Fig 7.

Etymology – name refers to the ascospore shape of the species.

Colonies on OA and PCA attaining a diameter of 11–12 mm and 6–9 mm, respectively, after 14 d at 25 °C, flat, initially somewhat velvety and white to cream-coloured, becoming granular and dark due to the presence of abundant black ascomata, with a white, regular and slightly lobulate margin; reverse grey to dark grey, paler towards the periphery. *Mycelium* with septate, hyaline, smooth-walled hyphae, 1–1.5 μm wide. *Ascomata* globose, 111–143 μm diam, with an ostiolar neck up to 46 μm long and 22.5–28 μm wide, occasionally without neck, black, glabrous; peridium with a *textura angularis*. *Asci* hyaline subglobose to ellipsoidal, 9.5–12.5 × 5–9 μm. *Ascospores* nearly lunate, 5–5.5 × 2.5 μm, yellowish, smooth-walled and without germ pores. *Asexual morph* not observed.

Temperature for growth – optimum 25 °C, maximum 30 °C.

Specimen examined: **Germany**: Hamburg, human tinea plantaris, Jan. 1985, *H. Listemann* (CBS-H 22297 – holotype, a dried culture on PDA; CBS 103.85 – culture ex-type).

Notes – The strain concerned (CBS 103.85), considered by von Arx *et al.* (1988) as representative of *Pithoascus nidicola*, was re-classified as *Microascus intermedius* by Abbott *et al.* (2002), now a species of the reinstated genus *Pithoascus* (Sandoval-Denis *et al.* 2016). The present study demonstrates that this strain belongs to the *Pithoascus* lineage, although with significant phylogenetic distance from the other species of the genus (i.e., *Pithoascus ater*, *Pithoascus exsertus*, *Pithoascus intermedious*, *Pithoascus nidicola*, and *Pithoascus stoveri*). *Pithoascus lunatus* differs morphologically from *P. stoveri*, the closest phylogenetically species, by the absence of an asexual morph and its larger ascomata with broadly lunate and smaller

