Four New Keratinophyton Species (Onygenaceae) From Europe

Roman Labuda (roman.labuda@vetmeduni.ac.at)
Veterinarmedizinische Universitat Wien https://orcid.org/0000-0003-0761-178X
Andreas Bernreiter
BOKU: Universitat fur Bodenkultur Wien
Doris Hochenauer
BOKU: Universitat fur Bodenkultur Wien
Christoph Schüller
BOKU: Universitat fur Bodenkultur Wien
Alena Kubatova
Charles University: Univerzita Karlova

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Four new *Keratinophyton* species (Onygenaceae) from Europe

R. Labuda[a,b*], A. Bernreiter[b,c], D. Hochenauer[b] , A. Kubátová[d], C. Schüller[b,c]. M. Wagner[a,b], J. Strauss[b,c]

[a]Department for Farm Animals and Veterinary Public Health, Institute of Food Safety, Food Technology and Veterinary Public Health; Unit of Food Microbiology, University of Veterinary Medicine Vienna, Veterinaerplatz 1, 1210 Vienna, Austria

[b]Research Platform Bioactive Microbial Metabolites (BiMM), Konrad Lorenz Strasse 24, 3430 Tulln a.d. Donau, Austria

[c]Department of Applied Genetics and Cell Biology, Fungal Genetics and Genomics Laboratory, University of Natural Resources and Life Sciences, Vienna (BOKU); Konrad Lorenz Strasse 24, 3430 Tulln a.d. Donau, Austria

[d] Charles University, Faculty of Science, Department of Botany, Culture Collection of Fungi (CCF), Benátská 2, 128 01 Prague 2, Czech Republic

*Corresponding author. Email: roman.labuda@vetmeduni.ac.at

Abstract: Four new *Keratinophyton* species (Ascomycota: Pezizomycotina, Onygenales), *K. gollerae*, *K. lemmensii*, *K. straussii* and *K. wagneri*, isolated from soil samples originating from Europe (Austria, Italy and Slovakia) are described and illustrated. The new taxa are well supported by phylogenetic analysis of the internal transcribed spacer region (ITS) region, the nuclear large subunit (LSU) rDNA, and their phenotype. Within the *Keratinophyton* clade, *K. lemmensii* is clustered with *K. durum, K. hubeiense*, *K. submersum* and *K. siglerae,* while *K. gollerae*, *K. straussii* and *K. wagneri* are resolved in a separate terminal cluster along with *K. minutisporosum*. All four new species can be well distinguished from the other asexual taxa in the genus *Keratinophyton* based on phenotypical characteristics alone. Ten new combinations are proposed for all other *Chrysosporium* asexual morphs which are resolved in the monophyletic *Keratinophyton* clade.

Key words: *Chrysosporium*, keratinophilic fungi, keratinolysis, one fungus = one name concept, new species, new combinations

Introduction

Keratinophyton is a genus of microscopic fungi (Ascomycota, Onygenales, Onygenaceae) comprising teleomorphic (sexual) and anamorphic (asexual) species that live mostly on the remains of hair and feather in soil as saprotrophs (Cano and Guarro 1990; Crous et al. 2016; Sutton et al. 2013; Vidal et al. 2000). Formerly, they were classified in *Aphanoascus* Zukal mainly based on the presence of ascomata (cleistoperidia) composed of a membranous peridium (Cano and Guarro 1990; Cano et al. 2002). In a review employing a phenotypic and phylogenetic approach, Cano *et al.* (2002) accepted 18 *Aphanoascus* species which are all sexual (i.e. teleomorphs - producing ascomata with asci and ascospores). Only recently, the polyphyletic status of *Aphanoascus sensu lato* has been resolved by Sutton *et al.* (2013) who established the genus *Keratinophyton* H.S. Randhawa & R.S. Sandhu encompassing and redisposing six species, namely *K. durum, K. hispanicum, K. multisporum, K. punsolae, K. saturnoideum and K. terreum* (all sexual), with *K. terreum* H.S. Randhawa & R.S. Sandhu taken as a type species. Ascospores of *Keratinophyton* species are characteristic by a conspicuous equatorial rim and pitted wall, while the genus *Aphanoascus* Zukal (*sensu stricto*) comprises species with reticulate ascospores and without a rim (Sutton et al. 2013). Within *Keratinophyton* species, only *K. multiporum* is connected with a *Malbranchea* anamorph, while the remaining sexual species hitherto known have a *Chrysosporium* asexual morph. In addition to the above mentioned sexual (teleomorphic) species, the monophyletic *Keratinophyton* clade currently encompasses also at least eleven species known only in their anamorphic (asexual) state (Cano and Guarro 1994; Crous et al. 2016; Crous et al. 2017; Liang et al. 2009; Oorschot 1980; Vidal et al. 2000; Vidal et al. 2002; Zhang et al. 2016; Zhang et al. 2017). Recently, Crous *et al.* (2017) introduced a new asexual species, namely *Keratinophyton turgidum* Rahul Sharma & Shouche, solely based on the morphology of its chrysosporium-like aleurioconidia. The same authors stated that all asexual species in this monophyletic clade which have a *Chrysosporium* asexual morph require redisposing in the genus *Keratinophyton*.

Regarding ecology and distribution, the presence of this large group of ubiquitous and often keratinolytic species is rather common especially in areas with high animal activity that results in transfer of the keratinous material (fur, hairs, etc.) to the soil (Papini et al. 1998; Vidal et al. 2000). The following reports confirm their world-wide distribution and occurrence

in different habitats usually associated with soil environments, e.g. soil in city parks (Papini et al. 1998; Vidyasagar et al. 2005), flower pots (Singh et al. 2009), sand in children´s sandpits (Labuda et al. 2008), mud (Zaki et al. 2005), poultry farms (Anbu et al. 2004; Cano and Guarro 1990), marshy meadows, salt pans, desert, cultivated or uncultivated soils (Cano and Guarro 1990; Chmel and Vláčilíková 1977; Deshmukh 2004; Deshmukh et al. 2008; Han et al. 2013; Javorekova et al. 2012; Zhang et al. 2016; Zhang et al. 2017) and river sediments (Ulfig et al. 1997; Vidal et al. 2000; Vidal et al. 2002). In general, these fungi are rarely reported as animal pathogens, and in fact, only two species (*C. echinulatum* and *C. pannicola*) have been involved in mycoses (Cabanes et al. 2014; Crous et al. 2016).

During a microbiological survey of environmental samples (soil and compost) in July 2019, interesting *Chrysosporium* asexual morphs were isolated being phenotypically similar to those ones previously isolated from the same samples in August 2015 by one of the authors (RL). The isolates were designated as BiMM-F76, BiMM-F77 (+ RL-07 strain isolated in July 2019), BiMM-F78 (+ RL-05 and RL-06 strains isolated in July 2019) and BiMM-F250. All strains were further characterized in terms of morphology, physiology and molecular phylogeny. Phylogenetically informative sequences were obtained from internal transcribed spacer (ITS) region and the nuclear large subunit (LSU) rDNA. Overall, the resulting data revealed that these isolates represent novel asexual species of the genus *Keratinophyton*, and they are described and illustrated here as *Keratinophyton gollerae*, *Keratinophyton lemmensii*, *Keratinophyton straussii* and *Keratinophyton wagneri* sp. nov.

Materials and Methods

Sample collection and isolation of the fungi

A sample of a garden soil in Vieste (Italy) was collected in July, 2004. A sample of a forest soil in Tatranská Lomnica (The Slovak Republic) was collected in August 2011. A sample of compost from an agricultural base at the Institute of Agrobiotechnology (IFA Tulln, Austria) was collected in August 2015. All three samples were collected from the surface layer (3–5 cm). The samples were dried and stored in plastic bags in a fridge (5-8 °C) till the time of analysis (August 2015 and July 2019). Isolation of the keratinophilic fungi was done as described previously (Javorekova et al. 2012). Each sample was divided into 10 subsamples.

The subsamples (20 g each) were poured into Petri dishes and soaked with antibiotic solution containing 500 ppm cycloheximide and 100 ppm chloramphenicol. Sterile defatted horse hair fragments (10 pieces of ca 2.0 cm per plate) were used as baits. The Petri dishes were then incubated at laboratory temperature (23-25 ± 1 °C), under ambient daylight, for a period of 2–3 months and remoistened with sterile deionized water whenever necessary. The Petri dishes were checked weekly for the presence of fungi, and isolates were cultured on Sabouraud 4% dextrose agar (SDA, VWR) supplemented with 500 ppm cycloheximide and 50 ppm chloramphenicol. Pure cultures were then transferred onto potato dextrose agar (PDA, Fluka). The preliminary identification of the resulting keratinophilic fungi (*Chrysosporium* asexual morphs) was based on their phenotypic characteristics according to van Oorschot (1980) and Vidal *et al.* (2000 and 2002).

Cultivation of a strain, media and morphological analysis

For phenotypic determination, the strains were transferred (three-point inoculation with a needle) on potato dextrose agar (PDA), malt extract agar (MEA, Merck), Sabouraud 4% dextrose agar (SDA) and incubated for 14 days in the dark at 25°C. Christensen´s urea agar (Sigma-Aldrich) was used for additional physiological and biochemical characteristics (25°C, 14 days, in the dark). Corn meal agar (CMA, Oxoid), potato carrot agar (PCA, (Samson et al. 2010)) and Emerson YpSs agar (Atlas, 1946) were used for stimulation of sexual reproduction (20°C, 25°C, and 28°C, up to 3 months, in the dark).

Colony size (in mm), colony structure and characteristics were noted after 14 days (PDA, MEA, SDA, PYE, YpSs, CMA and PCA), however, the cultivation was prolonged up to 3 months in order to observe and record changes in pigmentation of the colonies as well as to determine the onset of sexual reproduction. In order to determine the optimal and minimal/maximal temperatures for growth, PDA, MEA and SDA at 5, 8, 10, 12, 15, 18, 20, 25, 28, 29, 30, 31, 32, 35 and 37 °C were used and measured at 14^{th} day of cultivation. For comparative description of the macroscopic and microscopic characteristics, PDA was used according to Vidal *et al.* (Vidal et al. 2002) and Crous *et al.* (Crous et al. 2016; Crous et al. 2017).

For determination of microscopic traits, PDA was used after 14-18 days. Conidiophore structures and conidia formation were observed *in situ* under low magnification (50 – 100x). Details of conidiophores, conidia (aleurioconidia) and other microscopic structures, such as

width of hyphae, were observed in mounts with Melzer´s reagent and lactic acid with cotton blue and were used also as mounting media for microphotography. The photomicrographs were taken using phase and Nomarski contrast on the Olympus BX51 microscope with Olympus DP72 camera and QuickPHOTO Micro 3.0 software. Photographs of the colonies were taken with a Sony DSC-RX100.

Scanning electron microscopy (SEM) was performed on a JEOL JSM-6380 LV microscope (JEOL Ltd. Tokyo, Japan). Fungal samples were prepared according to a simplified method (Samson et al. 1979). Pieces of colonies (ca. 3x5 mm) growing on PDA were fixed in 6% glutaraldehyde overnight in the refrigerator (ca. 20 h), then dehydrated in 2-methoxyethanol for 10 min. This was followed by drying at a critical point and gold coating in BAL-TEC SCD 050 Sputter Coater. The samples were observed with spot size 35-39 and accelerating voltage 20-23 kV.

Dried herbarium specimens of the holotypes were deposited in the herbarium of the Mycological Department, National Museum in Prague, Czech Republic (PRM); the ex-type cultures were deposited in the Bioactive Microbial Metabolites (BiMM) Fungal Collection, UFT- Tulln (AT) and in the Culture Collection of Fungi (CCF), Prague (CZ).

Keratinolytic activity

The keratinolytic activity (Oorschot 1980) was tested by placing a few sterilized blond hairs of a five years old child on the PDA plate 1 cm away from the point of inoculation. Ability to digest keratin was observed after 21 days after incubation at 25 °C, in darkness. In addition, a hair perforation test according to de Hoog *et al.,* (2000) using 25 mL water containing 2-3 drops 10% yeast extract (YEW) was used as well (De Hoog et al. 2000). The children hairs were examined microscopically after 14 and 21 days after inoculation at 25 °C, in darkness. By the end of the cultivation a few pieces of hairs were taken out from the testing media (PDA and YEW). Overgrown fungus was deactivated with 70% ethanol and consequently discarded from the hair surface mechanically in the stream of a tap water. A degree of hair digestion-degradation (keratinolytic activity) was observed in a light microscope under 100x and 400x magnification. For the observation and microphotography of the hairs water was used as mounting fluid. Intensity of attack on the hair was estimated on a scale of 0 to 4 (Marchisio et

al. 1994): 0, no attack; 0-1, light attack on the cuticle; 1, moderate attack on the cuticle and/or rare formation of boring hyphae; 2, attack on cuticle and cortex, with about 20% destruction of the hair; 3, attack on cuticle and cortex, with about 50% destruction of the hair; 4, attack on cuticle and cortex, with about 80 % destruction of the hair. The photomicrographs of the hairs were taken using a Motic BA 310 microscope with Motic Image Plus 3.0 software. Final microscopic pictures were black and white inverted.

