Persoonia 38, 2017: 1–19
www.ingentaconnect.com/content/nhn/pimj

RESEARCH ARTICLE

ISSN (Online) 1878-9080
http://dx.doi.org/10.3767/003158517X692779

Biodiversity and human-pathogenicity of *Phialophora verrucosa* and relatives in *Chaetothyriales*

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Key words

Chaetothyriales
chromoblastomycosis
phaeohyphomycosis
Phialophora
phylogeny
taxonomy

Abstract *Phialophora* as defined by its type species *P. verrucosa* is a genus of *Chaetothyriales*, and a member of the group known as 'black yeasts and relatives'. *Phialophora verrucosa* has been reported from mutilating human infections such as chromoblastomycosis, disseminated phaeohyphomycosis and mycetoma, while morphologically similar fungi are rather commonly isolated from the environment. Phenotypes are insufficient for correct species identification, and molecular data have revealed significant genetic variation within the complex of species currently identified as *P. verrucosa* or *P. americana*. Multilocus analysis of 118 strains revealed the existence of five reproductively isolated species apparently having different infectious potentials. Strains of the sexual morph *Capronia semiimmersa* cluster within *P. americana*. The newly defined taxa differ markedly in their predilection for the human host.

Article info Received: 2 January 2016; Accepted: 5 May 2016; Published: 2 August 2016.

INTRODUCTION

Phialophora verrucosa is the type species of the genus *Phialophora*, which belongs to the family *Herpotrichiellaceae* (*Chaetothyriales*) comprising the black yeasts and relatives. This phylogenetic affiliation excludes numerous species that have been classified in older literature in *Phialophora* on the basis of the combination of morphological characters of a melanised thallus and one-celled, sticky conidia that are produced through large phialidic collarettes in a poorly differentiated conidial apparatus. Gams (2000) provided an overview of phialophora-like fungi and found that according to current standards belong in nine orders of *Ascomycota*; for nearly all of these, separate generic names are available at present.

Numerous asexual species in the *Chaetothyriales* classified in *Cladophialophora*, *Exophiala* or *Fonsecaea* show presence of phialophora-like synasexual morphs on nutritionally poor media, demonstrating the taxonomic coherence of species belonging to this order (De Hoog et al. 1999). Such phialidic synasexual morphs are also known in *Cladophialophora carrionii*, the agent of human chromoblastomycosis in arid climates and one of the nearest neighbours of *P. verrucosa* in molecular phylogeny. Although strictly monomorphic for phialides, *P. verrucosa* phylogenetically belongs to a group as a whole as the 'carrionii-clade' with *Cladophialophora carrionii* as the core species.

Several other but unrelated monomorphic phialophora-like lineages are known in the *Chaetothyriales*. *Phialophora europaea*, *P. reptans*, known from superficial skin infections in humans (Saunte et al. 2012), *P. attae* and *P. capiguarae*, from ant nests (Attili-Angelis et al. 2014), *P. sessilis* from inert surfaces (Caretta et al. 2006, Zhuang et al. 2010), *P. livistonae*, from living plant leaves (Crous et al. 2012) and *P. oxyspora* all are members of the 'europaea-clade' (De Hoog et al. 2011, Feng et al. 2012). This clade was given family status as *Cyphellophoraceae* by Réblová et al. (2013) and as a consequence some of the member species were reclassified in *Cyphellophora*.

As a result of the above rearrangements, the genus *Phialophora*, for which the Index Fungorum lists 92 species names (as per 01-01-2016), from a phylogenetic viewpoint is restricted to *P. verrucosa* and its sister species *Phialophora americana*, as they both cluster in the 'carrionii-clade'. Species of this clade, i.e. *Cladophialophora carrionii*, *Cl. samoensis* and *P. verrucosa* have been reported from mutilating cases of chromoblastomycosis, disseminated phaeohyphomycosis and mycetoma, which all can be chronic and refractory to therapy (McGinnis 1983, Turiansky et al. 1995, Hofmann et al. 2005, Seyedmousavi et al. 2014). *Phialophora americana*, a sister species of *P. verrucosa* is mostly regarded as being environmental. Also *Cl. carrionii* has an environmental sibling, viz. *Cladophialophora yegresii* (De Hoog et al. 2007). The bipartition clinical / environmental is however ambiguous. *Phialophora verrucosa* was first reported as a human pathogen a century ago (Lane 1915, Medlar 1915a, b), but fungi under this name have also been isolated from natural soils and plant debris (Gezuele et al. 1972). For most of these reports no material is known to be preserved and misidentifications with numerous phialophora-like fungi may have been concerned (Gams 2000, Lopez Martinez & Mendez Tovar 2007).

Recent studies have proven that molecular techniques have a higher precision in segregating phenotypically similar species that may differ in pathogenicity (Marimón et al. 2006, 2007). In black yeasts and allied fungi, molecular siblings may differ significantly in virulence; compare for example the neurotrope *Cladophialophora bantiana* and the gasoline-associated fungus *Cl. psammophila* (Badali et al. 2011). Internal transcribed spacer (ITS) sequencing is effective for species identification among black yeasts, as has been proven with the aid of multilocus studies (Zeng & De Hoog 2008, Heinrichs et al. 2012). No multilocus verification is available for the *P. verrucosa / P. americana* complex (Untereiner et al. 2008). Molecular typing of mitochondrial DNA using restriction fragment length polymorphisms

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(RFLP) suggested that *P. verrucosa* comprised three groups, while analyses of group 1 introns in the 28S ribosomal RNA gene divided the species into five genotypes (Yamagishi et al. 1997, Takizawa et al. 2011). Given this genetic variation a study of phylogenetic relationships is overdue.

Patients infected by *P. verrucosa* showed significant differences in treatment outcomes. This may be due to hidden genetic immune disorders of the host, but the possibility that different *Phialophora* species were concerned cannot be excluded (Tong et al. 2013, Wang et al. 2014). The aim of the present study was to explore the taxonomy of the *P. verrucosa* complex and to determine whether genetic diversity was associated with differences in pathogenicity. Sequence analyses of the ribosomal internal transcribed spacers (ITS), and partial β-tubulin (*BT2*), translation elongation factor 1 alpha (*TEF1*) and the small and large subunits of the nuclear ribosomal RNA (SSU / LSU) regions were used alone or in combination. Additionally, phenotypic characters of morphology and physiology were included along with ecological data.

MATERIALS AND METHODS

Strains studied

One hundred and twenty-six isolates that were initially identified as *P. verrucosa* based on morphology from across the world and including 32 from clinical samples, 89 from the environment, and five from unknown sources were analysed (Table 1). Strains were obtained from the Research Center for Medical Mycology at Peking University from 1997 to 2014, and from the reference collection of the Centraalbureau voor Schimmelcultures Fungal Biodiversity Centre (CBS), Utrecht, The Netherlands. *Phialophora americana*, *Capronia semiimmersa*, *Ca. svrcekiana*, *Cl. carrionii* and *Cl. yegresii* were also included in the study.

Morphology and physiology

For microscopy, small blocks were inoculated with three-point on slants of potato dextrose agar (PDA; Difco, Detroit, USA) at 30 °C for up to 7 d until rich sporulation was obtained. Observations were done with slide cultures using corn meal agar (CMA; Difco). Agar blocks of ~ 0.5 cm^2 were placed on the agar plate and inoculated at the four sides. The block was subsequently covered with a sterile cover slip (~ 2 cm^2). Plates were incubated at 30 °C for 7, 14 or 21 d in a closed plastic box with sterile gauze soaked with 5 mL sterile water to ovoid drying of the culture. Slides were made by Shear's mounting medium without pigments. Micrographs were taken using a Nikon Eclipse 80i microscope and DS Camera Head DS-Fi1/DS-5 m/DS-2Mv/DS-2MBW using NIS-Element freeware package (Nikon Europe, Badhoevedorp, The Netherlands).

Cardinal growth temperatures were determined in triplicate on 2 % malt extract agar (MEA; Difco) by measuring colony diameters for a selection of 28 strains based on phylogenetic results. Plates were incubated in the dark for 3 wk at 21, 24, 27, 30, 33, 37 and 40 °C. In order to evaluate whether 37 °C and 40 °C was fungicidal, cultures were returned to 30 °C and incubated for two additional weeks. In addition, gross morphology was observed both on MEA and OA.

DNA extraction

Genomic DNA was extracted and purified from approximately 1 cm^2 of fungal elements according to the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) with disruption of cells by glass beads (425–600 µm) (Sigma-Aldrich, Zwijndrecht, The Netherlands) and TissueLyser II (Qiagen). Extraction was according to the cetyltrimethylammonium bromide (CTAB) protocol according to Feng et al. (2012).

DNA amplification and sequencing

The following nuclear genes were amplified by PCR: ITS and partial *TEF1*, *BT2*, SSU and LSU. PCR amplifications and sequencing primers are shown in Table 2. Amplifications were done by the 2×EasyTaq PCR Super Mix protocol (TransGen Biotech, Beijing, China). Fifty to 100 ng of DNA template and a 0.2–0.4 µM concentration of forward and reverse primers were added in a total volume of 25 µL. Amplification was performed in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) and included initial denaturation at 94 °C for 5 min, followed by 30 cycles consisting of denaturation at 94 °C for 30 s, annealing for 30 s at 54 °C (ITS, *BT2*, SSU and LSU) or 52 °C (*TEF1*), and extension for 30 s (ITS, *BT2* and *TEF1*) or 1 min (SSU and LSU) at 72 °C. A final extension step of 72 °C for 10 min was included. Reading was done with Gel Doc XR+ system (Biorad, Hercules, CA, USA) with Trans2K Plus DNA Marker (TransGen Biotech) as size and concentration marker. Purification was performed with Silica Bead DNA Gel Extraction Kit (Thermo Fisher Scientific, Vilnius, Lithuania), sequencing with an ABI 3730 automatic sequencer (Applied Biosystems, Foster City, CA, USA) and sequence data were adjusted by SeqMan Pro (DNAStar, Madison, WI, USA). GenBank accession numbers are given in Table 1 except for the *TEF1* region because the sequence length was less than 200 bp.

Alignment and phylogenetic reconstruction

Sequence data were aligned with Clustal W v. 1.6. Alignments were deposited in TreeBASE (number: 19135). Phylogenetic reconstructions were done for each locus and ITS-*TEF1*-*BT2* combined using neighbour-joining (NJ), maximum likelihood (ML) and maximum parsimony (MP) implemented in MEGA v. 6.06 (Kimura 1980, Felsenstein 1985, Saitou & Nei 1987), and MrBayes trees were done by the CIPRES portal (http://www.phylo.org/). MEGA v. 6.06 selected the K2+G model was the most appropriate model of DNA substitution for NJ and ML analysis. Support for the internodes was assessed by bootstrap analysis from 1 000 replicates. MP heuristic search was performed for each dataset with 100 random taxon additions and tree bisection and reconstruction (TBR) as the branch-swapping algorithm. Partition Homogeneity Test (PHT) based on sequences of 3 loci (ITS, *TEF1* and *BT2*) was done by PAUP v. 4.0 b10 with 1 000 replicates for the congruence of gene genealogies. Trees were viewed and edited with MEGA v. 6.06, FigTree v. 1.4.2 and Adobe Illustrator CS6.