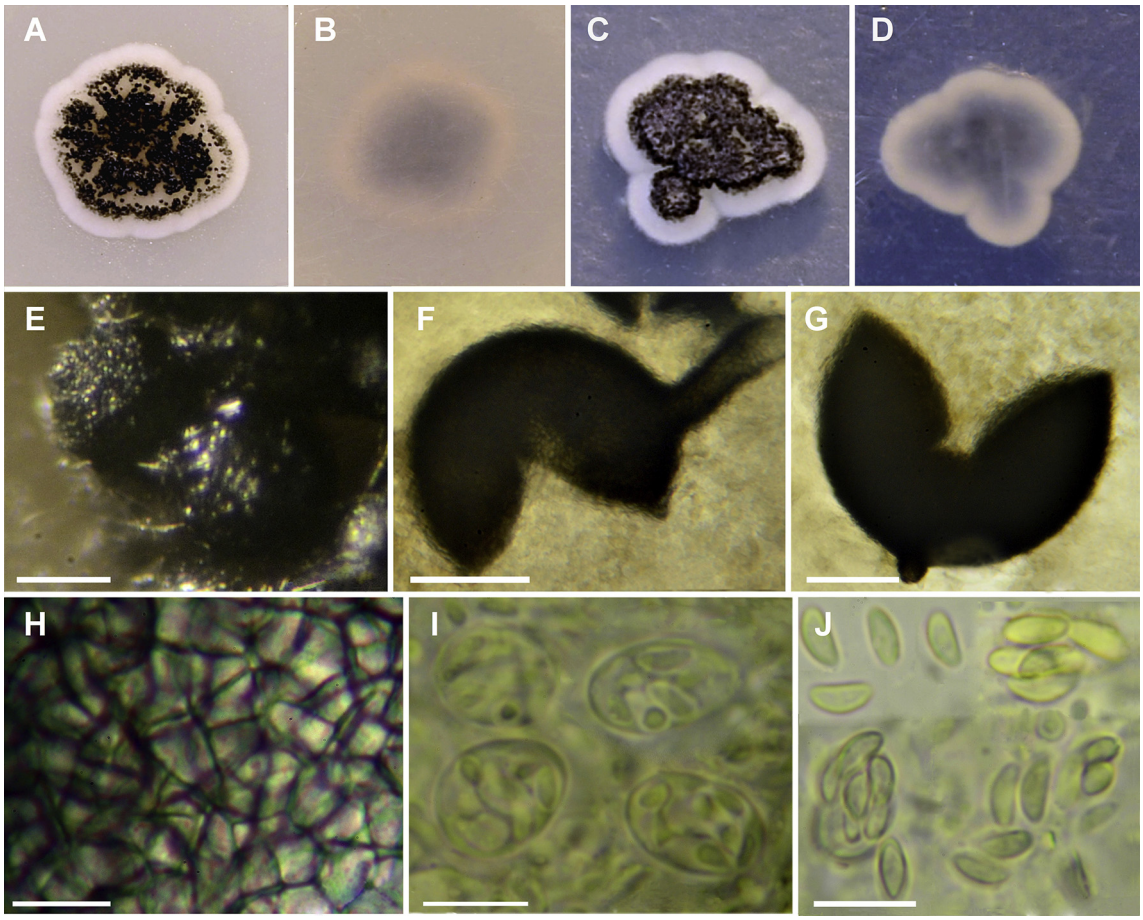

Fig 7 – *Pithoascus lunatus* (CBS 103.85). Colony morphology after 14 d at 25 °C on OA (A, B – reverse) and PCA (C, D – reverse). Ascomata (E–G). Detail of the peridium (H). Asci and ascospores (I, J). Scale bars: E–G = 100 μm; H–J = 10 μm.

ascospores; the ascospores of *P. stoveri* are navicular and measure 6–7.5 × 2–3 μm. The other two *Pithoascus* species with no asexual morph (i.e., *P. nidicola* and *P. exsertus*) differ mainly by having longer ascospores (6–8 μm in *P. nidicola*, 6.5–12 μm in *P. exsertus* and 5–5.5 μm in *P. lunatus*).

Scopulariopsis alboflavescens Zach, Oesterr. Bot. Z. 83: 177. 1934 – MycoBank No.: MB256268.

Specimens examined: **Austria**: Hamburg, human skin disease, Nov. 1934, *F. Zach* (CBS 399.34 and IMI 147446 – cultures ex-type). **France**, unknown substrate, 1922, *G. Bainier* (doubtfully type of *Scopulariopsis rufulus* and re-identified as *Scopulariopsis koningii*, CBS 152.22, IMI 086928, MUCL 9044; UAMH 9140, LSHB Sc-62). Unknown geographical origin, Elephant, 1951, *I.M. Scott* (as *S. koningii* CBS 208.61, IMI 086926, MUCL 9039; UAMH 952, LSHB Sc-8). **USA**, human toe nail, 2006, *D.A. Sutton* (FMR 12211, UTHSC 06-619).

Notes – The taxonomy of *S. alboflavescens* is somewhat controversial. It was considered a species synonym of *Scopulariopsis candida* (Morton & Smith 1963; Abbott & Sigler 2001), but Ropars *et al.* (2012) and Sandoval-Denis *et al.* (2016) have both recently shown that it is phylogenetically distant to that species and closely related to *Scopulariopsis brevicaulis*. The latter authors considered *S. alboflavescens* synonymous with *S. brevicaulis* despite subtle morphological differences between the two species. In the present study, the reference strain received as *S. koningii* (CBS 152.22), with subhyaline and smooth to finely roughened conidia, matches phylogenetically with the ex-type strain of *S. alboflavescens*, and together form a clade distant from *S. brevicaulis*. Interestingly, the mentioned strain CBS 152.22 has also been identified in different culture collections as a probable authentic strain of *S. rufulus*, a species described by Bainier (1907) and considered a synonym of *S. brevicaulis* (Morton & Smith 1963). However, since the type material of *S. rufulus* is not available and the origin of the mentioned strain cannot be established, we have reintroduced *S. alboflavescens* as a valid species for *Scopulariopsis*. The other strains studied showed morphological features similar to those of the protologue of *S. alboflavescens*, a species that morphologically differs from *S brevicaulis* in its white-cream to pale yellowish colonies and subhyaline mostly smooth-walled conidia, measuring 6.5–8.5 × 4.3–7.5 μm. Zach (1934) also described a sexual morph for *S. alboflavescens* with broadly reniform ascospores, 4.2–5 × 2.5–3.8 μm, which was observed in our study only in the ex-type strain. *Scopulariopsis alboflavescens* also resembles *Scopulariopsis macurae*, but can be easily differentiated from the latter by its growth at 37 °C, while the maximum temperature for growth of *S. macurae* is 30 °C.