DNA extraction, PCR amplification and sequencing

DNA was extracted using a standard cetyltrimethyl ammonium bromide (CTAB) procedure, as described previously (Doyle and Doyle 1987). The internal transcribed spacer (ITS) region with primers ITS1-F (Gardes and Bruns 1993) and ITS4 (White et al. 1990) was amplified with Taq-polymerase. The D1/D2 domains of the large-subunit (28S) rRNA gene (LSU) were amplified and sequenced using the primer pair ITS1/TW14 (White et al., 1990; Mori et al., 2000). All reactions were performed in an Eppendorf Gradient *MasterCycler* (Eppendorf, Hamburg). Conditions for amplification of ITS and LSU domains: 95 °C for 5 min; 35 cycles of 95 °C for 30 s, 54 °C for 30 s, 72 °C for 90 s and finally 5 min at 72 °C.

The PCR products were sequenced with the same primers used for the PCR amplifications (*Microsynth* AG, Balgach, Switzerland). All sequences obtained in this study were deposited in GenBank. For information on fungal strains used in this study see Table 1. This table provides GenBank accession numbers to ITS sequences for all accepted species in the genus *Keratinophyton* (except *K. multiporum*) and for selected ones in the genus *Aphanoascus*. The LSU sequences were provided to all new species and to majority of *Keratinophyton* and *Aphanoascus* species used for phylogeny.

Phylogenetic analysis

For phylogenetic analysis, sequences were aligned with ClustalX (Larkin et al. 2007). Phylogenetic analysis was done with SEAVIEW 4.6 (Gouy et al. 2010) software. The phylogenetic tree was constructed using Maximum Likelihood method (ML) method in SEAVIEW and genetic distances were computed with the Kimura-2-parameter (K2P) model.

Bootstrap analyses were performed in ML with 1000 bootstrap replicates. For ITS, *Chrysosporium merdarium* ex-neotype strain CBS 408.72 and *Uncinocarpus queenslandicus* ex-type strain IMI 121675 were selected as outgroups for phylogenetic evaluation. For LSU, *Ctenomyces serratus* ex-type strain CBS 187.61 was used as an outgroup.

Table 1 List of strains included in the study.

Species name	Strain[a]	Source	GenBank accession	
			ITS	LSU
K. gollerae sp. nov.	**BiMM-F250^{T}**	**forest soil, Slovakia**	**MN633084**	**MT874997**
K. lemmensii sp. nov.	**BiMM-F76^{T}**	**compost soil, Austria**	**MN633082**	**MT874998**
K. straussii sp. nov.	**BiMM-F78^{T}**	**garden soil, Italy**	**MN633081**	**MT874996**
K. wagneri sp. nov.	**BiMM-F77^{T}**	**forest soil, Slovakia**	**MN633083**	**MT874999**
A. canadensis	UAMH 4574^{T}	carnivore dung, Canada	AJ439435	
A. clathratus	IMI 329400^{T}	arable soil, Spain	AJ439436	
A. cubensis	FMR 4220^{T}	soil of tabacco field, Cuba	AJ439432	
A. foetidus	CBS 452.75	*Myomys daltoni* coat, unknown	AJ439448	
A. fulvescens	UAMH 5117	toe cleft, New Zealand	AF038357	JN941548
A. keratinophilus	IFM 55159^{T}	soil, Papua New Guinea	AB361655	AB361655
A. mephitalis	IMI 151084^{T}	dung of wolf, Canada	AJ439439	AY176725
A. orissi	CBS 340.89	soil in animal husbandry, Kuwait	AJ390393	
A. pinarensis	FMR 4221^{T}	forest soil, Cuba	AJ439433	
A. reticulisporus	NBRC 32373^{T}	soil, Spain	JN943435	JN941550
A. verrucosus	NBRC 32382^{T}	Soil, Spain	JN943439	JN941554
C. keratinophilum	IFO 7584^{T}	soil, New Guinea	AJ131681	AB361655
C. lucknowense	IMI 112798^{T}	soil, India	AJ131682	
C. merdarium	CBS 408.72^{N}	dung, USA	AJ390384	
C. tropicum	UAMH 691^{T}	Woolen cloth, Guadalcanal	AJ131685	MH869718
K. clavisporum (= *C. clavisporum*)	G80.1^{T}	soil, China	KY026601	
K. echinulatum (= *C. echinulatum*)	CCF 4652^{T}	sole of the foot, Czechia	LT548276	LT548276
K. pannicola (= *C. evolceanui*)	CBS 116.63^{T}	soil, India	AJ005368	MH869834
K. fluviale (= *C. fluviale*)	FMR 6005^{T}	river sediments, Spain	AJ005367	**MT875000**
K. hubeiense (= *C. hubeiense*)	EM66601^{T}	soil, China	KJ849227	
K. linfenense (= *C. linfenense*)	GZUIFR-H31^{T}	soil, China	FJ392561	
K. minutisporosum (= *C. minutisporosum*)	IMI 379912^{T}	river sediments, Spain	AJ131689	**MT875001**
K. qinghaiense (= *C. qinghaiense*)	GZUIFR-Chry-11^{T}	soil, China	JX868607	
K. siglerae (= *C. siglerae*)	UAMH 6541^{T}	Garden soil, Spain	AJ131684	**MT875002**
K. submersum (= *C. submersum*)	IMI 379911^{T}	river sediments, Spain	AJ131686	
K. durum	FMR 5651	unknown, Australia	AJ439434	AB075345
K. hispanicum	CBS 456.90^{T}	beach soil, Spain	KT155910	**MT875003**
K. punsolae	IMI 334818^{T}	soil, Spain	AJ439440	
K. saturnoideum	CBS 628.88^{T}	soil, Spain	NR_077135	AB075347
K. terreum	NBRC 32655^{T}	soil, India	JN943438	JN941552
K. turgidum	CBS 142596^{T}	barber shop soil, India	KY290503	KY962732
C. serratus	CBS 187.61^{T}	Soil, Australia		AY 176733
U. queenslandicus	IMI 121675^{T}	feather of domestic fowl, Australia	AJ390394	

[a] BiMM, Bioactive Microbial Metabolites Unit, UFT-Tulln, Austria; UAMH, University of Alberta Microfungus Collection and Herbarium; IMI, The International Mycological Institute in Kew, England; FMR, Facultat de Medicina in Ciències de la Salut, Reus, Spain; CBS, Westerdijk Fungal Biodiversity Centre, Utrecht, the Netherlands; NBRC, Culture Collection, Japan; IFO, Institute for Fermentation, Osaka, Japan; G, EM, and GZUIFR strains, The Institute of Fungus Resource, Guizhou University, China; A= *Aphanoascus*, K= *Keratinophyton*, C= *Chrysosporium*, U= *Uncinocarpus*, ^{T} ex-type, ^{N} ex-neotype. Newly obtained data are in bold.

Results

Phylogenetical analysis

Based on a search of NCBI's GenBank nucleotide database, the closest hit for *K. lemmensii* using the ITS sequence is *K. durum* (FMR5651; Genbank: AJ439434; Identities = 568/577 (98%), gaps 0/577 (0%)). The closest hits for *K. straussii*, *K. gollera*e and *K. wagneri* using the ITS sequence is *K. minutisporosum* (as *Chrysosporium minutisporosum* CBS 101577; Genbank: KT155616); with identities = 489/543 (90%), gaps 10/543 (1%); 487/543 (90%), gaps 11/543 (2%), and 486/541(90%), gaps 11/541(2%), respectively. The ITS similarity within the new species is as follow: *K. lemmensii/K. straussii* (86% and 4/515 (<1%) gaps), *K. lemmensii/K. gollerae* (86,63% and 5/515 (<1%) gaps, *K. lemmensii/K. wagneri* (87,45 and 5/514 (<1%) gaps; *K. strausii/K. gollerae* (98,26%) and 1/512 gaps, *K. straussii/ K. wagneri* (97,85% and 1/512 (<1%) gaps, and *K. gollerae/K.wagneri* (98,04%) and 2/512 (<1%) gaps.

The phylogenetic tree built by ITS sequence datasets (Fig. 1a) indicates the presence of seven terminal clusters in the monophyletic *Keratinophyton* clade with high bootstrap support and low interspecific sequence divergence. The isolate BiMM-F76 represents a new species being closest to *K. durum*, named here as *K. lemmensii sp. nov*. It clusters also with *K. hubeiense* and *K. submersum*. In addition, *K. straussii sp. nov*., *K. gollerae sp. nov*., and *K. wagneri sp. nov*., represented by the ex-type strains BiMM-F78, BiMM-F250 and BiMM-F77, respectively, are resolved in a separate terminal cluster-lineage. Similar topology is evident also based on LSU phylogeny (Fig. 1b), where the three new species (*K. straussii, K. gollerae* and *K. wagneri*) form a separate lineage and *K. lemmensii* clusters with *K. submersum*. The description of all four new species is based on phenotypic features of their *Chrysosporium* asexual morphs.

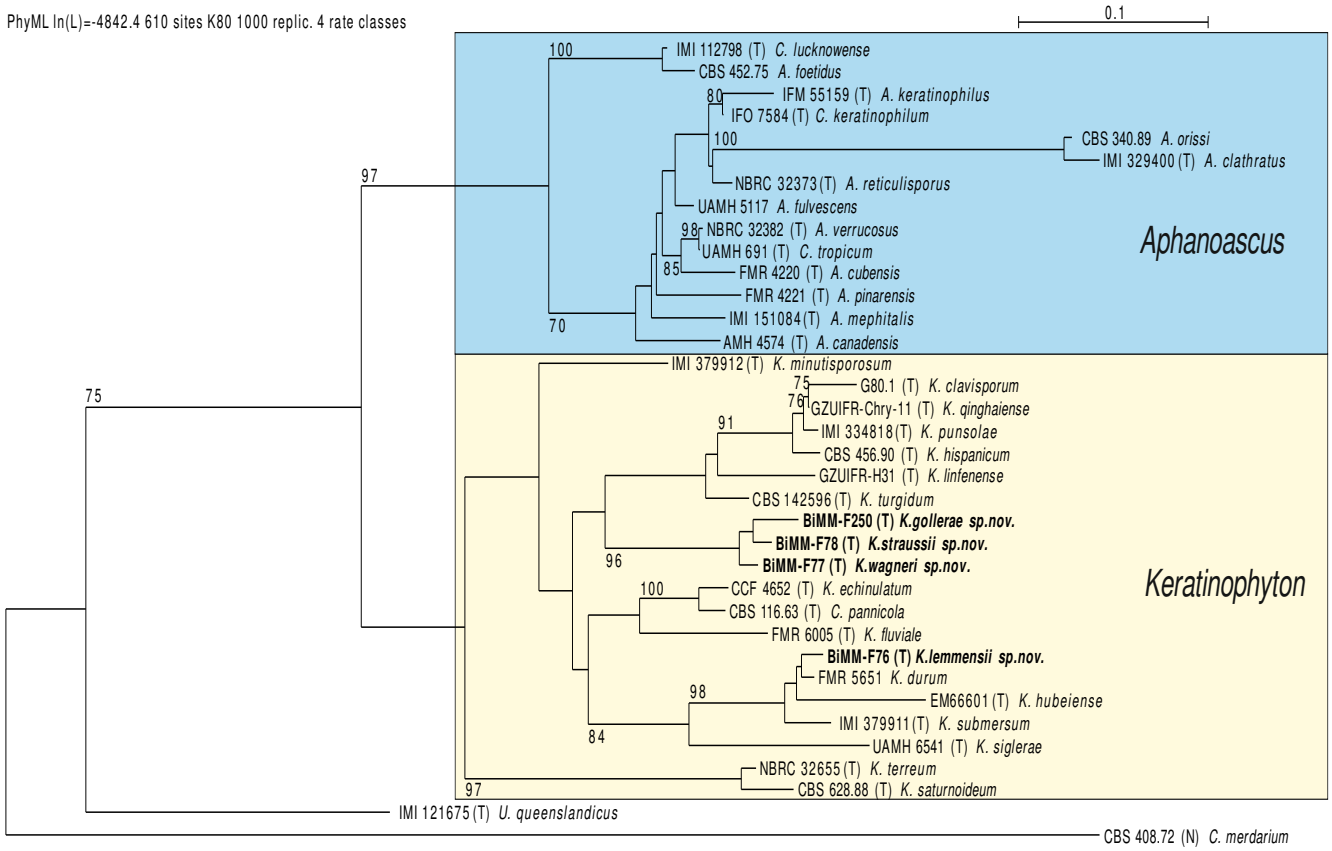

Fig. 1a Maximum Likelihood (ML) tree based on ITS sequence for the new taxa of *Keratinophyton* is compared with available sequences of the other related species. Numbers at nodes indicate bootstrap values (expressed as percentages of 1000 replications). *Uncinocarpus queensladicus* IMI 121675^{T} and *Chrysosporium merdarium* CBS 408.72^{N} were used as outgroups. A sequence for *K. multiporum* was not available for the study. Scale bar indicates 0.01 substitutions per nucleotide position. *A= Aphanoascus, K= Keratinophyton*, *C= Chrysosporium*, *U= Uncinocarpus.* (T) ex-type, (N) ex-neotype strain. New species are in Bold.