RESULTS

Phenotypic data

Based on morphology test results of 50 strains, we found that the conidial system of the strains identified to belong to the *Phialophora verrucosa* complex invariably showed limited differentiation. Phialides were inserted directly on branched hyphae or sometimes occurring as intercalary adelophialides without basal septum and continuous with the supporting hypha. Sometimes phialides were inserted on a lateral supporting cell, or were part of a poorly branched system; conidiophores were not recognized. Discrete phialides were flask-shaped to subcylindrical. Collarettes were always discernible, but varied from short frills to funnel- or vase-shaped, occasionally with a thin-walled, extremely fragile balloon-like extension. Microscopic observation indicated that conidia showing a variety of forms. Conidia were produced in slimy heads with irregular arrangement, were one-celled, and varied in shape from subspherical, tear-shaped to short-cylindrical with rounded ends. Significant variation in shape could often be noted within a single strain.

Table 1 List of strains analysed with isolation data.

Species	Culture no.[1]	Other reference	Source	Geography	GenBank accession numbers[2]		References
					ITS	*BT2*	
P. americana	CBS 400.67		Soil	Brazil	EU514695	EU514708	Untereiner et al. (2008)
	CBS 281.35	ATCC 4806; IMI 021191; MUCL 41728; NIH 8719; UAMH 9609	Chromoblastomycosis, verrucous	USA	EU514694	EU514707	Untereiner et al. (2008)
	NYS 323-90		–	–	U31840	–	Yan et al. (1995)
	CBS 221.97	CDC B-2723; IHM 1700; MUCL 40613	–	Uruguay	U31839	KU306350	Yan et al. (1995)
	CBS 220.97	ATCC 51962; CDC 5; MUCL 40612	Tree	Virginia, USA	U31837	KU306348	Yan et al. (1995)
P. americana, originally identified as *Capronia semiimmersa*	UAMH 10875 (T)	CDC 10; Conant 333	Woodpulp	USA	EU514696	EU514712	Untereiner et al. (2008)
	UAMH 10876	C.J.K. Wang 1050; WUC 402	Wood	USA	EU514697	EU514713	Untereiner et al. (2008)
	MUCL 40572	AFTOL 658	–	France	AF050259	EU514703	Untereiner & Naveau (1999), Untereiner et al. (2008)
	MUCL 39979		Rotten wood	USA	AF050260	EU514702	Untereiner & Naveau (1999), Untereiner et al. (2008)
P. americana, originally identified as *Capronia svrcekiana*	UAMH 10874		Wood	Czech Republic	EU514693	EU514706	Untereiner et al. (2008)
	UAMH 10873		Wood	Czech Republic	EU514692	EU514705	Untereiner et al. (2008)
	UAMH 10872		Wood	Czech Republic	EU514691	EU514704	Untereiner et al. (2008)
P. americana, originally identified as *P. verrucosa*	BMU 01246	CBS 140292	Chromoblastomycosis	North China	KF881941	KF971741	This study
	BMU 01244	CBS 140291; DCU-600, ATCC 38561, IFM 4928	Subcutaneous cyst	Japan	AB190375	KF971743	Iwatsu & Miyaji (1978); this study
	IFM 5089		Human	Japan	AB550776	–	Takizawa et al. (2011)
	CBS 225.97	CDC B-2152	Keratomycosis	Texas, USA	U31847	KU306353	Yan et al. (1995)
	FMC 2214		Human	Colombia	AF397136	–	Heinrichs et al. (2012)
	BMU 00125	CBS 140309	Tree bark	Jiamusi, northeast China	KF881947	KF971748	This study
	BMU 05998	CBS 140311	Soil of patient's garden	Hebei, north China	KF881949	KF971750	This study
	BMU 05996		Tree bark of patient's garden	Hebei, north China	KF881950	KF971751	This study
	BMU 04541	CBS 140312	Leaf of Changbaishan	Changchun, northeast China	KF881951	KF971752	This study
	BMU 00131		Dead wood	Beijing, north China	KF881952	KF971753	This study
	BMU 06000		Soil of patient's garden	Hebei, north China	KF881953	KF971766	This study
	BMU 00132		Wheat	Jiamusi, northeast China	KF881954	KF971754	This study
	BMU 05997		Soil of patient's garden	Hebei, north China	KF881956	KF971756	This study
	BMU 04493		Soil	Changchun, northeast China	KF881958	KF971758	This study
	BMU 04507		Tree bark	Changchun, northeast China	KF881961	KF971762	This study
	BMU 04522		Leaf	Changchun, northeast China	KF881962	KF971763	This study
	BMU 00121		Leaf	Jiamusi, northeast China	KF881963	KF971764	This study
	BMU 00101	CBS 140307	Soil	Xingjiang, northwest China	KJ700965	KM658141	This study
	BMU 00107		Soil	Xian, northwest China	KJ700946	KM658122	This study
	BMU 00109		Soil	Xian, northwest China	KJ700945	KM658121	This study
	BMU 00110		Soil	Xian, northwest China	KJ700949	KM658125	This study
	BMU 00111		Soil	Xian, northwest China	KJ700951	KM658127	This study
	BMU 00114		Soil	Haerbin, northeast, China	KJ700956	KM658132	This study
	BMU 00117		Soil	Xian, northwest China	KJ700962	KM658138	This study
	BMU 00118		Soil	Xian, northwest China	KJ700964	KM658140	This study
	BMU 00147		Soil	Xian, northwest China	KJ700957	KM658133	This study
	BMU 00170		Soil	Beijing, north China	KJ700944	KM658120	This study
	BMU 00206		Soil	Beijing, north China	KJ700958	KM658134	This study
	BMU 00432		Soil	Jiamusi, northeast China	KJ700967	KM658143	This study
	BMU 04506	CBS 140329	Soil	Changchun, northeast China	KJ700955	KM658131	This study
	BMU 04524		Soil	Changchun, northeast China	KJ700963	KM658139	This study
	BMU 04528		Soil	Changchun, northeast China	KJ700950	KM658126	This study
	BMU 04532		Soil	Changchun, northeast China	KJ700968	KM658144	This study
	BMU 04538		Soil	Changchun, northeast China	KJ700954	KM658130	This study
	BMU 04554		Tree bark	Changchun, northeast China	KJ700959	KM658135	This study
	BMU 07607		Soil	Shanghai, east China	KJ700969	KM658145	This study
	BMU 07608		Soil	Shanghai, east China	KJ700970	KM658146	This study
	BMU 07617		Coal	Shanghai, east China	KJ700976	KM658082	This study

Table 1 (cont.)

Species	Culture no.[1]	Other reference	Source	Geography	GenBank accession numbers[2]		References
					ITS	*BT2*	
P. americana, originally identified as *P. verrucosa* (cont.)	BMU 07625	CBS 140305	Leaf	Huangzhou, east China	KJ700981	KM658086	This study
	BMU 07626	CBS 140327	Leaf	Changsha, central China	KJ700982	KM658089	This study
	BMU 07645		Leaf	Chongqing, southwest China	KJ700985	KM658092	This study
	BMU 07650		Leaf	Chongqing, southwest China	KJ700987	KM658094	This study
	BMU 07653		Leaf	Chongqing, southwest China	KJ700988	KM658095	This study
	BMU 07640		Leaf	Chongqing, southwest China	KJ700993	KM658102	This study
	BMU 07641	CBS 140313	Leaf	Chongqing, southwest China	KJ700994	KM658103	This study
	BMU 07647		Leaf	Chongqing, southwest China	KJ700996	KM658105	This study
	BMU 07652		Leaf	Chongqing, southwest China	KJ700997	KM658106	This study
	BMU 07660		Leaf	Chongqing, southwest China	KJ701000	KM658109	This study
	BMU 07693		Leaf	Chongqing, southwest China	KJ701005	KM658081	This study
	BMU 07696	CBS 140315	Wood	Lijiang, southwest China	KJ701009	KM658116	This study
	BMU 07695	CBS 140301	Decaying wood	Lasa, southwest China	KJ701010	KM658117	This study
	BMU 07610		Bamboo	Shanghai, east China	KJ701011	KM658118	This study
	CBS 840.69	FMR 3247; MUCL 15537; LM 342; VTT D-96477; A. Salonen No 501	Decaying timber	Finland	AF050283	EU514711	Untereiner & Naveau (1999), Untereiner et al. (2008)
	IFM 41871		Soil	Colombia	AB550778	–	Takizawa et al. (2011)
	gi281331169		Japanese flounder	Japan	AB538235	–	
	CBS 102234		Decaying trunk (*Gochnathia polymorpha*)	Brazil	KU306358	KU306351	
P. chinensis, originally identified as *P. verrucosa*	BMU 02669	CBS 140300	Chromoblastomycosis	Guangdong, south China	KF881930	KF971731	This study
	BMU 01890 (T)	CBS 140326	Chromoblastomycosis	Guangdong, south China	KF881964	KF971765	This study
	IFM 51934		Human	China	AB550779	–	Takizawa et al. (2011)
	BMU 00441	CBS 140310	Wood	Haikou, south China	KF881948	KF971749	This study
	BMU 00127		Tree bark	Haikou, south China	KF881955	KF971755	This study
	BMU 00447	CBS 140308	Bark	Zhanjiang, south China	KF881957	KF971757	This study
	BMU 00104		Soil	Xian, northwest China	KJ700960	KM658136	This study
	BMU 00112		Soil	Haerbin, northeast China	KJ700966	KM658142	This study
	BMU 00150	CBS 140306	Soil	Haerbin, northeast China	KJ700947	KM658123	This study
	BMU 01057	CBS 140328	Soil	Xian, northwest China	KJ700953	KM658129	This study
	BMU 07609		Wood	Shanghai, east China	KJ700971	KM658147	This study
	BMU 07612		Wood	Shanghai, east China	KJ700972	KM658148	This study
	BMU 07613	CBS 140314	Wood	Shanghai, east China	KJ700973	KM658149	This study
	BMU 07615		Bamboo	Shanghai, east China	KJ700974	KM658150	This study
	BMU 07616		Soil	Shanghai, east China	KJ700975	KM658088	This study
	BMU 07621		Soil	Guangzhou, south China	KJ700978	KM658083	This study
	BMU 07622		Soil	Guangzhou, south China	KJ700979	KM658084	This study
	BMU 07623		Banyan leaves	Guangzhou, south China	KJ700980	KM658085	This study
	BMU 07642		Leaf	Chongqing, southwest China	KJ700983	KM658090	This study
	BMU 07643		Leaf	Chongqing, southwest China	KJ700984	KM658091	This study
	BMU 07649		Leaf	Chongqing, southwest China	KJ700986	KM658093	This study
	BMU 07654		Leaf	Chongqing, southwest China	KJ700989	KM658096	This study
	BMU 07627		Soil	Nanning, south China	KJ700990	KM658097	This study
	BMU 07629		Soil	Nanning, south China	KJ700991	KM658098	This study
	BMU 07636		Dead wood	Nanning, south China	KJ701012	KM658099	This study
	BMU 07637		Dead wood	Nanning, south China	KJ700992	KM658100	This study
	BMU 07661	CBS 140304	Wheat straw	Guangzhou, south China	KJ701013	KM658101	This study
	BMU 07646		Leaf	Chongqing, southwest China	KJ700995	KM658104	This study
	BMU 07656	CBS 140303	Leaf	Chongqing, southwest China	KJ700998	KM658107	This study
	BMU 07657		Leaf	Chongqing, southwest China	KJ700999	KM658108	This study
	BMU 07630		Soil	Nanning, south China	KJ701001	KM658110	This study
	BMU 07639		Molded leaf	Nanning, south China	KJ701002	KM658151	This study
	BMU 07664	CBS 140302	Molded leaf	Nanning, south China	KJ701003	KM658111	This study
	BMU 07692		Leaf	Chongqing, southwest China	KJ701004	KM658112	This study