Scopulariopsis macurae Jagielski, Sandoval-Denis & Gené, **sp. nov.** – MycoBank No.: MB814504; Fig 8.

Etymology – named in honour of the eminent Polish mycologist Anna B. Macura.

Colonies on OA attaining a diameter of 45–47 mm after 14 d at 25 °C, flat, powdery, cream-coloured to beige, with abundant ascomata at the centre, white towards the periphery, with a regular margin; reverse dark grey at the centre, cream-coloured at the periphery. On PCA at 25 °C attaining 57–58 mm diam, flat, powdery, whitish to cream-coloured, with few ascomata at the centre, and a regular fimbriate margin; reverse cream-coloured at the centre, whitish towards the periphery. *Mycelium* with septate, hyaline, smooth-walled, hyphae, 3–5 μm wide. *Ascomata* superficial or partly immersed, globose, 144–188 μm diam, with a papillate ostiolar neck, 19.5–26.5 × 38–50.5 μm, black, glabrous; peridium with a *textura angularis*. *Asci* globose, broadly ellipsoidal or pear-shaped, 12.5–16.5 × 8.5–10 μm. *Ascospores* broadly reniform, 4–5.5 × 3.5–4 μm, straw coloured, yellowish brown (color in the web version) in mass. *Conidiophores* simple as single lateral annellides growing directly on vegetative hyphae, or branched, 70–160 μm long. *Annellides* cylindrical, 14.5–31 × 3.5–4.5 μm. *Conidia* globose or subglobose, 6.5–9 × 7–8.5 μm, with a truncate base of 3.5–4.5 μm wide, hyaline or subhyaline, smooth-walled, arranged in chains.

Temperature for growth – optimum 25 °C, maximum 30 °C.

Specimen examined: **Canada**: Ontario, Guelph, chicken litter, Jan. 1966, *GL Barron* (CBS-H 22298 – holotype, a dried culture on PDA; CBS 506.66 – culture ex-type).

Notes – This species is phylogenetically and morphologically close to *S. candida*; in fact the isolate CBS 506.66 was previously identified as *Microascus manginii*, a specific name