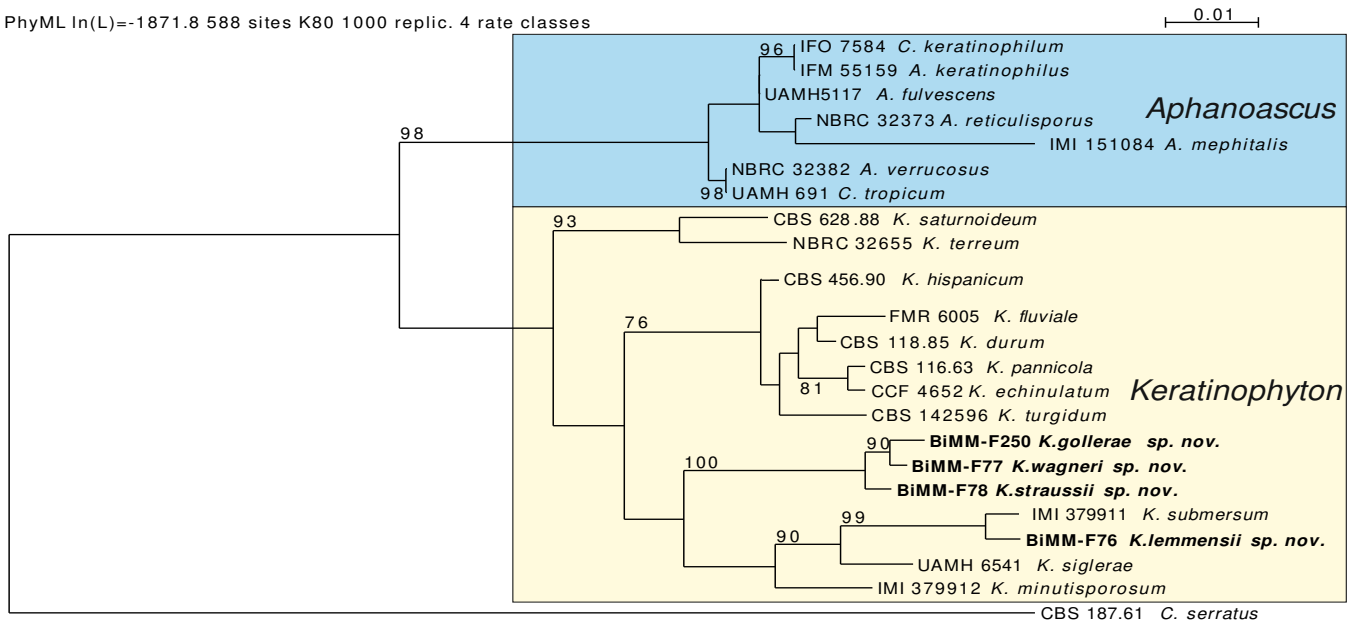

Fig. 1b. Maximum Likelihood (ML) tree based on LSU rDNA sequences for new taxa of *Keratinophyton* is compared with available sequences of the other related species (ex-type strains). Numbers at nodes indicate bootstrap values (expressed as percentages of 1000 replications). Scale bar indicates 0.01 substitutions per nucleotide position. *Ctenomyces serratus* CBS 187.61 was used as outgroup. New species are in Bold.

Taxonomy

Keratinophyton lemmensii Labuda, Bernreiter, Kubátová & C. Schüller **sp. nov.** - Figs. 2-3

MycoBank MB833632

Etymology: Latin, *lemmensii* = in honour of Professor Marc Lemmens, Laboratory of Plant Protection (IFA, BOKU, AT), an expert in the fungal plant pathology.

Culture characteristics (Fig. 2a)- Colony on potato dextrose agar (PDA) attaining 28-35 mm in diam. at 25 °C, in 14 days, floccose, with good sporulation, white, flat, slightly elevated (umbonate) at the center, with irregular margin, reverse lemon yellow, soluble pigment bright yellow, a few small clear to yellow-orange exudate droplets produced. At 30 °C, colonies attaining 38-45 mm in 14 days, white, flat, floccose and radially sulcate with good sporulation only at the center, and with lemon yellow reverse. Colony on Sabouraud dextrose agar (SDA) attaining 28-35 mm in diam. at 25 °C, in 14 days, morphology similar to PDA, without exudate and with pale yellow reverse. At 30 °C, colonies attaining 30-32 mm in 14 days, white, flat, floccose with good sporulation, with pale yellow reverse. Colonies on malt extract agar (MEA) attaining 20-25 mm in diam. at 25 °C in 14 days, morphology similar to PDA, exudate absent, and pale yellow reverse. At 30 °C, colonies attaining 18-20 mm in 14 days, white, floccose and radially sulcate, with good sporulation, and with pale yellow reverse. Colonies on CMA and PCA attaining 45-50 mm in diam. at 25 °C, in 21 days, white, flat and spread with poor sporulation, reverse white. No ascomata observed after prolonged incubation (3months).

The optimum temperature for growth on PDA, SDA and MEA was between 25-30 °C (Table S2a-c). Minimum growth (1-2 mm in diam.) was observed at 8°C, and the maximum temperature for growth was 32 °C (microcolonies to 1 mm in diam.). Keratinolytic activity very weak (Fig 10c), with hair attack intensity = 0-1. Positive urease activity (after 3 days of incubation).

Micromorphology (Fig. 2b-d, 3) - *Hyphae* hyaline, septate, smooth-walled, 1.5-5.0 µm wide, sparsely branched. *Racquet hyphae* present. *Conidia*, hyaline, white in mass, thin-walled, smooth to sparsely irregularly ornamented with minute warts. Terminal and lateral conidia (aleurioconidia) born on main fertile hyphae as sessile or on short protrusions, solitary, 1-3 (5)

per conidiogenous cell, obovate to clavate, 1-celled, (3.0-)4.5-6.5(-7.5) x (1.5-)2.0-2.5(-4.0) µm (mean = 4.9±0.8 x 2.4±0.4 µm, n= 120), and filiform, often sinusoidal, 25-35(-40) µm long conidia also present. Intercalary conidia (arthroconidia) present, 10-15 µm long. *Chlamydospores* absent. *Sexual morph* not observed on any of the media used.

Holotype: **Austria**, Tulln and der Donau, isolated from a compost soil at IFA Tulln, August 2015, isolated by Roman Labuda; PRM 952498 [Holotype, dried culture].

Ex-type strain: BiMM-F76 = CCF 6359.

DNA sequences: GenBank MN633082 (ITS) and MT874998 (LSU).

Distinguishing characteristics. Presence of filiform often sinusoidal conidia and abundant arthroconidia, production of bright yellow pigment on PDA and good growth at 30°C.

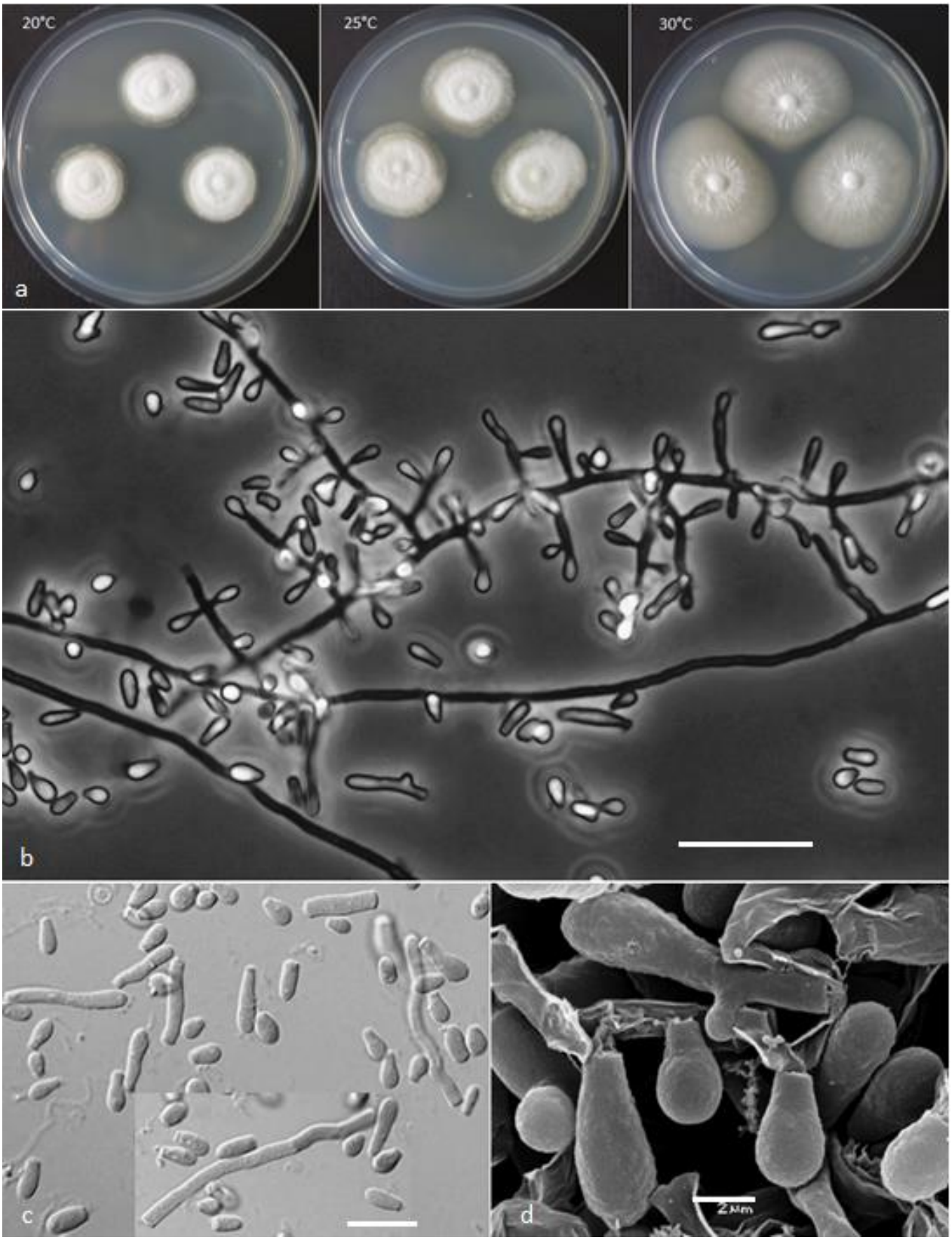

Fig. 2 *Keratinophyton lemmensii* (BiMM-F76). (a) Colonies on PDA (14 days old) at 20, 25 and 30 °C; (b) conidiophores with aleurioconidia; (c) aleurioconidia and arthroconidia (on PDA, 14 days old); (d) Scanning electron microscopy (SEM) of aleurioconidia (on PDA, 14 days old). Scale bars = 20 µm (b), 10 µm (c), 2 µm (d).

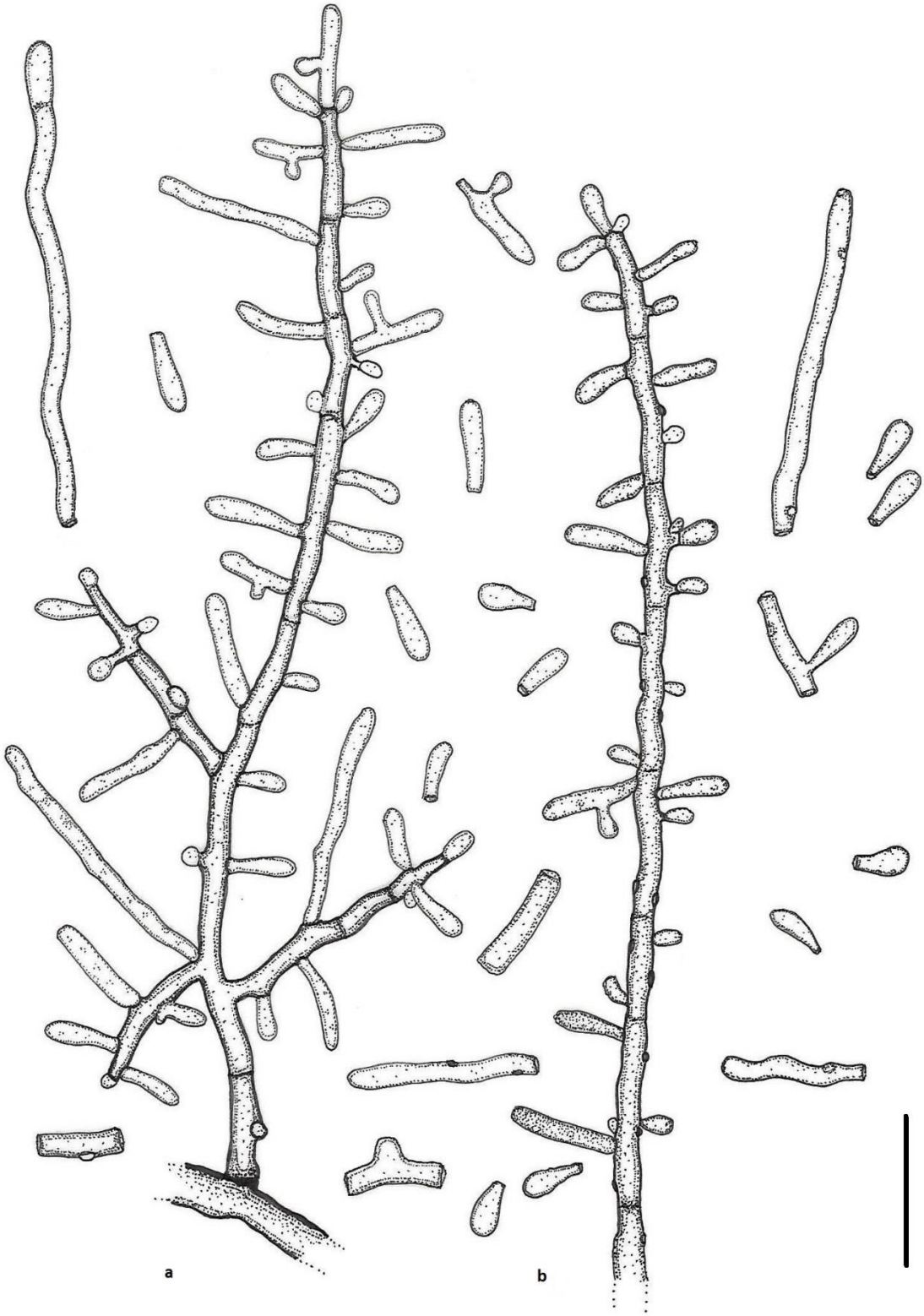

Fig. 3 Line drawing of micromorphology of *Keratinophyton lemmensii* (BiMM-F76). (a, b) conidiophores with young and mature aleurioconidia, including arthroconidia on PDA (14 days). (a) branched conidiophore. (b) unbranched conidiophore with sessile aleurioconidia. Scale bar = 10 µm (a-b).