	NH 258		Environment	Japan	AB498920	–	Hamada & Abe (2010)
	R70D1		Leaf of living tree	Brazil, Bahia state, Saubara, Bahia state, Brazil	KC445295	–	Research database
	WM 04.477		Environment	–	KU306361	–	Research database
P. ellipsoidea, originally identified as	CBS 286.47 (T)	ATCC 9541; MUCL 9768; UAMH 3635	Human	Brazil	AF050282	EU514715	Untereiner & Naveau (1999), Untereiner et al. (2008)
P. verrucosa	CBS 224.97	NIH 8701	Mycetoma hand	Texas, USA	U31848	KU306354	Yan et al. (1995)
P. expanda, originally identified as	BMU 01245	CBS 140322	Chromoblastomycosis	China	KF881934	KF971734	This study
P. verrucosa	BMU 02323 (T)	CBS 140298	Chromoblastomycosis	China	KF881937	KF971737	This study
P. macrospora, originally identified as *P. verrucosa*	BMU 07676	CBS 140320	Facial phaeohyphomycosis (patient 4 case 5)	Wuhan, central China	KJ701006	KM658113	This study; Tong et al. (2013), Wang et al. (2014)
	BMU 07163	CBS 140293	Phaeohyphomycosis skin; case 2	Hebei, north China	KF360975	KF971725	This study; Zhang et al. (2015)
	BMU 04480	CBS 140296	Chromoblastomycosis face	North China	KF881927	KF971726	This study
	BMU 03356	CBS 140295	Chromoblastomycosis hand	East China	KF881928	KF971727	This study
	BMU 03082	CBS 140321	Chromoblastomycosis	East China	KF881938	KF971738	This study
	BMU 00849	CBS 140297	Chromoblastomycosis	East China	KF881945	KF971746	This study
	BMU 07066	CBS 140294	Chromoblastomycosis upper limb	Tianjin, north China	KF881933	KF971759	This study
	CBS 226.97	NYS 303A	Human, facial burn	Tennessee, USA	U31846	KU306349	Yan et al. (1995)
	CBS 273.37 (T)	ATCC 10223; MUCL 9760; IHEM 5639; UAMH 3964	Chromoblastomycosis	Brazil	AF050281	EU514714	Untereiner & Naveau (1999), Untereiner et al. (2008)
	IMTSP.800		Human	Uruguay	AF397135	–	Heinrichs et al. (2012)
	dH 12667		Human	Mexico	KU317088	–	Research database
	dH 12665		Human	Mexico	KU306363	–	Research database
	BMU 00106		Soil	Xian, northwest China	KJ700948	KM658124	This study
	BMU 00115		Soil	Xian, northwest China	KJ700961	KM658137	This study
	BMU 00149		Soil	Xian, northwest China	KJ700952	KM658128	This study
	CBS 839.69	ATCC 34159; MUCL 15541	Wood	Sweden	EU514701	EU514716	Untereiner et al. (2008)
	CBS 138.67	LCP 971; dH 15384	–	France	KU306356	KU306355	This study
	WM 08.287		Environment	–	KU306357	–	Research database
	LY2		–	–	KU306359	–	Research database
	CBS 273.57		–	–	KU306360	–	Research database
P. tarda, originally identified as *P. verrucosa*	CBS 111589 (T)		Invasive Chromoblastomycosis; case 13	Libiya	KU306362	KU306347	This study
	BMU 07506 (ET)	CBS 140325	Phaeohyphomycosis leg	Anhui, east China	KF881960	KF971761	This study; Hu et al. (2011), Wang et al. (2014)
	BMU 07678	CBS 140299	Phaeohyphomycosis skin	Jinan, east China	KJ701008	KM658115	This study; Xu et al. (2011), Wang et al. (2014)
	BMU 04928		Phaeohyphomycosis back	Hebei, north China	KF881965	KF971730	This study; Gao et al. (2013), Wang et al. (2014)
	BMU 05960	CBS 140323	Phaeohyphomycosis skin	Hebei, north China	KF881935	KF971735	This study; Gao et al. (2013), Wang et al. (2014)
	BMU 07712	CBS 140324	Chromoblastomycosis skin	Chengdu, southwest China	KJ700942	KM658087	This study
	CBS 115956		Chromoblastomycosis	–	KU306364	KU306352	This study
Cladophialophora carrionii	CBS 160.54(T)	ATCC 16264	Chromoblastomycosis	Australia	EU137266	EU137201	This study; De Hoog et al. (2007)
	CBS 117906	UNEFM 0014-96 = dH 14504	Chromoblastomycosis hand	Venezuela	EU137288	EU137171	De Hoog et al. (2007)
	CBS 114402	UNEFM 9902 = dH 13271	Chromoblastomycosis arm	Venezuela	EU137275	EU137158	De Hoog et al. (2007)
Cladophialophora yegresii	CBS 114406	UNEFM SgSR1 = dH 13275	*Stenocereus griseus* cactus	Venezuela	EU137323	EU137208	De Hoog et al. (2007)
	CBS 114405(T)	UNEFM SgSR3 = dH 13274 (ex-T of *C. yegresii*)	*Stenocereus griseus* cactus	Venezuela	EU137322	EU137209	De Hoog et al. (2007)

[1] CBS: Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Utrecht, The Netherlands; ATCC: American Type Culture Collection, Virginia, USA; MUCL: Mycotheque de l'Université de Louvain, Louvain-la-Neuve, Belgium; UAMH: Microfungus Herbarium and Collection, Edmonton, Canada; NIH: National Institutes of Health, Bethesda; WC: Wadsworth Center for Laboratory and Research, New York; NYS: New York State Department of Health, New York; CDC: Centers for Disease Control and Prevention, Atlanta, USA; AFTOL: Assembling the Fungal Tree of Life; BMU; Department of Dermatology, Beijing Medical University, Beijing, China; DCU: Department of Dermatology, School of Medicine, Chiba University, Chiba, Japan; IFM: Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, Chiba, Japan; FMR: Facultat de Medicina i Ciencies de la Salut, Reus, Spain; FMC: Faculdade de Medicina, Caracas, Venezuela; UTHSC: University of Texas Health Science Center, San Antonio, TX, USA; IMTSP: Instituto de Medicina Tropical, São Paulo, Brazil; IHEM: The BCCM/IHEM Biomedical Fungi and Yeasts Collection, Brussels, Belgium; Conant: research collection of N.F. Conant; MR: research collection of M. Réblová; WUC: research collection of W.A. Untereiner; CJK Wang: research collection of C.J.K. Wang; dH: research collection of G.S. de Hoog.

[2] ITS: internal transcribed spacer; *BT2*: β-tubulin; *TEF1*: translation elongation factor 1-α.

Table 2 Primers used for PCR amplification and sequencing.

Gene region	Primer name	Primer sequence (5′- > 3′)	Reference
ITS	V9G	5′-TTACGTCCCTGCCCTTTGTA-3′	De Hoog & Gerrits van den Ende (1998)
	LS266	5′-GCATTCCCAAACAACTCGACTC-3′	Masclaux et al. (1995)
	ITS1	5′-TCCGTAGGTGAACCTGCGG-3′	White et al. (1990)
	ITS4	5′-TCCTCCGCTTATTGATATGC-3′	
BT2	Bt2a	5′-GGTAACCAAATCGGTGCTGCTTTC-3′	O'Donnell et al. (2000)
	Bt2b	5′-ACCCTCAGTGTAGTGACCCTTGGC-3′	
TEF1	EF1-728F	5′-CATCGAGAAGTTCGAGAAGG-3′	Carbone & Kohn (1999)
	EF1-986R	5′-TACTTGAAGGAACCCTTACC-3′	
LSU	NL1	5′-GCATATCAATAAGCGGAGGAAAAG-3′	O'Donnell (1993)
	LR5	5′-TCCTGAGGGAAACTTCG-3′	
SSU	NS1	5′-GTAGTCATATGCTTGTCTC -3′	White et al. (1990)
	NS24	5′-AAACCTTGTTACGACTTTTA-3′	Gargas & Taylor (1992)

Growth at different temperatures indicated an optimum at 27–30 °C (Fig. 1) for most of the strains. No growth was observed at 40 °C. The following eight isolates were unable to grow at 37 °C: *P. americana* BMU 01246, BMU 04506, BMU 04541, CBS 220.97 and CBS 102234; *P. verrucosa* BMU 07506, BMU 07678; *P. tarda* CBS 111589. When returned at 30 °C for 3 wk all eight isolates grew well, and all except one (CBS 840.69) isolates originated from patients.

Molecular phylogeny

Phylogenetic reconstruction based on the ITS region and using NJ (Fig. 2), ML, MP and BI algorithms showed similar, more or less congruent topologies (data not shown), but generally with poor resolution. Three main aggregates of strains were recognizable, while seven branches were bootstrap-supported, with a single strain, CBS 111589 located in an isolated position. As a tendency, isolates from human infections were clustered. The preponderantly environmental clades also contained five clinical strains, while conversely, the mainly clinical clusters comprised four environmental isolates. Strains identified as *Ca. semiimmersa* (UAMH 10875, UAMH 10876, MUCL 40572 and MUCL 39979) and *P. americana* were preponderantly found in the environmental clades. The study set also contained the type strain of *P. macrospora*, CBS 273.37; it was located in a cluster that mainly contained strains from clinical samples. Strains of *Ca. semiimmersa* were indistinguishable from those of *P. americana*; a small group of strains denominated *Ca. svrcekiana* took an unresolved position paraphyletic to the *P. americana / Ca. semiimmersa* clade. *Cladophialophora carrionii* and *Cl. yegresii*, which are known to be phylogenetically close to *P. verrucosa*, were selected as out-groups and were clearly distinguishable by ITS (Fig. 2). Phylogenetic reconstruction based on SSU and LSU did not distinguish species of the *P. verrucosa* complex or related groups (data not shown).

To verify the ITS results and to explore a more detailed clustering, we analysed the *BT2* and *TEF1* regions of 118 strains phenotypically identified as *P. verrucosa / P. americana*, with the addition of CBS reference strains. Topologies were congruent with that of ITS, but at a higher level of resolution. Results of PHT showed that three gene lineages were congruent ($P > 0.01$). The tree of the combined 3-gene locus dataset (Fig. 3) revealed a topology similar to those of individual ITS, *TEF1* and *BT2* genes.