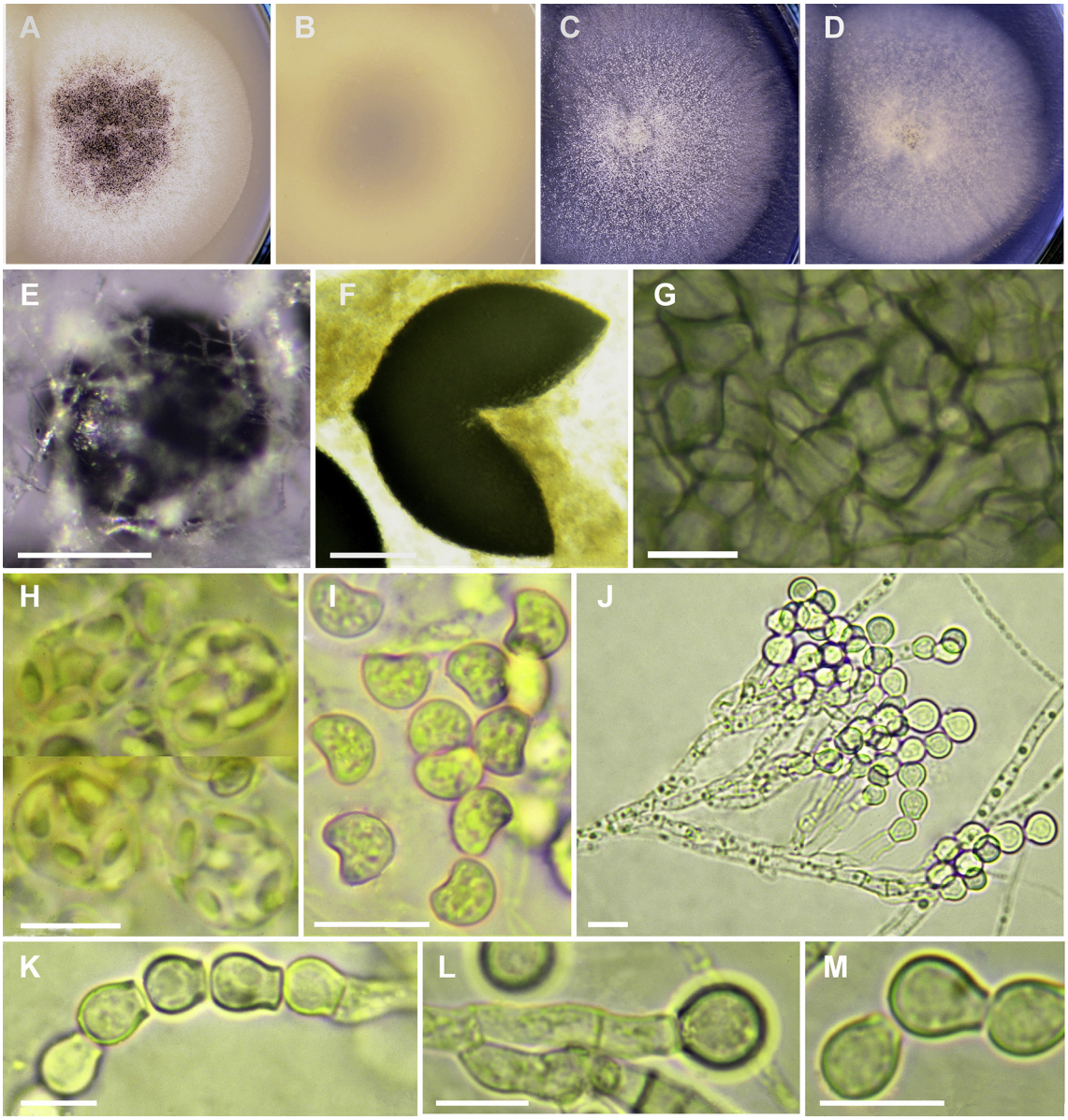

Fig 8 – *Scopulariopsis macurae* (CBS 506.66). Colony morphology after 14 d at 25 °C on OA (A, B – reverse) and PCA (C, D – reverse). Ascomata (E, F). Detail of the peridium (G). Asci (H). Ascospores (I). A branched conidiophore (J). Conidia (K–M). Scale bars: E, F = 100 μm; G–M = 10 μm.

commonly attributed to the sexual state of *S. candida* (De Hoog *et al.* 2011; Sandoval-Denis *et al.* 2016). These species are only distinguished by their ascospores; in *S. macurae* they are broadly reniform and measure 4–5.5 × 3.5–4 μm, but those of *S. candida* are reniform or heart-shaped and slightly larger (4–6 × 5–6 μm). The other species phylogenetically related to *S. macurae* is *S. asperula*, but the latter is easily recognized by its dark brown colonies (Morton & Smith 1963; Abbott & Sigler 2001, Sandoval-Denis *et al.* 2016).