Keratinophyton gollerae Labuda, Bernreiter, Kubátová, C. Schüller & J. Strauss **sp. nov.**- Figs. 4 -5

MycoBank MB 833633

Etymology: Latin, *gollerae* = in honour of Dr. Sabine Strauss-Goller, the Department of Applied Genetics and Cell Biology, Fungal Genetics and Genomics Laboratory (BOKU, AT), an expert in the fungal genetics and indoor mold analytics.

Culture characteristics (Fig. 4a)- Colonies on potato dextrose agar (PDA) attaining 20-22 mm in diam. at 25 °C, in 14 days, powdery to downy (mealy), with abundant sporulation, white to creamy, flat, umbonate at the center, with regular colony margin submersed into agar, reverse white to slightly yellowish, no pigment or exudate produced. At 30 °C, no growth (spore germination only). Colony on Sabouraud dextrose agar (SDA) attaining 23-25 mm in diam. at 25 °C, in 14 days, morphology similar to PDA with more floccose colony margin and more yellowish colonies, with dark yellow reverse. At 30 °C, no growth (no spore germination). Colonies on malt extract agar (MEA) attaining 14-16 mm in diam. at 25°C in 14 days, morphology similar to PDA with more floccose colonies and with yellow reverse. At 30 °C, no growth (no spore germination). Colonies on CMA and PCA attaining 15-20 mm in diam at 25 °C, in 21 days, white, granular, with good sporulation, reverse yellowish. No ascomata observed after prolonged incubation (3 months).

The optimum temperature for growth on PDA, SDA and MEA was between 15 and 25 °C (Table S2a-c). Minimum growth (microcolonies to 1 mm in diam.) was observed at 10°C. Germination of the spores was observed at 8 °C. The maximum temperature for growth on PDA was 29 °C, while 27 °C and 28 °C on MEA and SDA, respectively (microcolonies to 1 mm in diam.). Keratinolytic activity very strong (Fig 10b), with hair attack intensity = 4. Negative urease activity (after 14 days of incubation).

Micromorphology (Fig. 4b-e, 5) - *Hyphae* hyaline, septate, smooth-walled, 1.0-5.0 µm wide, sparsely to pronouncedly branched, often in right angles. *Racquet hyphae* present. *Conidia* (aleurioconidia), hyaline, white in mass, thin-walled, mostly smooth to finely roughened, some also verrucose (under light microscope) and irregularly ornamented with minute warts (visible under SEM). Terminal and lateral conidia born on main fertile hyphae or from side branches of variable length, sessile or on short protrusions, occasionally only very slightly swollen and

of variable length, solitary, 1-3 (5) per conidiogenous cell, obovate to clavate, mostly 1-celled, (3.5-)5.0-7.0(-10.0) x (1.5-)2.0-2.5(-3.0) µm (mean = 5.2±0.9 x 2.2±0.2 µm, n = 120). Intercalary conidia absent. *Chlamydospores* absent. *Sexual morph* not observed on any of the media used.

Holotype: **The Slovak Republic**, Tatranská Lomnica, isolated from a forest soil, July 2019, isolated by Roman Labuda; PRM 952499 [Holotype, dried culture].

Ex-type strain: BiMM-F250 = CCF 6360.

DNA sequences: GenBank MN633084 (ITS) and MT874997 (LSU).

Distinguishing characteristics. Production of slender and mostly smooth-walled conidia, no growth at 30°C, and strong keratinolytic ability.

Fig. 4 *Keratinophyton gollerae* (BiMM-F250). (a) Colonies on PDA (14 days old) at 20, 25 and 30 °C; (b) conidiophores with aleurioconidia; (c) aleurioconidia and arthroconidia (on PDA, 14 days old); (d) Scanning electron microscopy (SEM) of aleurioconidia (on PDA, 14 days old). Scale bars = 20 µm (b), 10 µm (c), 2 µm (d).

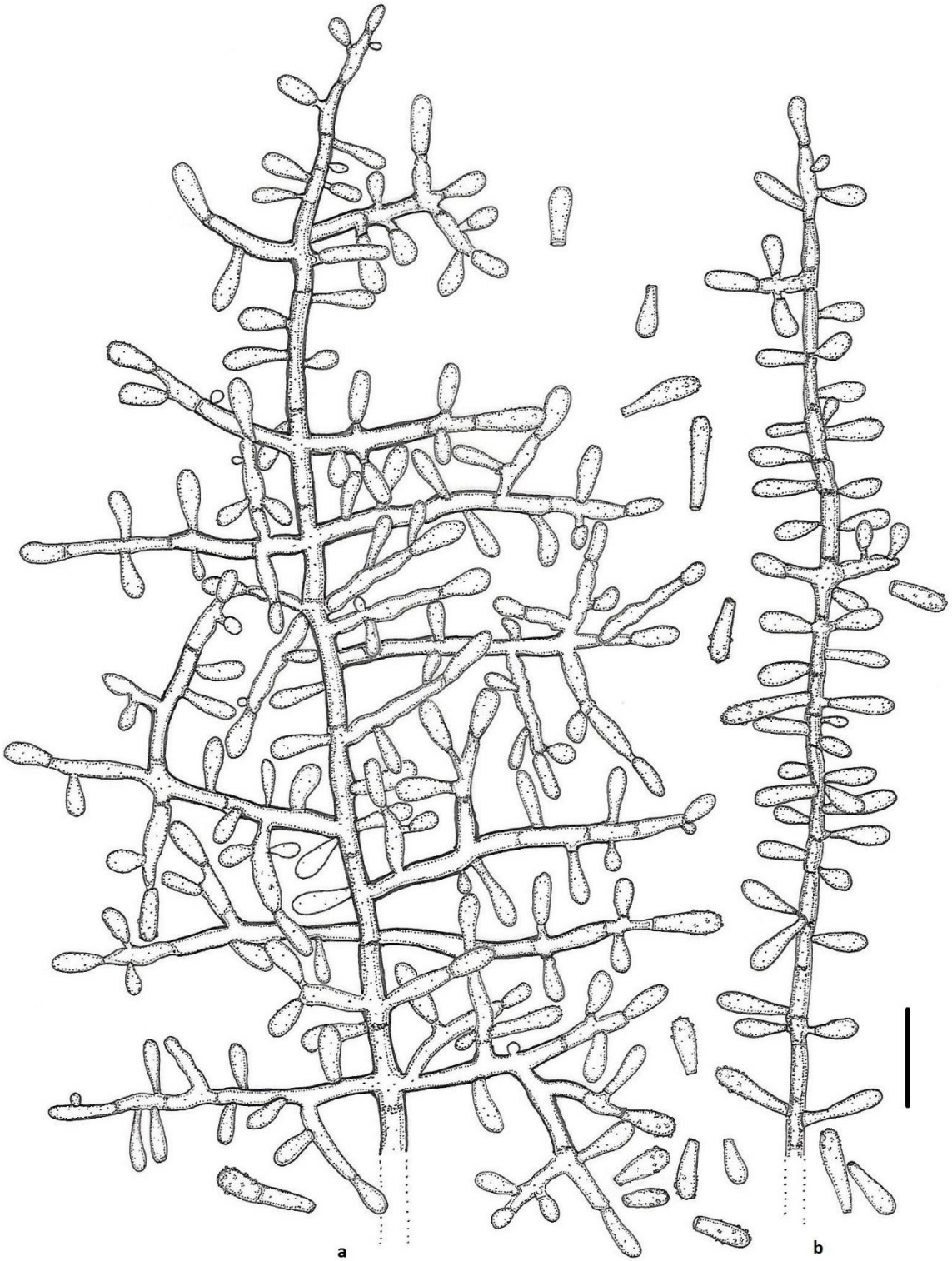

Fig. 5 Line drawing of micromorphology of *Keratinophyton gollerae* (BiMM-F250). (a, b) conidiophores with young and mature aleurioconidia on PDA (14 days old). (a) branched conidiophore. (b) unbranched conidiophore with sessile aleurioconidia. Scale bar = 10 µm (a-b).

Keratinophyton straussii Labuda, Bernreiter, Kubátová & C. Schüller **sp. nov.** - Figs 6- 7

MycoBank MB 833634

Etymology: Latin, *straussii* = in honour of Professor Joseph Strauss, a head of the Department of Applied Genetics and Cell Biology, founder of the Fungal Genetics and Genomics Laboratory (BOKU, AT), an expert in the fungal genetics, epigenetics and functional genomics.

Culture characteristics (Fig. 6a)- Colony on potato dextrose agar (PDA) attaining 24-28 mm in diam. at 25 °C, in 14 days, powdery to downy (mealy), with abundant sporulation, white to very slightly creamy yellowish, flat, slightly elevated (umbonate) remaining powdery at the center, with irregular margin, reverse white with slightly yellowish center, no pigment or exudate produced. At 30 °C, colonies attaining 15-20 mm in 14 days, white to creamy yellowish, flat, powdery to downy (mealy) with very good sporulation, and with white to yellowish reverse. Colony on Sabouraud dextrose agar (SDA) attaining 16-20 mm in diam. at 25 °C, in 14 days, morphology similar to PDA with dark yellow reverse. In age (after 5 weeks) yellow pigment is produced and colony reverse becomes bright reddish-yellow to orange. At 30 °C, colonies attaining 15-20 mm in 14 days, white to creamy yellowish, umbonate, with strong sporulation, and with yellowish reverse. Colonies on malt extract agar (MEA) attaining 18-20 mm in diam. at 25 °C in 14 days, morphology similar to PDA with more floccose and yellowish colonies. At 30°C, colonies attaining 5-10 mm in 14 days, slightly umbonate, floccose to granular, with very good sporulation white to yellowish, and with yellow reverse. Colonies on CMA and PCA attaining 18-20 mm in diam. at 25 °C, in 21 days, white, granular, good sporulation, reverse yellowish. No ascomata observed after prolonged incubation (3months).

The optimum temperature for growth on PDA, SDA and MEA was between 20 and 25 °C (Table S2a-c). Minimum growth (microcolonies to 1-2 mm in diam.) was observed at 10 °C. No germination of the spores was observed at 8 °C. The maximum temperature for growth was 32 °C (microcolonies to 1-2 mm in diam.). Keratinolytic activity very strong (Fig 10c), with hair attack intensity = 4. Negative urease activity (after 14 days of incubation).

Micromorphology (Fig. 6b-e, 7) - *Hyphae* hyaline, septate, smooth-walled, 1.5-4.0 µm wide, sparsely to pronouncedly branched, usually in right angles. *Racquet hyphae* present. *Conidia* (aleurioconidia), hyaline, white to yellowish in mass, thin-walled and regularly ornamented with minute warts (visible under SEM) and coarsely roughened (under light microscope). Terminal and lateral conidia born on main fertile hyphae or from side branches of variable length, sessile or on short protrusions, commonly slightly swollen and of variable length, solitary, 1-3 (5) per conidiogenous cell, obovate to clavate, 1-celled, (3.5-)4.5-5.0(-6.5) x (2.0-)2.5-3.0(-3.5) µm (mean = 4.9±0.4 x 2.6±0.2 µm, n = 120), very rarely 2- to 3-celled, up to 12 µm large aleurioconida also present. Intercalary conidia absent. *Chlamydospores* absent. *Sexual morph* not observed on any of the media used.

Holotype: **Italy**, Vieste, isolated from a garden soil, August 2015, isolated by Roman Labuda; PRM 952500 [Holotype, dried culture].

Ex-type strain: BiMM-F78 = CCF 6361.

DNA sequences: GenBank MN633081 (ITS) and MT874996 (LSU).

Additional strains: RL-05 (GenBank ITS: MT898644, LSU: MT898648) and RL-06 (GenBank ITS: MT898645, LSU: MT898649) referring to personal collection of RL, isolated from different sub-samples in July 2019. At 30 °C they grew somewhat better (up to 5 mm larger in diam.) than the ex-type strain.

Distinguishing characteristics. Coarsely roughened conidia produced from swollen conidiogenous cells, good growth at 30°C, and strong keratinolytic ability.