The multilocus tree was used as a basis for a new taxonomic system for the *P. verrucosa* complex. The complex contained seven species, consistently separated with all partitions at high statistical support. Only a single cluster contained a type strain, i.e. CBS 273.37 of *Phialophora macrospora*. Strains generally identified and published in the literature with case reports as *P. verrucosa* comprised a small group of strains from five patients, two of which had been proven to have a CARD9 immunodeficiency, the two strains are BMU 07678 and BMU 07506, and this group kept the species name *P. verrucosa* (Gao et al. 2013, Tong et al. 2013, Wang et al. 2014, Zhang et al. 2015). A single, slow-growing isolate from a girl with a disseminated, severely mutilating chromoblastomycosis-like infection in Libya (Hofmann et al. 2005) took an isolated position in all analyses. One environmental cluster with 32 isolates

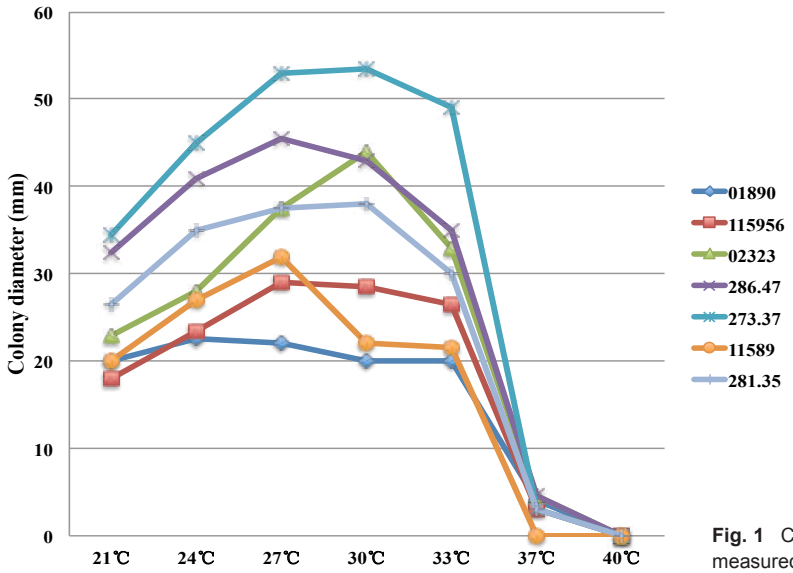

Fig. 1 Colony diameters at various temperatures ranging from 21–40 °C, measured after 3 wk on 2 % MEA.

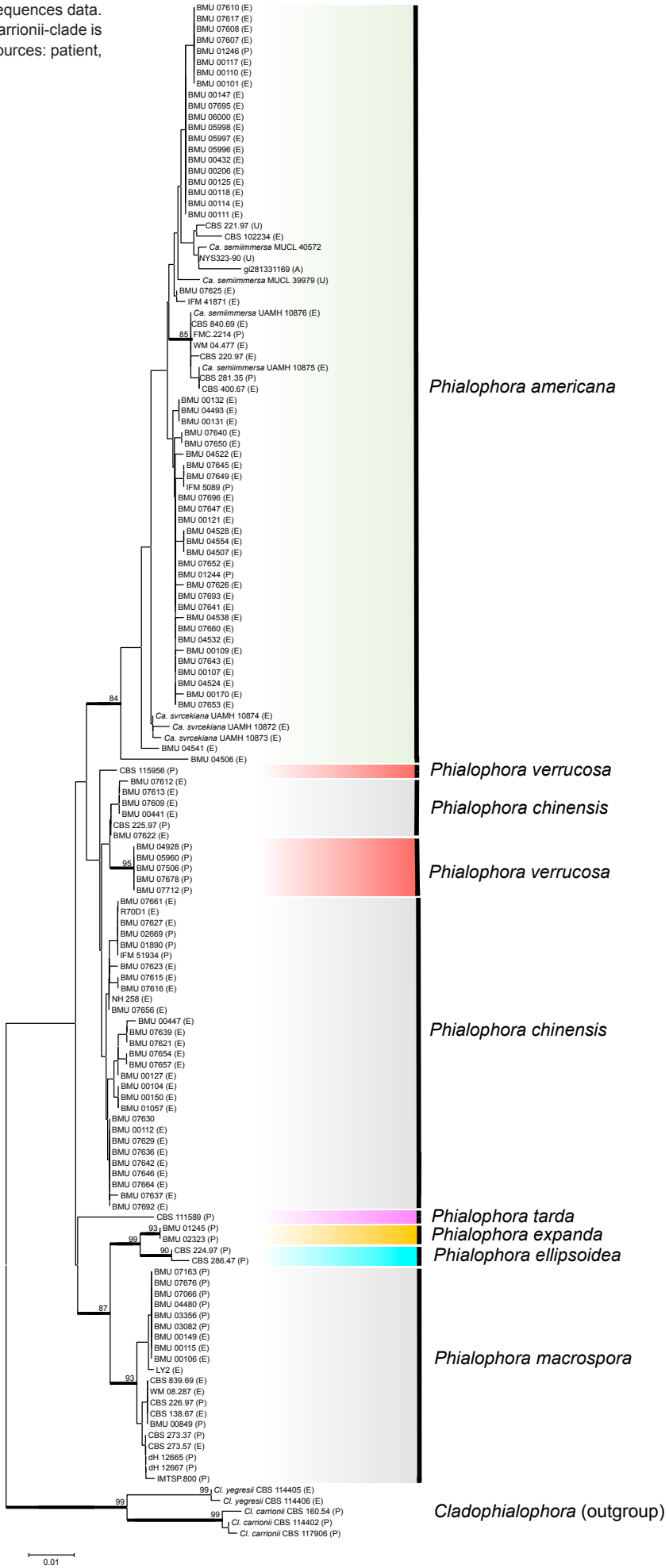

Fig. 2 Neighbour-Joining tree obtained from the 141 ITS sequences data. Bootstrap values above 80 % are shown at the nodes. The carrionii-clade is selected as outgroup. P, E, A, U after strain number mean sources: patient, environment, animal and unknown.

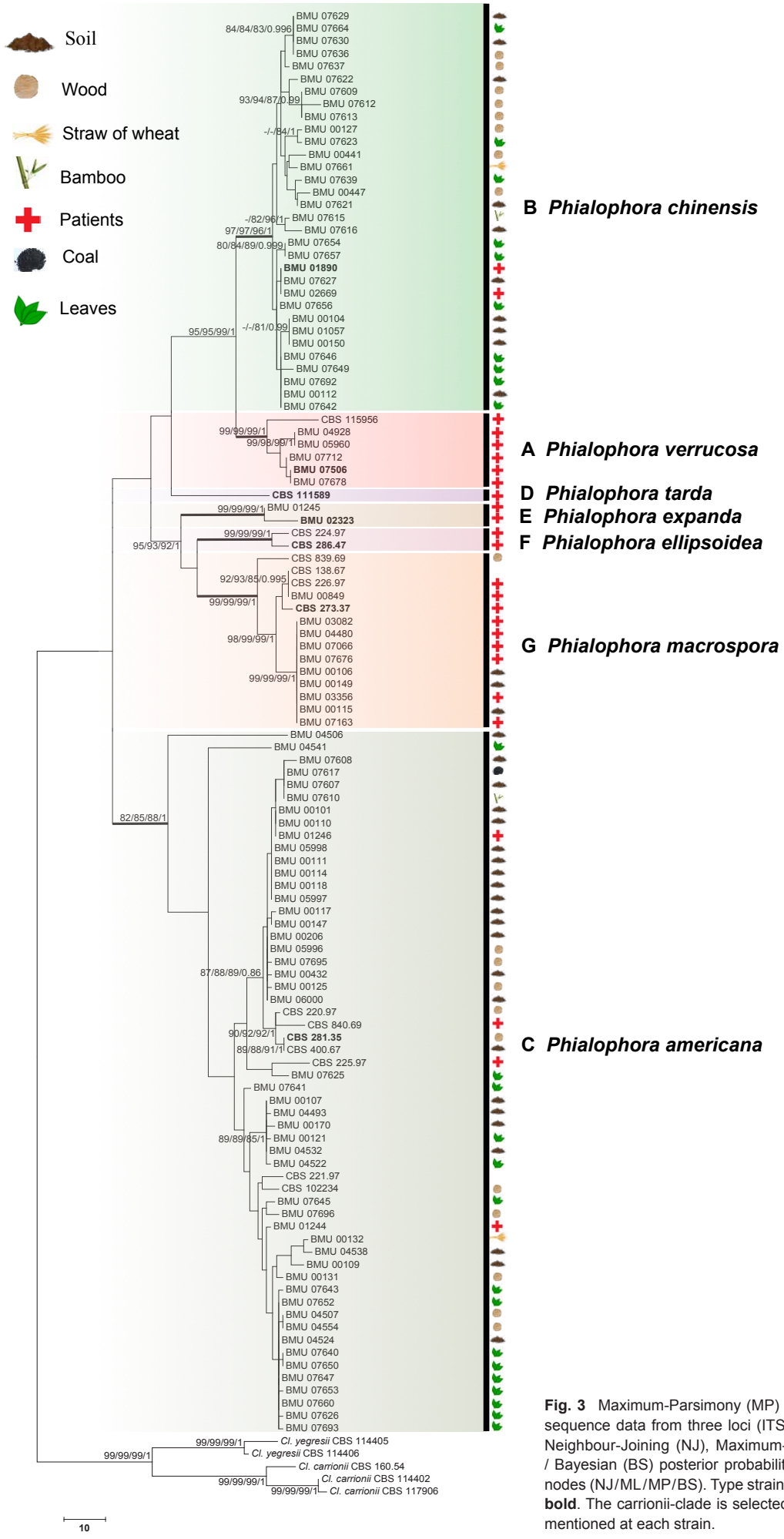

Fig. 3 Maximum-Parsimony (MP) tree obtained from the combined DNA sequence data from three loci (ITS, *BT2* and *TEF1*). Bootstrap values of Neighbour-Joining (NJ), Maximum-Likelihood (ML) and MP above 80 % / Bayesian (BS) posterior probability value above 0.80, are shown at the nodes (NJ/ML/MP/BS). Type strains and supported branches are drawn in **bold**. The carrionii-clade is selected as outgroup. Sources of isolation are mentioned at each strain.