Discussion

The molecular taxonomy of scopulariopsis-like fungi has rarely been a subject of rigorous and comprehensive research. A pioneering work by Issakainen *et al.* (2003), using LSU rDNA sequences as a marker, provided an initial insight into the genetic composition of *Scopulariopsis* species and allied fungi. These were split into 12 lineages, with most of the human pathogenic species being accommodated in a single clade, designated the *Microascus manginii* clade. More than a decade later, investigation on the phylogenetic relationships within the Microascaceae was resumed with an extensive monographic study by Sandoval-Denis *et al.* (2016). The typing strategy used by those authors, and adopted also for the present study, involved sequencing of four different loci, i.e., LSU, ITS, *EF1-α*, and *TUB* genes, producing unambiguous resolution and high branch support. This multi-gene approach allowed a clear demarcation between four already accepted (viz. *Microascus*, *Pithoascus*, *Pseudoscopulariopsis*, and *Scopulariopsis*) and one newly-erected (*Fuscoannellis*) genera, as well as separation of 41 individual species within those genera (Fig 1). The success of the four-locus typing strategy for Microascaceae is quite consistent with previous reports that demonstrated a high resolution and discriminatory capacity of other multigene datasets for studying the systematics of the Sordariomycetes. For instance, Walker *et al.* (2012) evaluated the phylogenetic performance of five genetic loci, i.e., ITS, *EF1-α*, *TUB*, and two newly identified single-copy protein-coding genes, FG1093 and MS204. Different combinations of these markers proved to be useful in resolving species affinities within the Gnomoniaceae (Diaporthales). Tang *et al.* (2007) tested the phylogenetic usefulness of four loci (i.e., LSU, SSU, *TUB*, and *RPB2*) across three different subclasses of the Sordariomycetes (Hypocreomycetidae, Sordariomycetidae, and Xylariomycetidae) and concluded that a combined set of LSU and SSU rDNA, with or without *RPB2*, provides the most reliable phylogeny.

In the present study, by using a polyphasic approach consisting of molecular and phenotypic data, not only was a high-confidence identification of a set of clinical isolates originating from China achieved, but a re-identification of several strains obtained from the CBS culture collection, representing different *Scopulariopsis* and *Microascus* species, was also carried out. The species diversity among Chinese isolates, which were predominantly recovered from nail lesions, was rather high. As expected, *Scopulariopsis brevicaulis* was the most frequently identified species, but many other species of *Microascus* were also detected. Apart from the known human opportunistic pathogen *M. cirrosus* (Krisher *et al.* 1995; De Hoog *et al.* 2011; Miossec *et al.* 2011), we identified *Microascus croci*, *Microascus gracilis*, and the newly described species *Microascus brunneosporus*, *Microascus intricatus*, and *Microascus restrictus*, most of them having been associated with clinical settings in the USA (Sandoval-Denis *et al.* 2013; Sandoval-Denis *et al.* 2016). Furthermore, two other novel species, i.e., *Microascus chinensis* and *Microascus onychoides*, were delineated among these isolates. The role of these newly discovered species in human disease, although plausible as they were all isolated from clinically affected human samples, has to be confirmed. Special attention should be paid when culturing *M. brunneosporus*, *M. intricatus*, *M. restrictus*, and *M. gracilis* from human-derived specimens. These four species have been isolated previously from respiratory specimens, but their implication in pulmonary disease has not been clearly determined (Sandoval-Denis *et al.* 2016). One characteristic of these fungi is that they are able to grow at 40 °C and, therefore, are potentially able to replicate in human tissues inflicting an infection (Seyedmousavi *et al.* 2013).

All CBS strains obtained for this study (n = 26) were sequenced at four loci (Table 1), and the resulting sequences were compared with those of the ex-type strains of all currently accepted species and genera well-delineated by Sandoval-Denis *et al.* (2016). Of the CBS strains investigated, only one-third (8/26; 30.7 %) had their taxonomic status confirmed, while the remainder were either re-classified as other known species (14/26; 53.8 %) or as new species (4/26; 15.38 %) (viz. *Microascus pseudolongirostris*, *Microascus terreus*, *Pithoascus lunatus* or *Scopulariopsis macurae*). In the light of these results, it is desirable to re-evaluate the taxonomic status of scopulariopsis-like fungi deposited in international culture collections, according to the molecular taxonomy proposed by Sandoval-Denis *et al.* (2016) and updated in the present study. Considering that the official fungal barcode was unable to discriminate among closely related species of the genera studied, we recommend the use of *EF1-α* locus as an alternative barcode for the correct identification of all these fungi as previously suggested by Ropars *et al.* (2012).

Acknowledgements

This study was in part supported by the Polish Ministry of Science and Higher Education, grant «Iuventus Plus» (Contract no: IP12013023672), by the Spanish Ministry of Economy and Competitivity, grant CGL 2011-27185, and by the Chinese National Natural Science Foundation, grant 31570015.

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