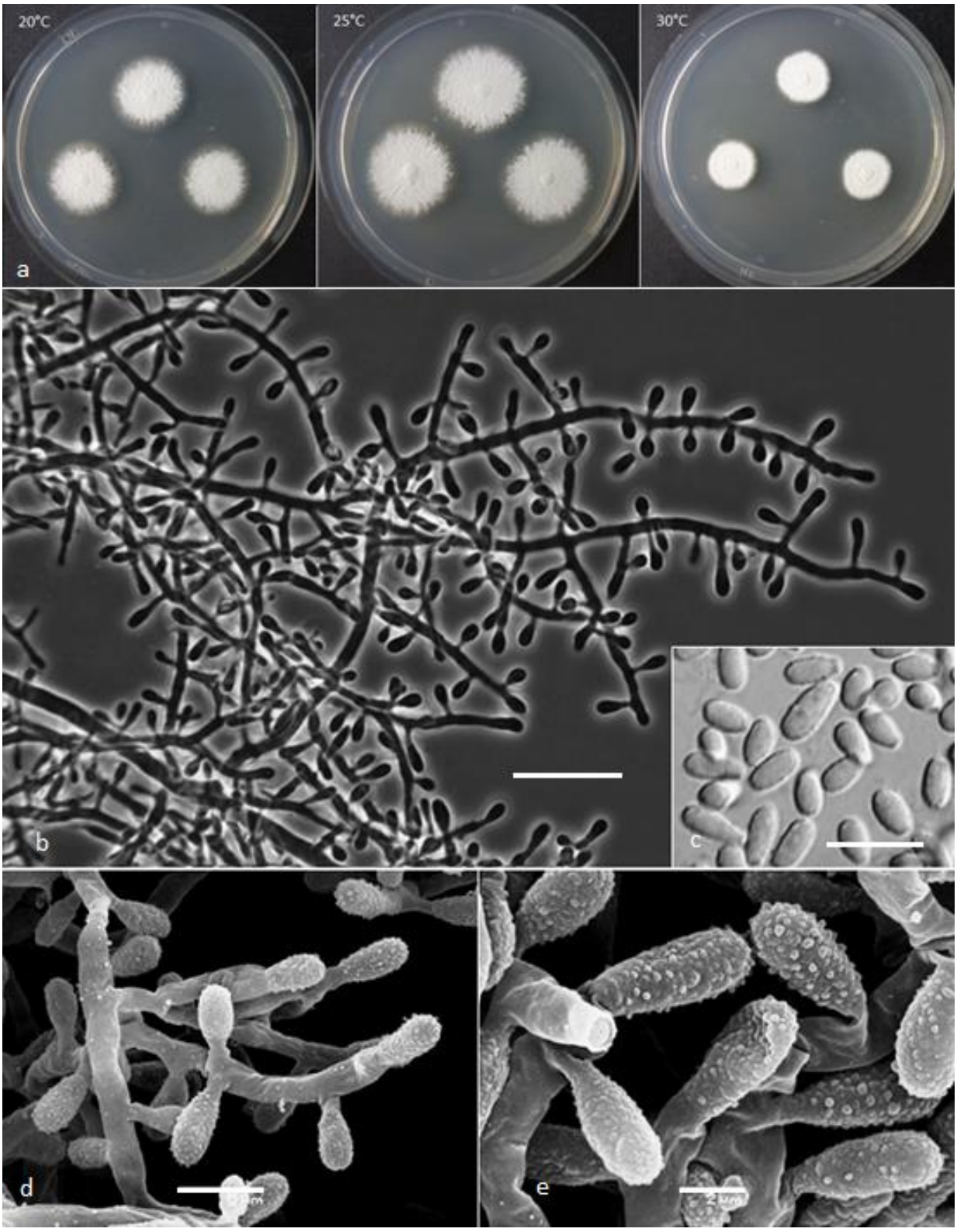

Fig. 6 *Keratinophyton straussii* BiMM-F78. (a) Colonies on PDA (14 days old) at 20, 25 and 30 °C; (b) conidiophores with aleurioconidia; (c) aleurioconidia (on PDA, 14 days old); (d-e) Scanning electron microscopy (SEM) of conidiogenous cells and aleurioconidia (on PDA, 14 days old). Scale bars = 20 µm (b), 10 µm (c), 5 µm (d), 2 µm (e).

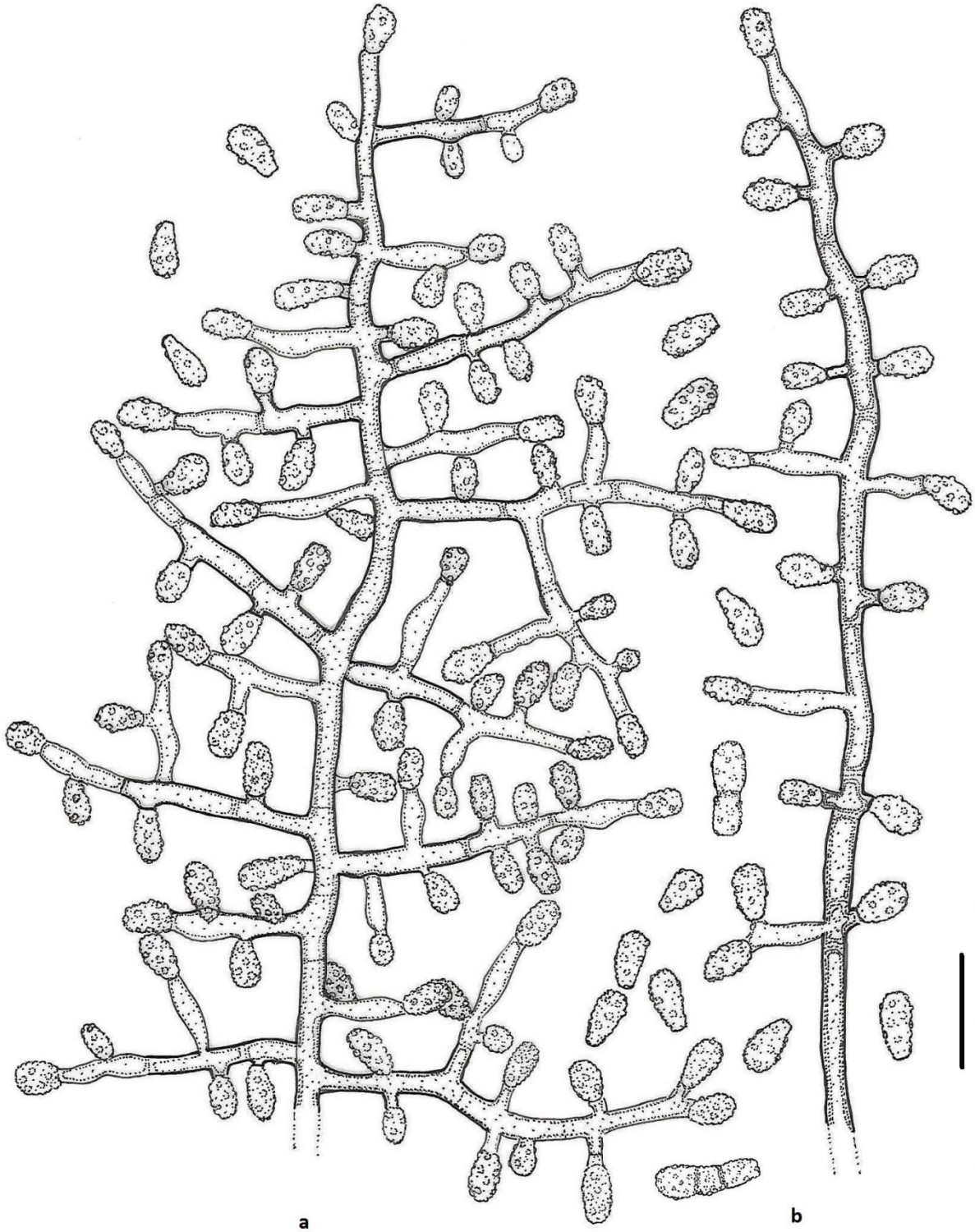

Fig. 7 Line drawing of micromorphology of *Keratinophyton straussii* (BiMM-F78). (a, b) conidiophores with young and mature aleurioconidia on PDA (14 days old). (a) branched conidiophore. (b) unbranched conidiophore with sessile aleurioconidia. Scale bar = 10 µm (a-b).

Keratinophyton wagneri Labuda, A. Bernreiter, Kubátová & C. Schüller **sp. nov.** – Figs. 8-9

MycoBank MB 833635

Etymology: Latin, *wagneri* = in honour of Professor Martin Wagner, Head of Unit for Food Microbiology and Head of Institute for Food Safety, Food Technology and Veterinary Public Health, University of Veterinary Medicine, Vienna (Austria), an expert in the veterinary microbiology.

Culture characteristics (Fig. 8a)- Colony on potato dextrose agar (PDA) attaining 25-30 mm in diam. at 25 °C, in 14 days, powdery to downy (mealy), with abundant sporulation, white to slightly yellowish, flat, slightly elevated (umbonate) and more floccose at the center, with irregular margin, reverse white with slightly yellowish center, no pigment or exudate produced. At 30 °C, colonies attaining 4-8 mm in 14 days, white, floccose with poor sporulation, and with yellowish reverse. Colonies on Sabouraud dextrose agar (SDA) attaining 14-18 mm in diam. at 25 °C, in 14 days, morphology similar to PDA. In age, yellowish-brown (amber) pigment is produced and colony reverse becomes dark reddish-brown (after 4 weeks). At 30 °C, no growth or only microcolonies observed. Colonies on malt extract agar (MEA) attaining 18-22 mm in diam. at 25 °C in 14 days, morphology similar to PDA with more yellowish colonies. At 30 °C, no growth or only micro-colonies observed. Colonies on CMA and PCA attaining 20-25 mm in diam. at 25 °C, in 21 days, white to yellowish, granular, good sporulation, reverse yellowish. Pinkish pigment production observed after 3-4 weeks on PCA (in both strains tested). No ascomata observed after prolonged incubation (3months).

The optimum temperature for growth on PDA, SDA and MEA was between 20 and 25 °C (Table S2a-c). Minimum growth (1-2 mm in diam.) was observed at 10 °C, and germination of majority of the spores was observed at 8 °C. The maximum temperature for growth was 31 °C (1-3 mm in diam.). Keratinolytic activity weak to moderate (Fig 10d), with hair attack intensity = 2. Negative urease activity (after 14 days of incubation).

Micromorphology (Fig. 8b-e, 9) - *Hyphae* hyaline, septate, smooth-walled, 2.0-6.0 µm wide, sparsely to pronouncedly branched. *Racquet hyphae* present. *Conidia* (aleurioconidia), hyaline, white to yellowish in mass, thin-walled and regularly ornamented with minute warts with minute warts (visible under SEM) and coarsely roughened (under light microscope).

Terminal and lateral conidia born on main fertile hyphae or from side branches of variable length, sessile or on short protrusions, occasionally swollen and of variable length, solitary, 1-4 (10) per conidiogenous cell, obovate to clavate, single celled, (4.0-) 5.5-6.5 (-8.0) x (2.5-) 3.0-3.5(-4.0) µm (mean = 5.7±0.4 x 3.2±0.2 µm, n= 120), rarely 2-celled, up to 12 µm large aleurioconida also present. Intercalary conidia absent. *Chlamydospores* absent. *Sexual morph* not observed on any of the media used.

Holotype: **The Slovak Republic**, Tatranská Lomnica, isolated from a forest soil, August 2015, isolated by Roman Labuda; PRM 952501 [Holotype, dried culture].

Ex-type strain: BiMM-F77 = CCF 6362.

DNA sequences: GenBank MN633083 (ITS) and MT874999 (LSU).

Additional strain: RL-07 (GenBank ITS: MT903275, LSU: MT903309) referring to personal collection of RL, isolated from a different sub-sample in July 2019, phenotypically identical with the ex-type strain.

Distinguishing characteristics. Robust and coarsely roughed conidia produced from non-swollen conidiogenous cells, none to very limited growth at 30°C, and production of pink pigment on PCA after prolonged incubation (3 weeks).

The main distinguishing phenotypic characteristics of the four new species compared with the other asexual members of the genus *Keratinophyton* are listed in the Table 2.