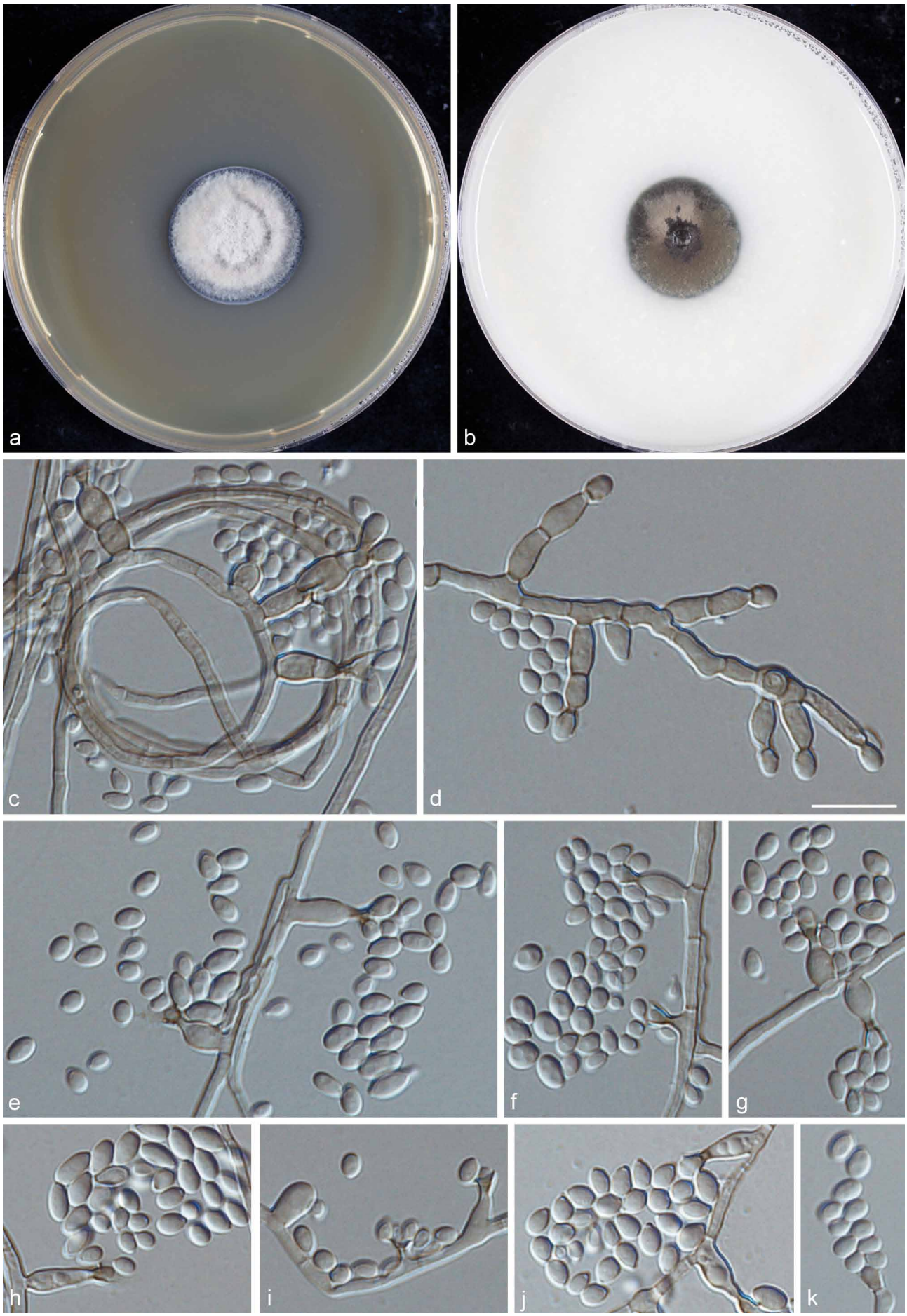

Fig. 4 *Phialophora verrucosa* (CBS 140325). a. Colonies grown on MEA for 3 wk; b. colonies grown on OA for 3 wk; c–k. micromorphology showing phialides and conidia. — Scale bar = 10 µm.

contained strains collected in China from diverse environments such as soil, wood and plant debris, in addition to two isolates (BMU 01890 and BMU 02669) from human patients. Two small groups of strains with human-derived strains only were clearly separate from the main groups at high statistical support. A further, predominantly environmental group (4 clinical of 56 in total) contained strains that were identified in the literature (Untereiner & Naveau 1999) as *P. americana* and its sexual morph *Ca. semiimmersa*. For sequences deposited under the name *Ca. svrcekiana* no multi-locus data were available, but the position of these strains in the ITS tree, i.e. unresolved and adjacent to the *P. americana* group, suggested that the same taxonomic entity was concerned; for extended data see Untereiner et al. (2008).

TAXONOMY

Clade A

Phialophora verrucosa Medlar, Mycologia 7: 203. 1915 — MycoBank MBT203396; Fig. 4

Typus. Lectotype designated herewith f. 1 in Medlar (1915b: 201), an illustration of the fungus from a culture derived from a lesion in the buttock of a 22-yr-old Italian immigrant to Boston, USA. Whether original material of this strain has been preserved could not be ascertained. China, from skin lesions of human disseminated phaeohyphomycosis patient with CARD9 deficiency, epitype designated here: CBS 140325 (preserved at CBS in metabolically inactive condition in liquid nitrogen). Living strain also deposited as BMU 07506.

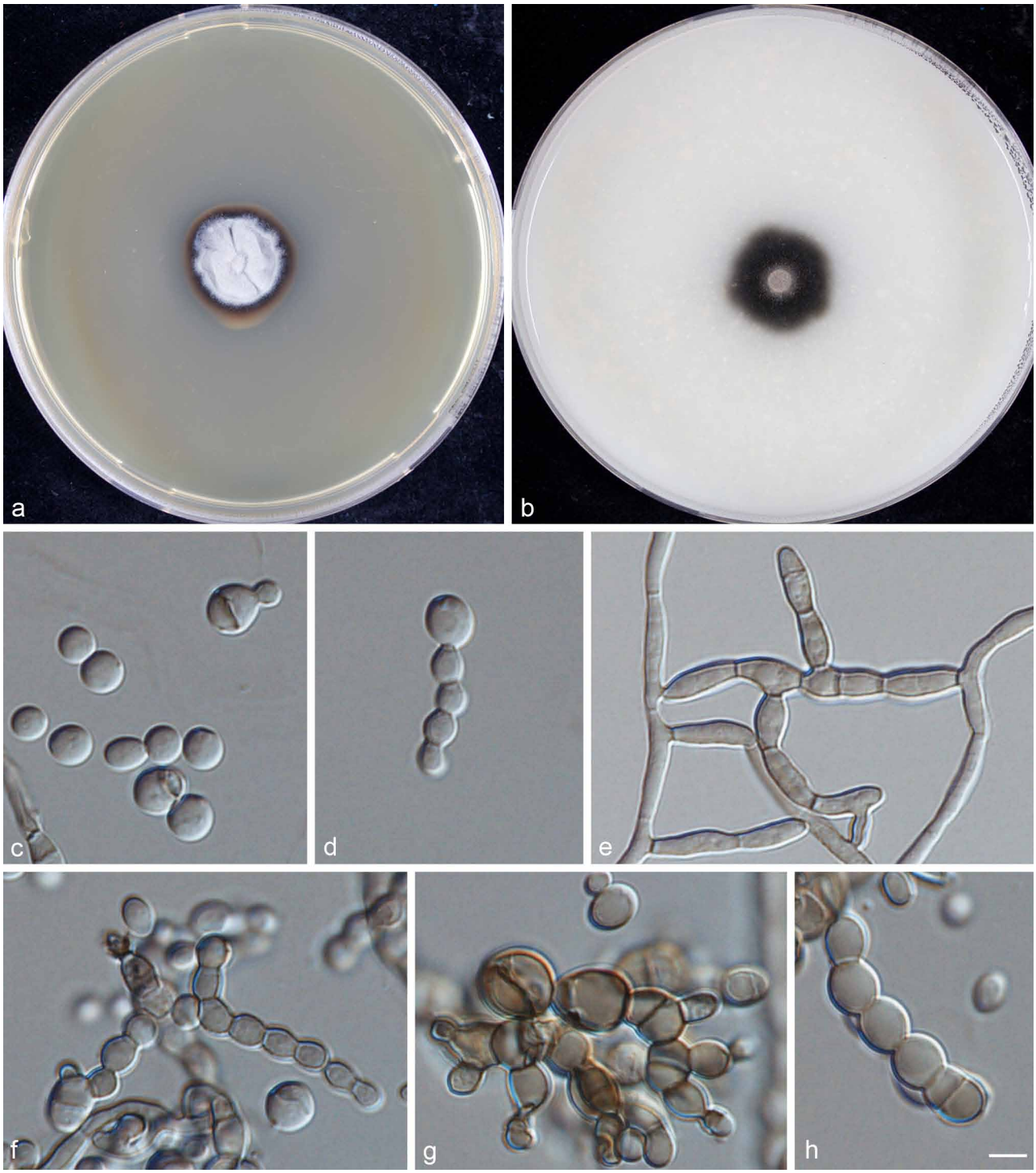

Fig. 5 *Phialophora chinensis* (CBS 140326). a. Colonies grown on MEA for 3 wk; b. colonies grown on OA for 3 wk; c–h. micromorphology showing phialides, conidia, torulose hypha and muriform-like cells. — Scale bar = 10 µm.

Description of CBS 140325 after 3 wk incubation on OA, 30 °C: Colonies growing slowly, olivaceous brown, with black olivaceous in the centre and slightly pink margin. Reverse olivaceous black. On MEA, 30 °C: Colonies growing slowly, pale grey, woolly with smooth, moist margin; reverse olivaceous brown. No diffusible pigment produced. *Hyphae* olivaceous brown, irregularly separate, flexuous, 2.5 ± 0.5 (1.5–3.5) µm wide. *Conidiophores* absent. *Phialides* broadly flask-shaped to elongate, of variable length, often inserted on a subtending cell; adelophialides without basal septum are common. *Collarettes* large, funnel-shaped, sometimes small, darker brown than the supporting phialide, producing conidia in heads. *Conidia* hyaline, 4.5 ± 0.5 (3.0–5.5) × 2.5 ± 0.5 (2.0–3.5) µm, smooth-walled, teardrop-shaped with protruding beak on one end and remain aggregated around the phialides. *Sexual morph* unknown. Cardinal temperatures: minimum below 21 °C, optimum 30 °C, maximum 37 °C.

Additional material examined. Table 1.

Notes — The type isolate has been preserved at Research Center of Medical Mycology, Peking University and at CBS. Isolates belonging to this species were derived from five patients, including four from China, and two of them concerned cases of CARD9-related immunodeficiency phaeohyphomycosis reported by Wang et al. (2014).

Clade B

Phialophora chinensis Yali Li, de Hoog & R.Y. Li, *sp. nov.* — MycoBank MB815345; Fig. 5

Typus. China, from skin lesions of human chromoblastomycosis patient, holotype CBS 140326 (preserved at CBS in metabolically inactive condition in liquid nitrogen). Living strain also deposited as BMU 01890.

Description of BMU 01890 after 3 wk incubation on OA, 30 °C: Colonies growing slowly, olivaceous black, with pale olivaceous centre. Reverse olivaceous black. On MEA, 30 °C: *Colonies* growing slowly, woolly, pale olivaceous grey with brown, smooth margin; reverse olivaceous brown. No diffusible pigment produced. *Hyphae* brown, regularly septate, 4.0 ± 0.5 (3.0–4.5) µm wide. *Conidiophores* absent. *Phialides* broadly flask-shaped. *Conidia* hyaline, smooth-walled, spherical to broadly ellipsoidal, 4.5 ± 0.5 (3.0–6.0) × 3.5 ± 0.5 (2.0–5.5) µm, some larger conidia developing a median septum resembling muriform cells, or show budding. *Sexual morph* unknown. Cardinal temperatures: minimum below 21 °C, optimum 24 °C, maximum 40 °C.