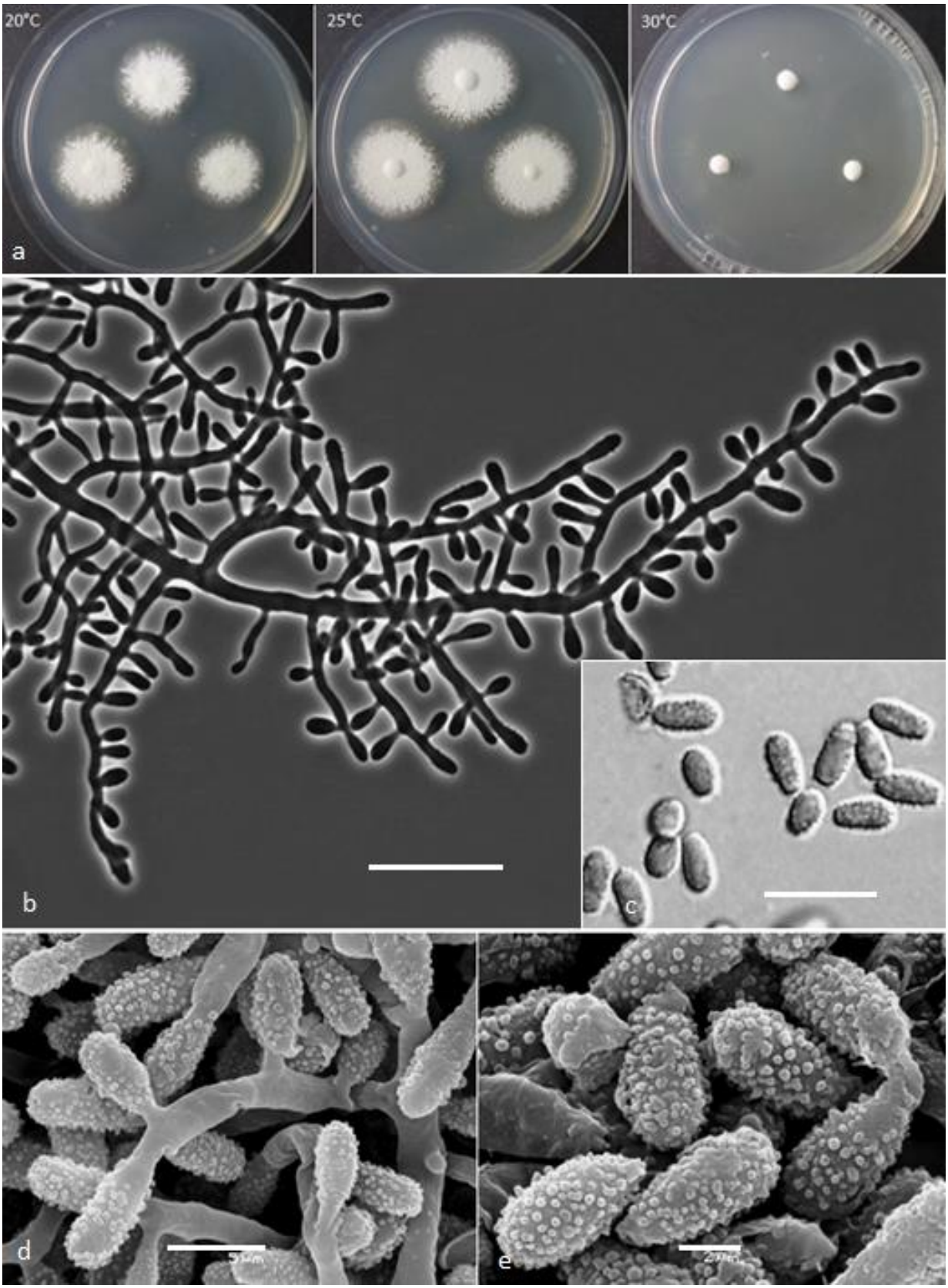

Fig. 8 *Keratinophyton wagneri* (BiMM-F77). (a) Colonies on PDA (14 days old) at 20, 25 and 30 °C; (b) conidiophores with aleurioconidia; (c) aleurioconidia (on PDA, 14 days old); (d-e) Scanning electron microscopy (SEM) of conidiogenous cells and aleurioconidia (on PDA, 14 days old). Scale bars = 20 µm (b), 10 µm (c), 5 µm (d), 2 µm (e).

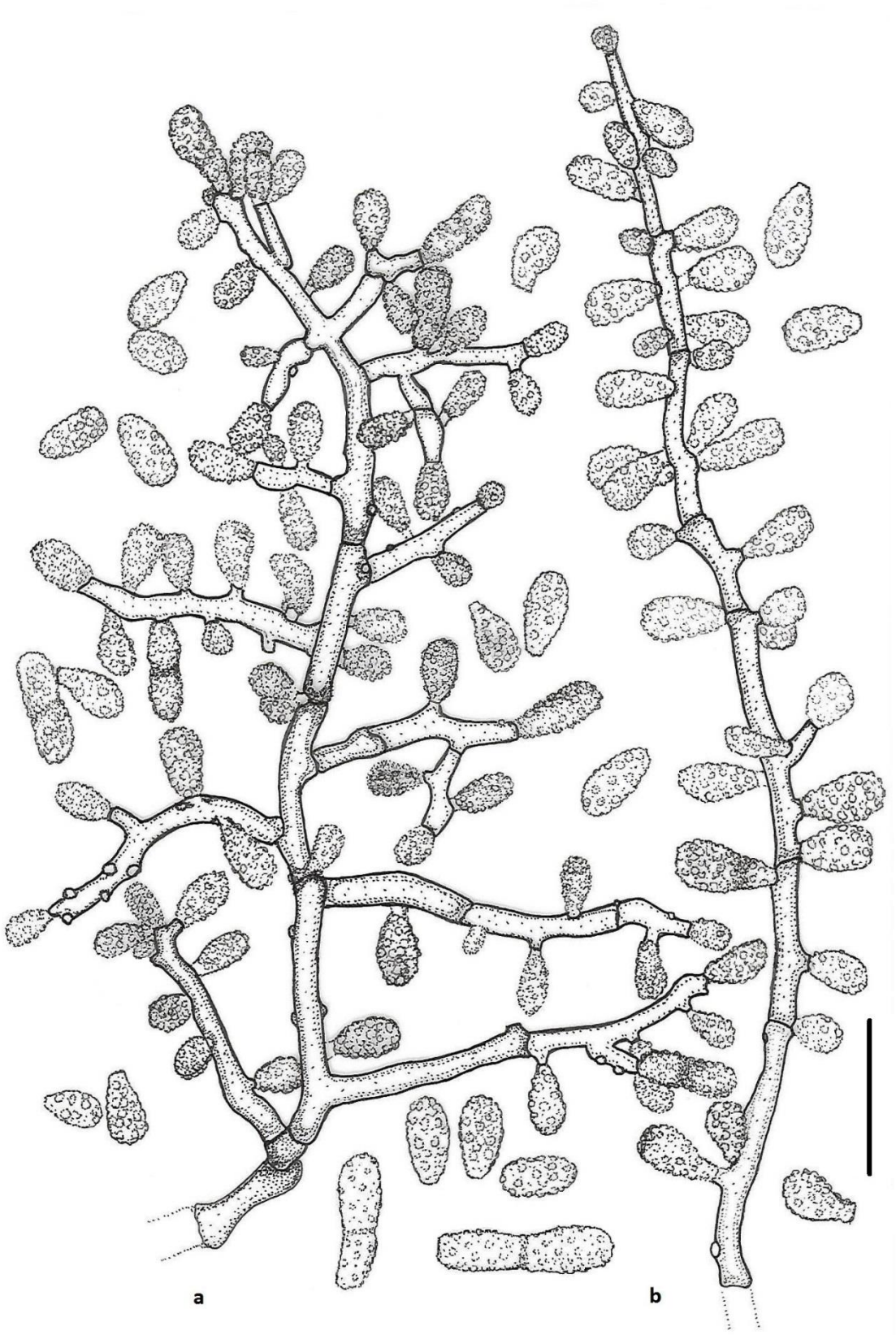

Fig. 9 Line drawing of micromorphology of *Keratinophyton wagneri* (BiMM-F77). (a, b) conidiophores

with young and mature aleurioconidia on PDA (14 days old). (a) branched conidiophore. (b) unbranched conidiophore with sessile aleurioconidia. Scale bar = 10 µm (a-b).

Table 2 Comparison of the main phenotypic characteristics of *Keratinophyton* spp. (asexual *Chrysosporium* morphs)

Species	Growth at 30 °C on PDA*	Colony Color Growth/ Reverse on PDA at 25 °C, after 14d**	Conidial Shape	Conidial dimensions (µm)	Conidial Surface	Intercalary conidia	References
***K. gollerae* sp. nov.**	None	White to creamy, 20-22 mm/ white to yellowish	Obovoid to clavate	5.0-7.0 x 2.0-2.5	Smooth to finely roughed	Absent	**This study**
***K. lemmensii* sp. nov.**	Present (good)	White, 28-35 mm/ lemon yellow	Clavate to filiform	3,0 – 40 µm	Smooth	Present	**This study**
***K. straussii* sp. nov.**	Present (good)	White to creamy, 24-28 mm/white to yellowish	Obovoid to clavate	4.5-5.0 x 2.5-3.0	Verrucose	Absent	**This study**
***K. wagneri* sp. nov.**	Present (inhibited)	White to yellowish, 25-30 mm/white to yellowish	Obovoid to clavate	4.0-8.0 x 2.5-4.0	Verrucose	Absent	**This study**
K. clavisporum	Present (inhibited)[x]	White, 53 mm (26°C)/ red-brown	Clavate to long - ellipsoidal	5-10 x 2.5-5	Smooth	Absent	Zhang *et al.,* 2017
K. echinulatum	Present (good)	Yellow to pale orange yellow, 28-45 mm/orange yellow	Obovoid to clavate	4.5-7 x 2.5-4.0	Echinulate	Present	Crous *et al.,* 2016
K. fluviale	Present (good)	White to yellowish white, 60-70mm (30°C) /brownish orange	Obovate, clavate, nearly ellipsoidal or pyriform	3.5-15 x 1-3.5 (1- and also 2-celled)	Verrucose	Present (very rare)	Vidal *et al.,* 2000
K. qinghaiense	Present (good) [x]	White to yellowish, 30 mm (7days)/yellowish	Clavate to cylindrical	3.6-13 x1.8-3.6	Smooth	Present	Han *et. al.,* 2013
K. hubeiense	Present (inhibited)[x]	Grey white to white, 65-67 mm/reverse yellowish	Obovoid to ellipsoidal	2.2-4.3 x 1.6-3.2	Smooth	Absent	Zhang *et al.,* 2016
K. linfenense	Present (good)	White to cream, 72 mm (30 °C)/white to light yellow	Ellipsoidal to fusiform, also clavate	3.2-5.4 x-1.4-2.2	Smooth	Absent	Liang *et al.,* 2009
K. minutisporosum	Present (inhibited)	White to yellowish white, 55-70 mm/white	Pyriform or subglobose, also clavate	3-4 (-11) x 1.5-3.5	Verrucose	Present (very rare)	Vidal *et al.,* 2002
K. pannicola	Present (good)	White to pale yellow, 20-38 mm (PYE***) / pale brown	Obovoid to clavate	6-11 x 3.5-4.5	Verrucose	Present (less abundant)	Oorschot, 1980
K. siglerae	Present (good)	Grisaceous orange, 15-20 mm (21 days)/ pale brown	Cylindrical to clavate	5-30 x 2-3.5 1-and 2-celled	Smooth to slightly verrucose	Present	Cano & Guarro, 1994
K. submersum	Present (inhibited)	Yellowish white, 50-60mm/ yellowish white	Clavate, also pyriform, obovoid and subglobose	4-35 x 2.5-5.0 (1- to 4-celled)	Smooth to verrucose-thick-walled	Present (in old cultures)	Vidal *et al.,* 2002
K. turgidum	Present (good)	White, 50-55 mm (SGA at 28°C) / pale brown	Pyriform to oval	5-7 x 3.5-5	Smooth	Present	Crous *et al.,* 2017

*if not stated other medium; ** if not stated otherwise; *** PYE phytone yeast extract agar; **** CZA Czapek agar; [x] Yanfeng Han personal communication

Table S1 a Temperature dependent growth of the new *Keratinophyton* species (in mm) on PDA

Species	Temperature (°C) after 14 days on PDA											
	8*	10	12	15*	18	20	25*	28	29	30*	31*	32
K. lemmensii	1-2	4-5	7-9	10-14	-	25-27	28-35	-	-	38-45	32-38	M-1
K. gollerae	SG-M	M-1	2-6	15-18	-	18-20	20-22	11-12	M-1	SG		
K. straussii	nSG	1-2	4-5	7-9	18-20	18-22	24-28	-	-	15-20	6-8	1-2
K. wagneri	SG	1-2	8-10	6-8	18-20	20-25	25-30	-	-	3-4	2-3	M-1

*crucial distinctive growth temperatures; SG – spore germination; nSG – no spore germination; M – microcolonies

Table S1 b Temperature dependent growth of the new *Keratinophyton* species (in mm) on MEA

Species	Temperature (°C) after 14 days on MEA											
	8*	10	12	15	18	20	25	28	29	30*	31*	32
K. lemmensii	1-2	2-4	5-7	7-10	-	15-17	20-25	-	-	18-20	12-15	SG
K. gollerae	SG-M	M	1-2	5-8	-	10-12	14-16	M	nSG			
K. straussii	nSG	M	M	4-7	10-12	10-12	18-20	-	-	5-10	3-4	M-1
K. wagneri	SG	M	M-2	3-5	10-13	12-15	18-20	-	-	M	M	SG

*crucial distinctive growth temperatures; SG – spore germination; nSG – no spore germination; M – microcolonies

Table S1 c Temperature dependent growth of the new *Keratinophyton* species (in mm) on SDA

Species	Temperature (°C) after 14 days on SDA											
	8*	10	12	15*	18	20	25	28	29	30*	31*	32
K. lemmensii	1-2	6-7	8-10	7-10	-	24-26	28-35	-	-	30-32	25-30	M-1
K. gollerae	SG-M	M-1	M-1	20-22	-	12-17	23-25	M-2	SG	SG	nSG	
K. straussii	nSG	M-1	2-3	3-4	-	14-16	16-20	-	-	15-20	1-2	nSG
K. wagneri	SG	M-1	1-3	3-4	-	12-14	14-18	-	-	M-1	SG-M	nSG

*crucial distinctive growth temperatures; SG – spore germination; nSG – no spore germination; M – microcolonies

Ability to digest keratin after 21 days was observed in all four species on both testing media (PDA and YEW). However, a value of attack intensity on the hair (scaling according to Marchisio *et al.,* (1994)) substantially differed from species to species, being very strong in *K. gollerae* and *K. straussii* (= 4), while moderate in *K. wagneri* (= 2) to only weak in *K. lemmensii* with a value of 0-1 (Fig. 10).