Additional material examined. Table 1.

Notes — The species grows with short cells producing thick-walled, swollen cells with median septa strongly resembling muriform cells on routine media. Nevertheless, nearly all strains known of *P. chinensis* are environmental, mostly being isolated from soil and plant debris. Two of the strains examined (Table 1) were derived from human patients, causing chromoblastomycosis.

Clade C

Phialophora americana (Nannf.) S. Hughes, Canad. J. Bot. 36: 795. 1958 — MycoBank MB203397; Fig. 6

Basionym. Cadophora americana Nannf., in Melin & Nannf., Svensk Skogsvårdsförening Tidskr. 3–4: 412. 1934.

= *Dictyotrichiella semiimmersa* Cand. & Sulmont, Rev. Mycol. 36: 242. 1972.

≡ *Capronia semiimmersa* (Cand. & Sulmont) Unter. & F.A. Naveau, Mycologia 91: 73. 1999.

= *Capronia svrcekiana* Réblová, Czech Mycol. 49: 82. 1996.

Typus. USA, Wisconsin, woodpulp, *A. Richards*, holotype of *P. americana* slide 6320-2 (UPS). Living strain also deposited as UAMH 10875 = CDC 10.

Description of CBS 281.35 after 3 wk incubation on OA, 30 °C: Colonies growing moderately rapidly, olivaceous brown and pale at the centre. Reverse olivaceous black. On MEA, 30 °C: woolly, olivaceous grey; reverse olivaceous black. No diffusible pigment produced. *Hyphae* irregular, 2.5 ± 0.5 (1–3) µm wide. Distinct conidiophores absent. *Phialides* variable, flask-shaped to cylindrical or elongated, with darker, vase-shaped or tubular collarettes, which may also be sessile directly on undifferentiated hyphae. *Conidia* hyaline, 5.0 ± 0.5 (3.5–7.0) × 3.0 ± 0.5 (2–4) µm, subspherical to broadly ellipsoidal, occasionally subcylindrical, of variable size, mostly adhering in loose clumps at the collarette openings, rarely arranged in loose strings. Cardinal temperatures: minimum below 21 °C, optimum 30 °C, maximum 37 °C.

Additional material examined. Table 1.

Notes — The strain taken by several authors as representative for the species, CBS 281.35 was derived from a verrucous dermatosis of the legs of a human chromoblastomycosis patient, USA. The isolate was first described as *Phialophora verrucosa* by Schol-Schwarz (1970) as representative of that species, but later it was redescribed as *P. americana* by Yamagishi (in Yamagishi et al. 1997), Untereiner (in Untereiner et al. 2008) and Takizawa (in Takizawa et al. 2011). The species was also reported as *Capronia semiimmersa* from a herbarium specimen by Candousseau & Sulmont (1971). Untereiner & Naveau (1999) judged living strain MUCL 40572, parasitizing a lichen on *Populus* wood in France, identical to the type specimen and provided an illustration of its monomorphic *Phialophora* asexual morph with deep, vase-shaped phialidic collarettes. Strains UAMH 10872, 10873, 10874 are representative of *Ca. svrcekiana* and are also identical to *P. americana* in the ITS tree (Fig. 2), confirming conclusions of Untereiner et al. (2008).

Clade D

Phialophora tarda Yali Li, de Hoog & R.Y. Li, *sp. nov.* — MycoBank MB815349; Fig. 7

Typus. Libya, from tissue of disseminated chromoblastomycosis-like infection in human patient (Hofmann et al. 2005), holotype CBS 111589 (preserved at CBS in metabolically inactive condition in liquid nitrogen).

Description of CBS 111589 after 3 wk incubation on OA, 30 °C: Colonies growing slowly, olivaceous brown, with black olivaceous centre. Reverse olivaceous black. On MEA, 30 °C: Colonies growing slowly, pale olivaceous grey, woolly, with narrow smooth margin; reverse olivaceous brown. No diffusible pigment produced. *Hyphae* olivaceous brown, flexuous, 2.0 ± 0.5 (1.5–2.5) µm wide. *Conidiophores* absent. *Phialides* regularly flask-shaped to elongate; adelophialides uncommon. *Collarettes* slightly darker than the rest of the phialide, narrow funnel-shaped to almost cylindrical, up to 5.6 µm long. *Conidia* hyaline, variable in shape, mostly broadly ellipsoidal, 3.5 ± 1.0 (2.0–5.5) × 2.5 ± 0.5 (1.5–3.5) µm, smooth-walled. *Sexual morph* unknown. Cardinal temperatures: minimum below 21 °C, optimum 27 °C, maximum 40 °C.

Notes — The species is known from a single strain causing a severely mutilating, disseminated infection in a girl from Libya, initially identified as *P. verrucosa* (Hofmann et al. 2005). The patient was judged to be immunocompetent, but at that time the existence of CARD9- or STAT1-based or other rare inherited genetic immune defects was not known. Muriform cells in tissue had a variable appearance without typical cruciate septation.

(text continues on p. 17)

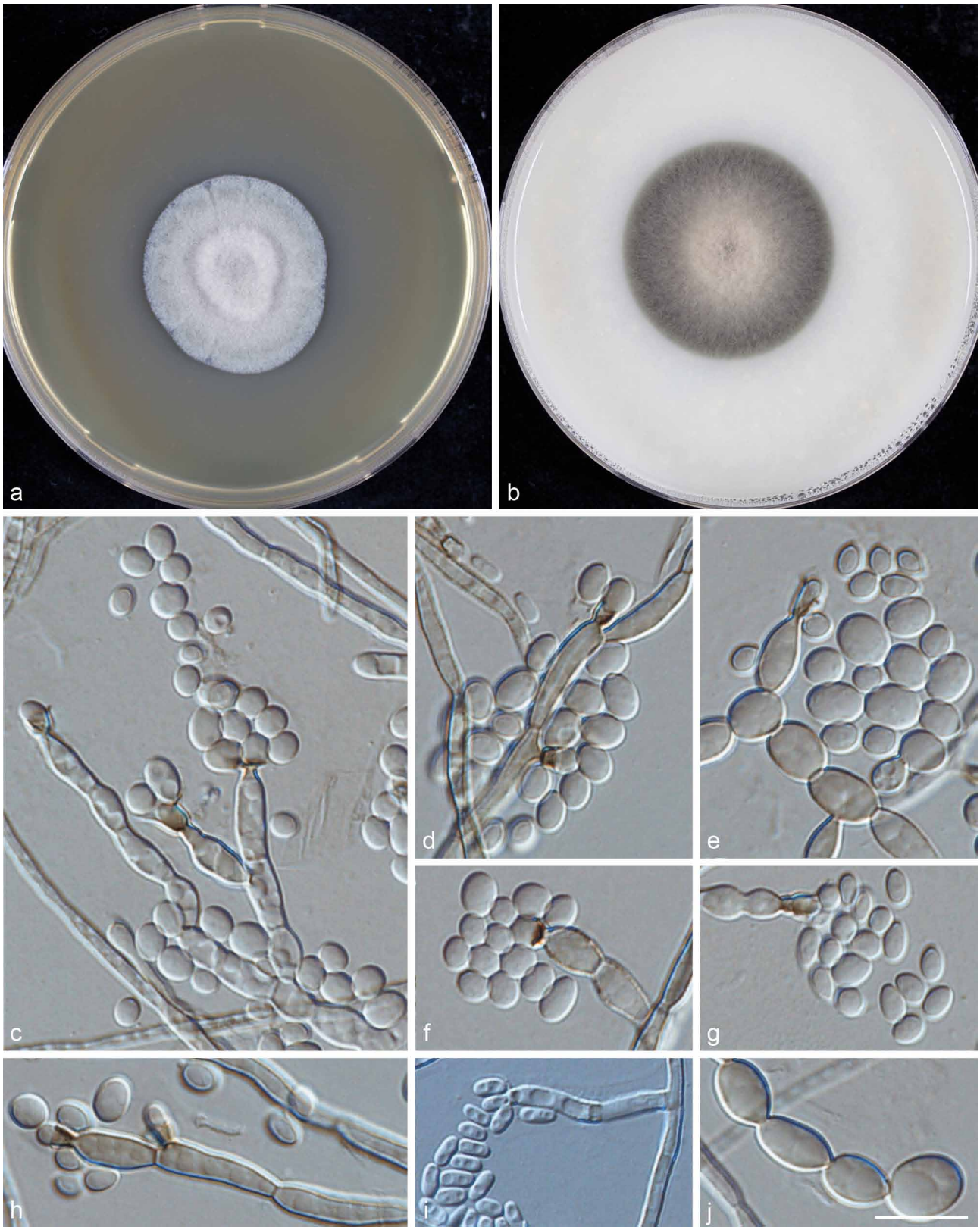

Fig. 6 *Phialophora americana* (CBS 281.35). a. Colonies grown on MEA for 3 wk; b. colonies grown on OA for 3 wk; c–j. micromorphology showing phialides, conidia and torulose hypha. — Scale bar = 10 µm.

Fig. 7 *Phialophora tarda* (CBS 111589). a. Colonies grown on MEA for 3 wk; b. colonies grown on OA for 3 wk; c–k. micromorphology showing phialides and conidia. — Scale bar = 10 µm.

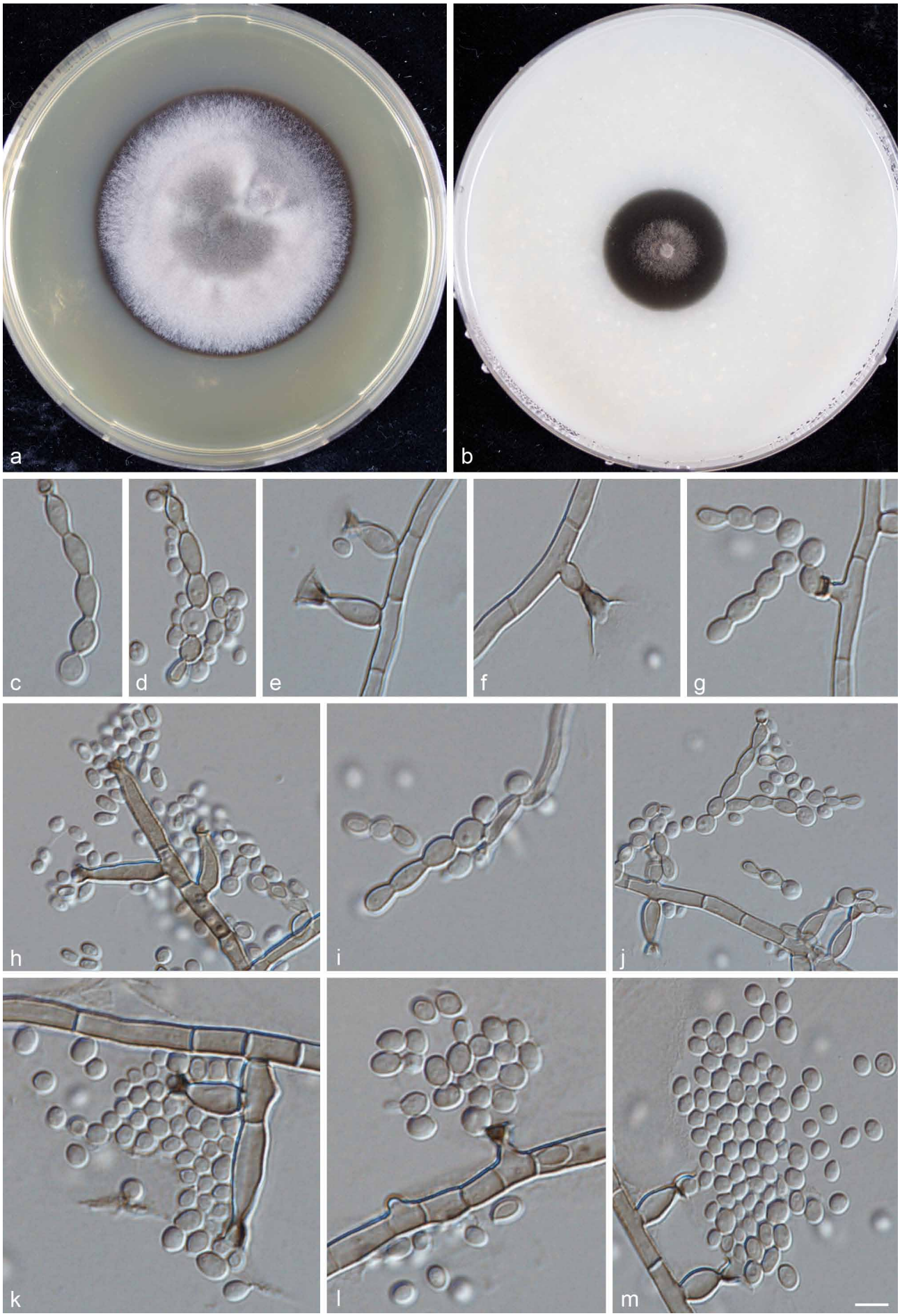

Fig. 8 *Phialophora expanda* (CBS 140298). a. Colonies grown on MEA for 3 wk; b. colonies grown on OA for 3 wk; c–l. micromorphology showing phialides, conidia and torulose hypha. — Scale bar = 10 µm.

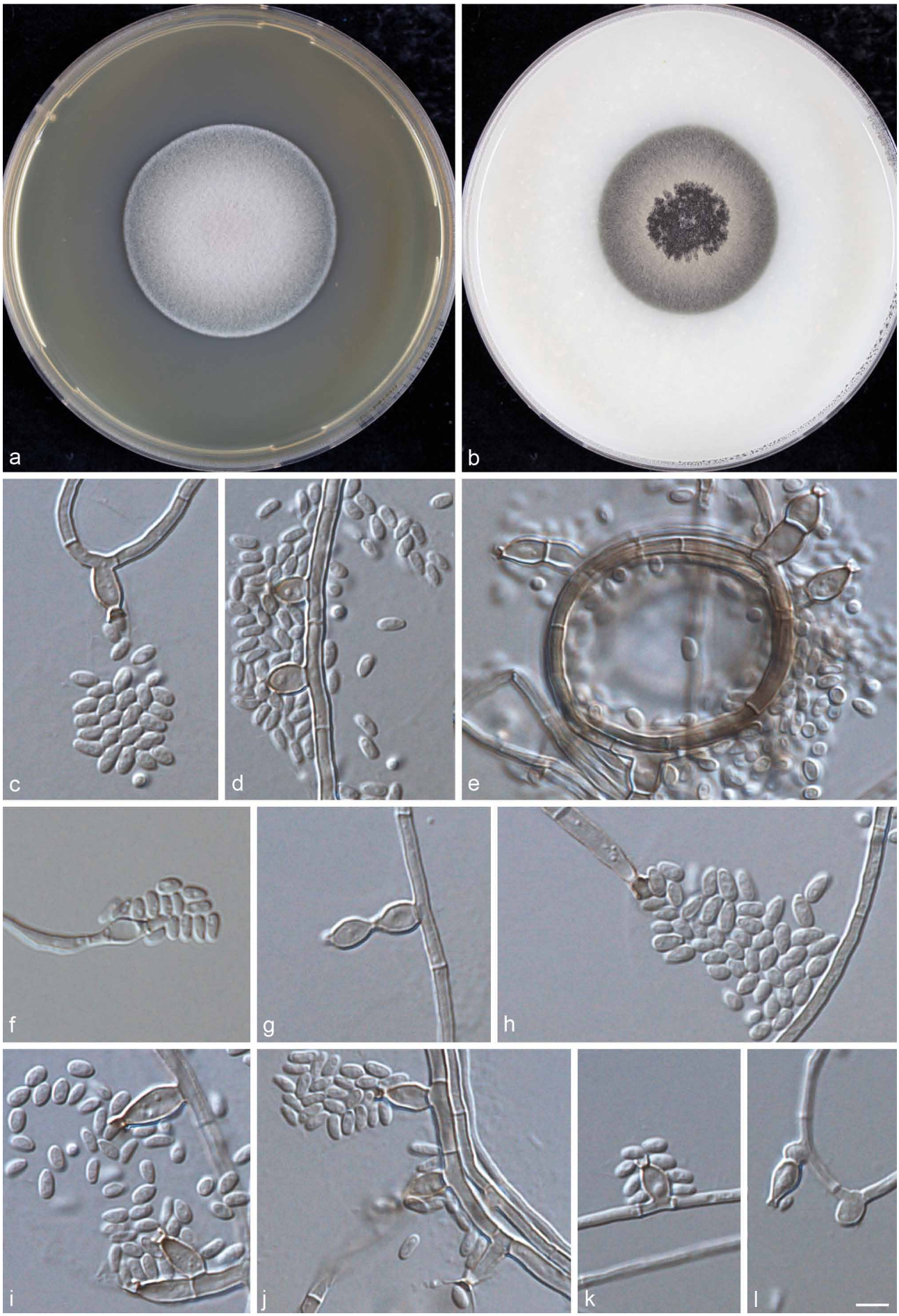

Fig. 9 *Phialophora ellipsoidea* (CBS 286.47). a. Colonies grown on MEA for 3 wk; b. colonies grown on OA for 3 wk; c–k. micromorphology showing phialides and conidia. — Scale bar = 10 µm.

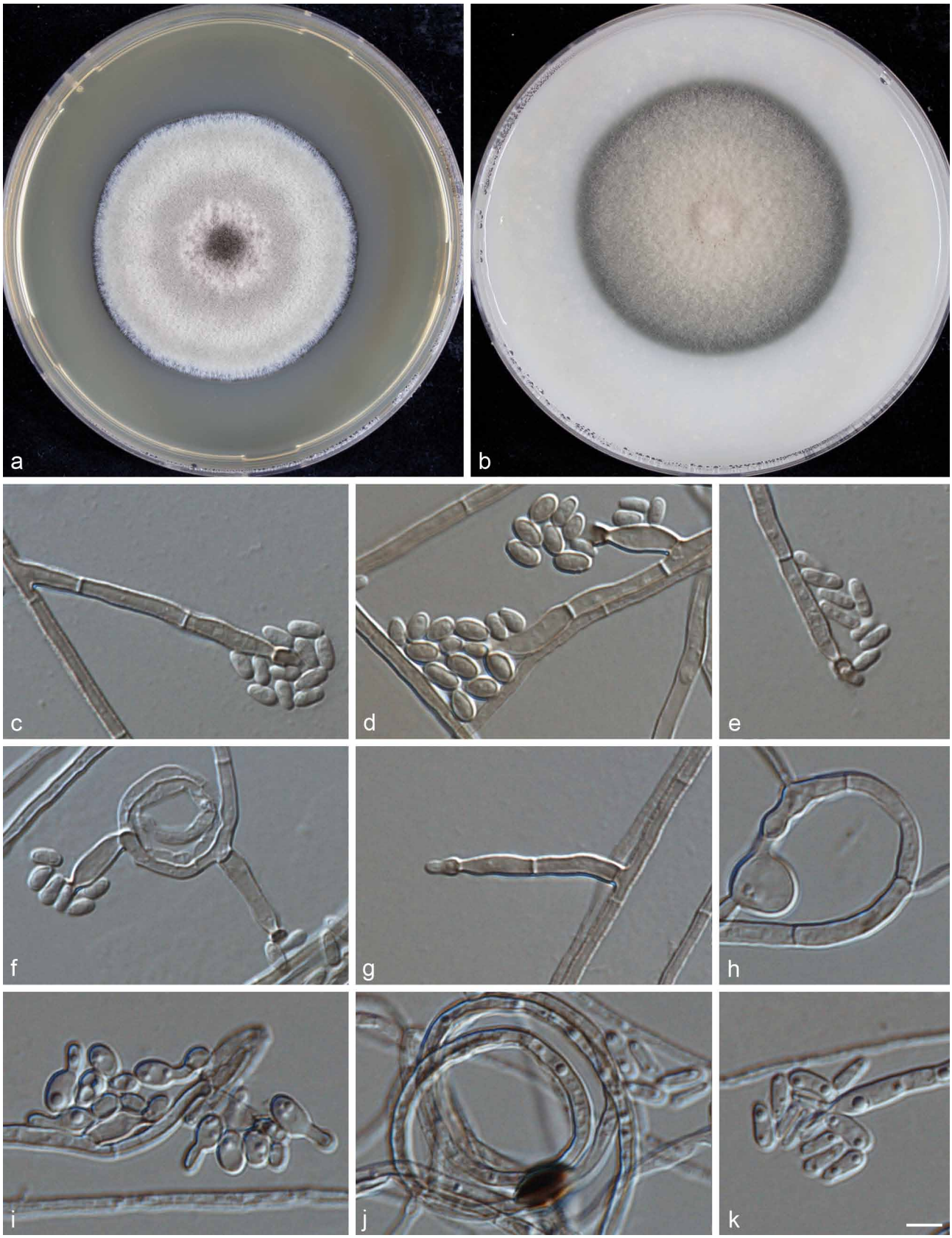

Fig. 10 *Phialophora macrospora* (CBS 273.37). a. Colonies grown on MEA for 3 wk; b. colonies grown on OA for 3 wk; c–k. micromorphology showing phialides and conidia. — Scale bar = 10 µm.

Clade E

Phialophora expanda Yali Li, de Hoog & R.Y. Li, *sp. nov.* — MycoBank MB815350; Fig. 8

Typus. China, from skin lesions of chromoblastomycosis patient, holotype CBS 140298 (preserved at CBS in metabolically inactive condition in liquid nitrogen). Also deposited as living strain CBS 140298 = BMU 02323.