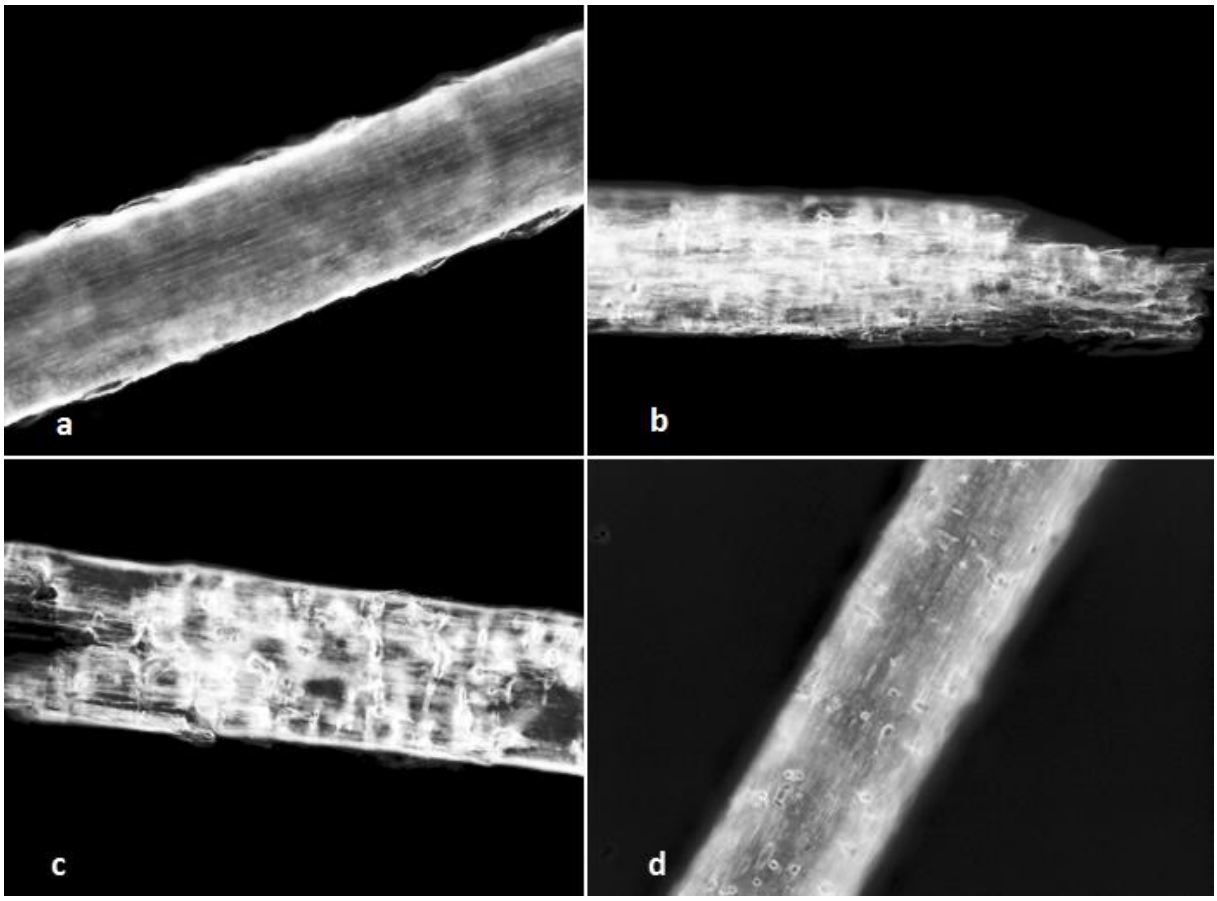

Fig. 10 Hair perforation *in vitro* - keratinolysis. A detail view on a child hair after colonization by the fungus on PDA (21 days old) at 25 °C. (a) *Keratinophyton lemmensii* (BiMM-F76). (b) *Keratinophyton gollerae* (BiMM-F250). (c) *Keratinophyton straussii* (BiMM-F78). (d) *Keratinophyton wagneri* (BiMM-F77). Intensity of attack on the hair was estimated on a scale of 0 to 4 (Marchisio et al., 1994). (a) 0-1 = light attack to cuticle, (b-c) 4 = cuticle and cortex attack with about 80% destruction, (d) 2 = cuticle and cortex attack with about 20% destruction.

Notes

All four new species are directly distinguishable from the other taxa in the genus *Keratinophyton*, known only as *Chrysosporium* asexual morphs, based on phenotypical characteristic alone. From the sexual *Keratinophyton* species, they are principally differing by inability to form ascomata in a culture (Cano et al. 2002), but also by overall phenotypic characters, such as growth at high temperature and/or conidia morphology (Cano and Guarro 1990; Cano and Guarro 1994; Currah 1985). The most solid species-specific phenotypic distinguishing characteristics are morphology of conidia (shape, surface and dimensions) and growth response to 30 °C exposure after 14 days on PDA.

Phenotypically, *K. lemmensii* is characteristic and differs from the relatives in the cluster (*K. durum*, *K. hubeiense*, *K. submersum*, and *K. siglerae*) by the combination of the following features: (1) presence of long filiform often sinusoidal uni- to bicellular conidia (up to 40 µm), (2) white, moderately growing colonies (28-35 mm in diam., on PDA at 25 °C), (3) production of lemon yellow pigment on PDA at 25°C, (4) minimum (8 °C) and maximum (32 °C) growth temperature, (5) very weak keratin digestion after 21 d. Along with foregoing features, *K. lemmensii* can be directly distinguished from the phylogenetically closest *K. durum* asexual morph also by the presence of numerous arthroconidia which are completely missing in the later species (Cano and Guarro 1990; Currah 1985).

Keratinophyton gollerae can be readily distinguished from the two other species in their joint cluster, i.e. *K. straussii* and *K. wagneri*, by its inability to grow at 30 °C, by narrower and mostly smooth to finally roughened conidia, and by its slower growth at 25 °C on PDA. Moreover, in comparison with *K. straussii*, *K. gollerae* grows substantially better at 15°C (on PDA and SDA) and its spores germinate at 8 °C (see Table S2a-c). The remaining two species in the cluster, *K. straussii* and *K. wagneri* seem to be morphologically very similar, however, they can be distinguished based on (1) size of conidia (av. = 4.9 x 2.5 µm vs 5.7 x 3.2 µm), (2) growth at 30 °C on PDA (15-20 mm vs 3-4 mm), (3) morphology of conidiogenous cells (commonly vs non– to occasionally swollen), (4) colony pigmentation on SDA after prolonged incubation (bright orange vs dark brown), and (5) keratinolytic ability after 3 weeks (very strong vs moderate). In addition, the production of a pinkish pigment on PCA after 3-4 weeks (at 20 and 25 °C) has been observed only in *K. wagneri*. Moreover, conidia of this species are more coarsely roughed (warty) than those in *C. straussii* (Figs 8 c-e).

New combinations

The phylogenetic analyses strongly supported the recent distinct classification of species previously classified as *Chrysosporium* asexual morphs into two phylogenetically different sexual genera, namely *Aphanoascus* and *Keratinophyton* (Crous et al. 2017; Sutton et al. 2013). In this study, ten new combinations are proposed for *Chrysosporium* asexual morphs resolved in a monophyletic clade affiliated to the later genus. Hence, the following *Chrysosporium* species: *C. hubeiense, C. submersum, C. siglerae, C. echinulatum, C. evolceanui, C. fluviale, C. minutisporosum, C. clavisporum, C. qinghaiense* and *C. linfenense* are redisposed in the genus *Keratinophyton* H.S. Randhawa & R.S. Sandhu.

Keratinophyton clavisporum (Y.W. Zhang, Y.F. Han & Z.Q. Liang) Labuda & Bernreiter, **comb. nov.**

Basionym: *Chrysosporium clavisporum* Y.W. Zhang, Y.F. Han & Z.Q. Liang, *Phytotaxa* 303:177, 2017

MycoBank MB833653

Keratinophiton echinulatum (Hubka, Mallátová, Cmoková & M. Kolařík) Labuda & Bernreiter, **comb. nov.**

Basionym: *Chrysosporium echinulatum* Hubka, Mallátová, Cmoková & M. Kolařík, *Persoonia* 36: 410-411, 2016

MycoBank MB833636

Keratinophyton fluviale (P. Vidal & Guarro) Labuda & Bernreiter, **comb. nov.**

Basionym: *Chrysosporium fluviale* P. Vidal & Guarro, *Mycol. Res.* 104(2): 245, 2000

MycoBank MB8333637

Keratinophyton qinghaiense (Y.F. Han, Y.R. Wang, J.D. Liang & Z.Q. Liang) Labuda & Bernreiter, **comb. nov.**

Basionym: *Chrysosporium qinghaiense* Y.F. Han, Y.R. Wang, J.D. Liang & Z.Q. Liang, Mycosystema 32 (4): 607, 2013

MycoBank MB833655

Keratinophyton hubeiense (Yan W. Zhang, Y.F. Han & Z.Q. Liang) Labuda & Bernreiter, **comb. nov.**

Basionym: *Chrysosporium hubeiense* Yan W. Zhang, Y.F. Han & Z.Q. Liang, *Phytotaxa* 270(3): 213, 2016

MycoBank MB833638

Keratinophyton linfenense (Z.Q. Liang, J.D. Liang & Y.F. Han) Labuda & Bernreiter, **comb. nov.**

Basionym: *Chrysosporium linfenense* Z.Q. Liang, J.D. Liang & Y.F. Han, *Mycotaxon* 110: 67, 2009

MycoBank MB833639

Keratinophyton minutisporosum (P. Vidal & Guarro) Labuda & Bernreiter, **comb. nov.**

Basionym: *Chrysosporium minutisporosum* P. Vidal & Guarro, *Stud. Mycol.* 47: 205, 2002

MycoBank: MB833640

Keratinophyton pannicola (Corda) Labuda & Bernreiter, **comb. nov.**

Basionym: *Capillaria pannicola* Corda, Icon. fung. (Prague) 1: 10, 1837

≡ *Chrysosporium pannicola* (Corda) Oorschot & Stalpers, *Stud. Mycol.* 20: 43, 1980

≡ *Chrysosporium evolceanui* (H.S. Randhawa & R.S. Sandhu) Garg, *Sabouraudia* 4(4): 262, 1966

≡ *Trichophyton evolceanui* H.S. Randhawa & R.S. Sandhu, *Mycopath. Mycol. appl.* 20: 232, 1963

≡ *Sporotrichum pannicola* (Corda) Rabenh., *Deutschl. Krypt.-Fl.* (Leipzig) 1: 78, 1844

MycoBank: MB8333643

Keratinophyton siglerae (Cano & Guarro) Labuda & Bernreiter, **comb. nov.**

Basionym: *Chrysosporium siglerae* Cano & Guarro, *Mycotaxon* 51: 75, 1994

MycoBank MB833641

Keratinophyton submersum (P. Vidal & Guarro) Labuda & Bernreiter, **comb. nov.**

Basionym: *Chrysosporium submersum* P. Vidal & Guarro, *Stud. Mycol.* 47: 200, 2002

MycoBank MB833642

Discussion

Phylogeny

Phylogenetic reconstruction applying ITS sequences resulted in clustering of a new species, *Keratinophyton lemmensii*, with *K. durum* (as *Aphanoascus durus*; (Cano and Guarro 1990)), *K. hubeiense* (as *Chrysosporium hubeiense*; (Zhang et al. 2016)) and *K. submersum* (as *Chrysosporium submersum*; (Vidal et al. 2002)), and forming a sister clade with *K. siglerae* (as *Chrysosporium siglerae*; (Cano and Guarro 1994)). The other three novel species, *K. gollerae*, *K. straussii* and *K. wagneri* were resolved in a separate terminal cluster (Fig 1a). Its sister cluster similarly encompasses mostly asexual *Chrysosporium* morphs belonging to *K. clavisporum* (as *Chrysosporium clavisporum*; (Zhang et al. 2017)), *K. quinghaense* (as *Chrysosporium quinghaense*; (Han et al. 2013)), *K. linfenense* (as *Chrysosporium linfenense*, (Liang et al. 2009)) and *K. turgidum* (Crous et al., 2017). Based on phylogeny and due to the one-fungus one name concept (Taylor 2011), asexual species (anamorphs) are now placed in genera conventionally comprising only sexual form (teleomorphs) regardless of whether they are sexual or asexual (Crous et al. 2017). Hence, following this concept also in this study, the generic name *Keratinophyton* is chosen to represent these new taxa instead of *Chrysosporium.* As pointed out by Crous *et al.,* (2017) the monophyletic *Keratinophyton* clade contains several asexual species which have a *Chrysosporium* morph, and require redisposing in the genus *Keratinophyton*. Likewise, they introduced a new asexual species, *Keratinophyton turgidum* Rahul Sharma & Shouche, solely based on the morphology of its chrysosporium-like aleurioconidia. As for *K. hubeiense, K. submersum, K. siglerae, K. echinulatum, K. pannicola, K. fluviale, K. minutisporosum, K. clavisporum, K. qinghaiense*, and *K. linfenense*, all these species are phylogenetically clustered in the *Keratinophyton* clade as it is evident from the current study analyzing and summarizing ITS and LSU datasets (Fig. 1a,b), as well from earlier studies (Crous et al. 2017; Li et al. 2019; Sutton et al. 2013; Zhang et al. 2016; Zhang et al. 2017) employing the ITS phylogenics. Therefore, it justifies redisposing these ten asexual taxa in the genus *Keratinophyton* H.S. Randhawa & R.S. Sandhu. At the time of this study, the monophyletic genus *Keratinophyton* contains 6 sexual (Sutton et al. 2013) and 15 asexual morphs (species), including recently described *K. turgidum* (Crous et al. 2017) and four novel taxa described here. Each of these *Chrysosporium* asexual morphs is characteristic by its

particular combination of the morphological traits (the colony color and growth rate, growth response on higher/lower temperature as well as morphology of conidia) and also easily distinguished from the four novel taxa on these phenotypic bases.