Description of BMU 02323 after 3 wk incubation on OA, 30 °C: Colonies growing slowly, olivaceous black, with brown, woolly hyphae near the centre. Reverse olivaceous black. On MEA, 30 °C: Colonies growing moderately rapidly, woolly, pale olivaceous grey, with smooth margin; reverse olivaceous brown. No diffusible pigment produced. *Hyphae* olivaceous brown, often emerging from torulose hyphae and flexuous, 2.0 ± 0.5 (2.0–3.5) µm wide. *Conidiophores* absent or poorly differentiated. Most phialides flask-shaped to elongate, narrowed towards the tip; short adelophialides without basal septa frequently present. *Collarettes* darker than the rest of the phialide, funnel-shaped to almost cylindrical, often with a large, less intensely pigmented and very fragile apical portion, which is widely open. *Conidia* hyaline, ellipsoidal, 3.5 ± 0.5 (2.0–5.0) × 2.5 ± 0.5 (1.5–3.5) µm, smooth-walled, occasionally budding, aggregated in heads, sometimes in short chains. *Sexual morph* unknown. Cardinal temperatures: minimum below 21 °C, optimum 30 °C, maximum 40 °C.

Additional material examined. Table 1.

Notes — This isolate was collected by Peking University First Hospital from a chromoblastomycosis patient in 2000. It always clustered with the isolate BMU 01245 that was collected in 1999 from another patient.

Clade F

Phialophora ellipsoidea Yali Li, de Hoog & R.Y. Li, *sp. nov.* — MycoBank MB815351; Fig. 9

Typus. Brazil, from human patient, holotype CBS 286.47 (preserved at CBS in metabolically inactive condition in liquid nitrogen). Living strain CBS 286.47 = ATCC 9541 = MUCL 9768 = UAMH 3635.

Description of CBS 286.47 after 3 wk incubation on OA, 30 °C: Colonies growing moderately rapidly, olivaceous brown, with black and purple granules at the centre. Reverse olivaceous black. On MEA, 30 °C: woolly, olivaceous grey; reverse olivaceous brown. No diffusible pigment produced. Hyphae pigmented with slightly brown, separate uniform with 2 ± 0.5 (1.5–2.5) µm wide. Distinct conidiophores absent. Part of the phialides flask-shaped, later enlarge to become subellipsoidal; some of the phialides give rise to a second phialide. *Collarettes* mostly small, sometimes longer, 1.5 ± 0.5 (0.5–2.0) µm. *Conidia* hyaline, ellipsoidal, 3.0 ± 0.5 (2.0–4.5) × 1.5 ± 0.5 (1.5–2.0) µm. *Sexual morph* unknown. Cardinal temperatures: minimum below 21 °C, optimum 27 °C, maximum 37 °C.

Additional material examined. Table 1.

Notes — This isolate had been identified as *P. verrucosa* all the time. Now, according to the ITS, *BT2* and *TEF1* gene analyses, it always clustered together with CBS 224.97 with high support value, and they are from human patients.

Clade G

Phialophora macrospora M. Moore & F.P. Almeida, Ann. Mo. Bot. Gdn 23: 545. 1936. — MycoBank MB270192; Fig. 10

Typus. Brazil, São Paulo, from human chromoblastomycosis-like infection, *M. Moore*, holotype CBS 273.37 = ATCC 10223 = MUCL 9760.

= *Fonsecaea pedrosoi* (Brumpt) Negroni var. *phialophora* Carrión, Mycologia 34: 432. 1942.

Description of CBS 273.37 after 3 wk incubation on OA, 30 °C: Colonies growing rapidly, olivaceous brown, pale at the centre. Reverse olivaceous black. On MEA, 30 °C: Colonies growing rapidly, pale grey, brown at the centre, woolly with smooth, moist margin; reverse olivaceous brown. No diffusible pigment produced. Hyphae brownish, regularly septate, 2.0 ± 0.3 (1.5–2.5) µm wide, flexuous. *Phialides* inserted directly on hyphae, flask-shaped. *Collarettes* small and short, vase- to funnel-shaped, part of them darker than the rest of the phialide. *Conidia* hyaline, sometimes showing some budding, 4.0 ± 0.5 (3.0–5.5) × 2.0 ± 0.5 (1.5–2.5) µm. *Sexual morph* unknown. Cardinal temperatures: minimum below 21 °C, optimum 30 °C, maximum 40 °C.

Notes — This isolate had been identified as *P. verrucosa* (Untereiner & Naveau 1999, Untereiner et al. 2008, Heinrichs et al. 2012), or *P. americana* (Yamagishi et al. 1997).

DISCUSSION

The present study aims to investigate the biodiversity and taxonomy of *Phialophora verrucosa*, which has been reported in older literature as one of the uncommon agents of human chromoblastomycosis (Guerriero et al. 1998). However, also other types of infection have been ascribed to this species, among which are mycetoma (Turiansky et al. 1995), disseminated (Hofmann et al. 2005, Tong et al. 2013) and particularly different kinds of subcutaneous infection, often with cystic encapsulation (Iwatsu & Miyaji 1978, Schnadig et al. 1986, Kimura et al. 2003). Most infections were noted in patients with apparently good health; the share of immunocompromised patients, such as transplant recipients (Lundstrom et al. 1997), those with AIDS (Duggan et al. 1995) or with chronic use of antibiotics (Hochfelder & Fetto 2013) are relatively limited. In addition to human infection, the species has also been isolated from the environment, by enrichment in a mammal vector (Gezuele et al. 1972) but also with methods that are standard for direct black yeast isolation (Iwatsu et al. 1981). The majority of these isolates have, however, not been preserved, and their identity thus can no longer be verified.

Our data provide evidence that separate species are concerned, with different predilection and possibly causing different disorders. The combined ITS-*TEF1-BT2* tree showed seven clades, six of which were supported by high bootstrap values and the seventh took an isolated position in all partitions. It was concluded that seven putative phylogenetic species exist in the *P. verrucosa* complex. Most of the recognised phylogenetic species exhibited a high degree of origin specificity, with species significantly differing in apparent pathogenicity. Two of the main environmental clusters contained 6.3–7.1 % strains from human patients, whereas in the combined 'pathogenic' clusters this ratio was 83.3 %.

Species identification for black yeasts in general (Zeng et al. 2013) including members of the genus *Phialophora* s.str. (Chowdhary et al. 2014) is possible by ITS sequencing. Our study shows the taxonomy of *P. verrucosa* in more detail. The phylogenetic trees of both ITS and *BT2* distinguish the *P. verrucosa* complex unambiguously from its close the relatives *Cl. carrioni* and *Cl. yegresii*. Within the complex, rDNA ITS provides insufficient resolution in that the seven species-clusters have statistical support due to strains in paraphyletic position. Nevertheless, characteristic ITS-profiles are recognizable for each species, so that ITS can be used as barcode for routine identification (Schoch et al. 2012).

Only a single sexual morph of *P. americana*, *Ca. semiimmersa*, is known in the *P. verrucosa* complex (Untereiner et al. 2008). Sexual connections (*Ca. semiimmersa* including *Ca. svrcekiana*) were made by isolation of ascospores from natural samples, and sequences of cultures invariably clustered in *P. americana* (Untereiner et al. 2008, Réblová 1996). *Phialophora americana* is a preponderantly environmental species and is predicted to have low human pathogenicity judging from isolation sources.

Pathogenicity and virulence is known to differ significantly between closely related species of black fungi (Chowdhary et al. 2014). Virulence factors listed thus far include melanin and carotene, thick cell walls, muriform cells, yeast-like phases, thermo- and perhaps also osmotolerance, adhesion, hydrophobicity, aromatic hydrocarbon assimilation, and production of siderophores, factors exerting variable influence upon location and severity of the infection (Seyedmousavi et al. 2014). These are general factors attributed to the entire family *Herpotrichiellaceae*; significant differences between species as yet have not been found. It remains difficult to explain why closely related species, as in *P. verrucosa* and its allies, differ significantly in this respect, while on the other hand agents of a highly specific disease as chromoblastomycosis are scattered over the family. Infections caused by members of the *P. verrucosa* complex can be destructive and highly refractory to therapy. Clinical isolates collected in the course of our study mostly were derived from patients with chromoblastomycosis or phaeohyphomycosis, while treatment outcomes of those patients were quite different (Tong et al. 2013, Wang et al. 2014). Remarkably, two patients were ultimately proven to have a mutation in the CARD9 signalling pathway interfering with Dectin-1 immunity. *Phialophora verrucosa* isolates caused recalcitrant infections, and a species named *P. tarda* was collected from an invasive disseminated mycosis in Libya (Hofmann et al. 2005) in a patient that may also have had a CARD9 immune defect. It is not understood why such patients acquire just a single mycotic infection, and why black fungi are relatively frequent in these hosts. Infections by *P. americana* and *P. chinensis* are environmental fungi with opportunistic behaviour after local trauma.

Isolates used in this study had been recovered from diverse environmental sources across the world such as plant debris, soil and rotten wood. These environmental isolates tended to aggregate in a limited number of clusters, different from the subgroups with preponderantly human sources of isolation according to the phylogenetic trees. The overabundance of Chinese strains probably is a sampling effect; we expect that all environmental species have a global distribution. The most enigmatic species in the complex is *P. tarda*, originating from a severe human infection and without known environmental source. Notably, despite extensive environmental sampling, *P. verrucosa* (s.str.), *P. expanda* and *P. ellipsoidea* were not encountered either.

CONCLUSIONS

Distinction of six clades described here and summarised in Fig. 1 was achieved with molecular characters, phenotype and ecology. Optimum temperatures differ between strains and are therefore compared below at an average of 27 °C after 3 wk. *Phialophora chinensis* (B), nearly exclusively derived from environmental sources, in culture nevertheless has a strong tendency to production of isodiametric cells resembling muriform cells of chromoblastomycosis, and shows some yeast-like cells; hyphae are scant and conidiophores are absent. Growth is moderately slow (19–42 mm). *Phialophora verrucosa* s.str. (A) contains clinical strains only. Phialides have a wide base and a dark, funnel-shaped collarette. Growth 22–31 mm. *Phialophora tarda* (D) is known only from a moderately slow-growing (32 mm) clinical strain with well-differentiated, flask-shaped phialides. *Phialophora expanda* (E), with slow or fast-growing colonies (15–44 mm), has more slender phialides and very dark collarettes which have a huge expansion when young. *Phialophora macrospora* (G) has expanding, woolly colonies; phialides are slender, nearly cylindrical, with ellipsoidal conidia. *Phialophora ellipsoidea* (F), known from two clinical cases grows moderately rapidly (22–45 mm), has flask-shaped phialides but with short collarettes. *Phialophora americana* (C) is an environmental species with moderate growth (26–37 mm), differentiated conidiogenous cells with dark, funnel- to vase-shaped collarettes and broadly ellipsoidal conidia. Several species need further study when additional material becomes available.

Acknowledgements We express our gratitude to all colleagues who provided us with fungal strains. This work was supported by the grants from the National Natural Science Foundation and the Ministry of Science and Technology of China (81520108026 and 2013ZX10004612-002). We declare that we have no relevant conflicts of interest.

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