Ecology and Distribution

Including this study, almost all known *Keratinophyton* species were isolated from soil or soil-like substrata, such as river sediment, compost and sand (Cano and Guarro 1990; Crous et al. 2017; Labuda et al. 2008; Liang et al. 2009; Oorschot 1980; Vidal et al. 2000; Vidal et al. 2002). Hubalek (2000) provided a list of keratinolytic fungi associated with free-living mammals and birds in which *Keratinophyton pannicola* (as *Chrysosporium evolceanui*) has been isolated from a variety of animals, namely from different species of rodents in Australia, Czechia, England, Germany, former Yugoslavia, from a rabbit in Canada, and birds in Czechia, Queensland, former Yugoslavia and India (Hubalek 2000). *Keratinophyton durum* (as *Aphanoascus durus*) has been isolated from a hedgehog in Ivory Coast, and *Keratinophyton terreum* (as *Aphanoascus terreus*) has been associated with a variety of rodents in Romania, Germany, India, Czechia, Yugoslavia and Nigeria, and birds in Czechia, former Yugoslavia, India, Queensland and USA (Hubalek 2000). To the best of our knowledge, only a single report dealing with a human clinical isolation is connected to the ex-type strain *K. echinulatum* (CCF 4652=CBS 141178) from the sole of the foot of a 35-years-old woman in the Czech Republic (Crous et al. 2016). However, these same authors indicated that the etiological significance of the fungus is unclear, and they concluded that the infection was in fact caused by a dermatophyte, which was not isolated or overgrown by this *K. echinulatum* isolate. A few other cases have been published in a small range of animals including *Keratinophyton pannicola* (as *Chrysosporium pannicola*) from affected skin of a dog in former Yugoslavia (Cabanes et al. 2014; Oorschot 1980) and from a case of keratomycosis in a horse (Cabanes et al. 2014). In her review on *Chrysosporium* and related genera in Onygenaceae, Sigler (2003) stated that some reports concerning *Chrysosporium* species as etiological agents must be viewed with caution, however, since the isolated organism has neither been identified to species nor documented well enough to confirm the etiology. In the follow up list of species of medical relevance, there is no species mentioned being currently affiliated within the genus *Keratinophyton* (Sigler 2003). On the other side, according to Papini *et al.,* (1998), every

keratinophilic fungus can be considered a potential pathogen. Thus, soil can be regarded as an epidemiological link, probably evolutionary as well, that relates geophilic, zoophilic, and anthropophilic keratinophilic fungi. In fact, during a mycological investigation of the samples (data not shown), there was a huge prevalence of geophilic dermatophytes such as *Nannizzia gypsea* (as *Microsporum gypseum*) in a soil sample from Italy (2004), *Arthroderma uncinatum* (as *Trichophyton ajelloi*) with co-occurrence of *Aphanoascus keratinophilus* (as *Chrysosporium keratinophilum*) in a soil sample from Slovakia (2011), and finally, *Trichophyton terrestre* along with abundant *A. uncinatum* co-population in a compost sample from Austria (2015).

Significance

As members of the genus *Keratinophyton* are considered as typical soilborne fungi (Cano and Guarro 1990; Cano et al. 2002; Sutton et al. 2013) and there is not any solid evidence of pathogenicity reported, it is likely that those above mentioned animal-associated cases reflect just a simple environmental pollution of the animals with soil particles present around their habitats and dwellings and ability to persistent in dormant stages on fur or feather of the animals. The ability of these fungi to persist and survive in soils in dormancy was observed also during the present study, as in case of *K. straussii*, the type strain (BiMM-F78) was isolated 11 years after the sample collection in 2004, and two more strains representing this taxon (RL-05 and RL-06 obviously clonal) were isolated in a repeated study even after 15 years since the sample collection. Likewise, a second strain (RL-07) used for description of *K. wagneri* and the type strain of *K. gollerae* (BiMM-F250) were both isolated after 8 years since the time of collection. In this study, the degree of keratin digestion by the tested strains varied and was found to be very strong in both *K. gollerae* and *K. straussii* evoking attack on cuticle and cortex with about 50-80% destruction of the hair. According to Marchisio *et al.,* (1994) and Mitola *et al.,* (2002) keratinolytic fungi share common properties with dermatophytes. Potential pathogenicity to humans and homoiotherm vertebrates (mammals or birds) by theses fungi is highly unlikely as they do not grow at temperatures above 32°C. Instead, their strong keratinolytic ability might be providing a competitive advantage in nature to acquire nutrients and may potentially be used in industry for production of proteolytic enzymes applied in degradation processes of keratinous material (hairs, fur, feather etc.). Furthermore, these fungi represent yet undiscovered source of new bioactive secondary metabolites as there is not much known from literature about these properties of the genus (e.g. Kushwaha and

Guarro 2000). Hence, all these newly described fungi are currently under investigation in our research facilities as for their ability to produce prospective bioactive secondary metabolites.

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Authors' Contributions

RL performed isolation and phenotypic research with the novel fungi and fungal illustration (line drawings). AB and DH performed molecular analysis and phylogenetic analysis was done by AB. AK performed all microscopical measurements and provided the microphotography (including SEM). The manuscript was written by RL and CS. All authors read and approved the final manuscript.

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Figures

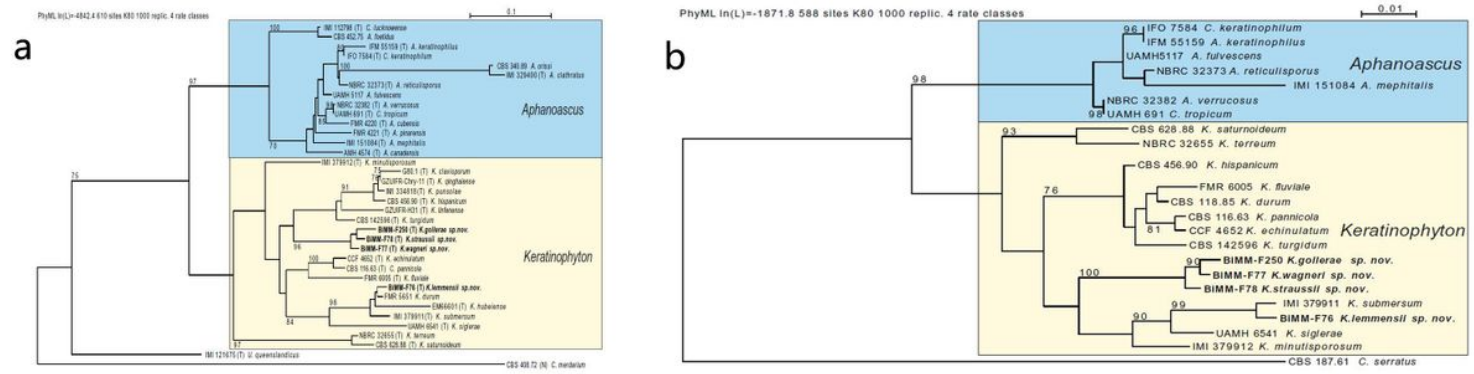

Figure 1

a Maximum Likelihood (ML) tree based on ITS sequence for the new taxa of Keratinophyton is compared with available sequences of the other related species. Numbers at nodes indicate bootstrap values (expressed as percentages of 1000 replications). Uncinocarpus queensladicus IMI 121675T and Chrysosporium merdarium CBS 408.72N were used as outgroups. A sequence for K. multiporum was not available for the study. Scale bar indicates 0.01 substitutions per nucleotide position. A= Aphanoascus, K= Keratinophyton, C= Chrysosporium, U= Uncinocarpus. (T) ex-type, (N) ex-neotype strain. New species are in Bold. b. Maximum Likelihood (ML) tree based on LSU rDNA sequences for new taxa of Keratinophyton is compared with available sequences of the other related species (ex-type strains). Numbers at nodes indicate bootstrap values (expressed as percentages of 1000 replications). Scale bar indicates 0.01 substitutions per nucleotide position. Ctenomyces serratus CBS 187.61 was used as outgroup. New species are in Bold.

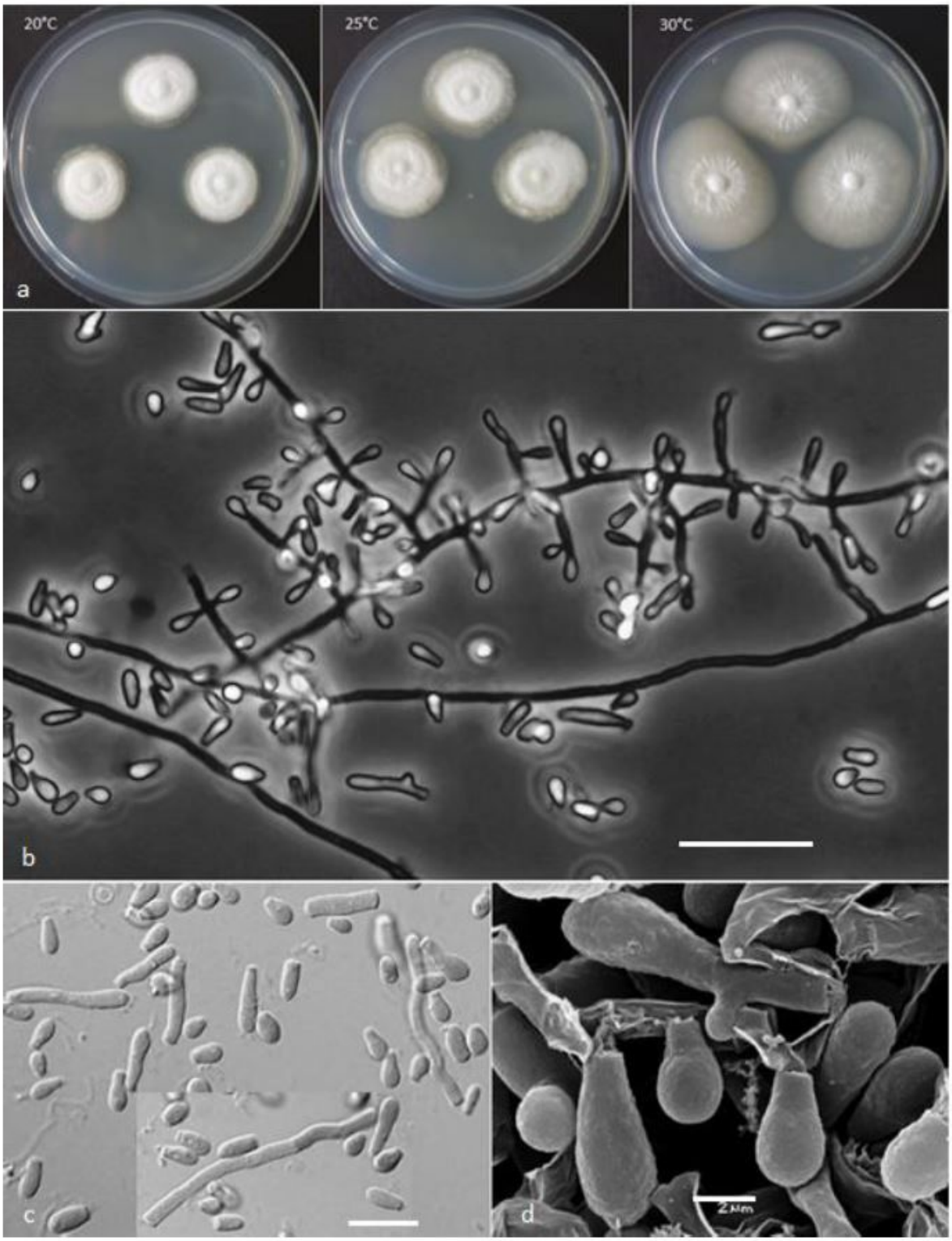

Figure 2

Keratinophyton lemmensii (BiMM-F76). (a) Colonies on PDA (14 days old) at 20, 25 and 30 °C; (b) conidiophores with aleurioconidia; (c) aleurioconidia and arthroconidia (on PDA, 14 days old); (d) Scanning electron microscopy (SEM) of aleurioconidia (on PDA, 14 days old). Scale bars = 20 µm (b), 10 µm (c), 2 µm (d).

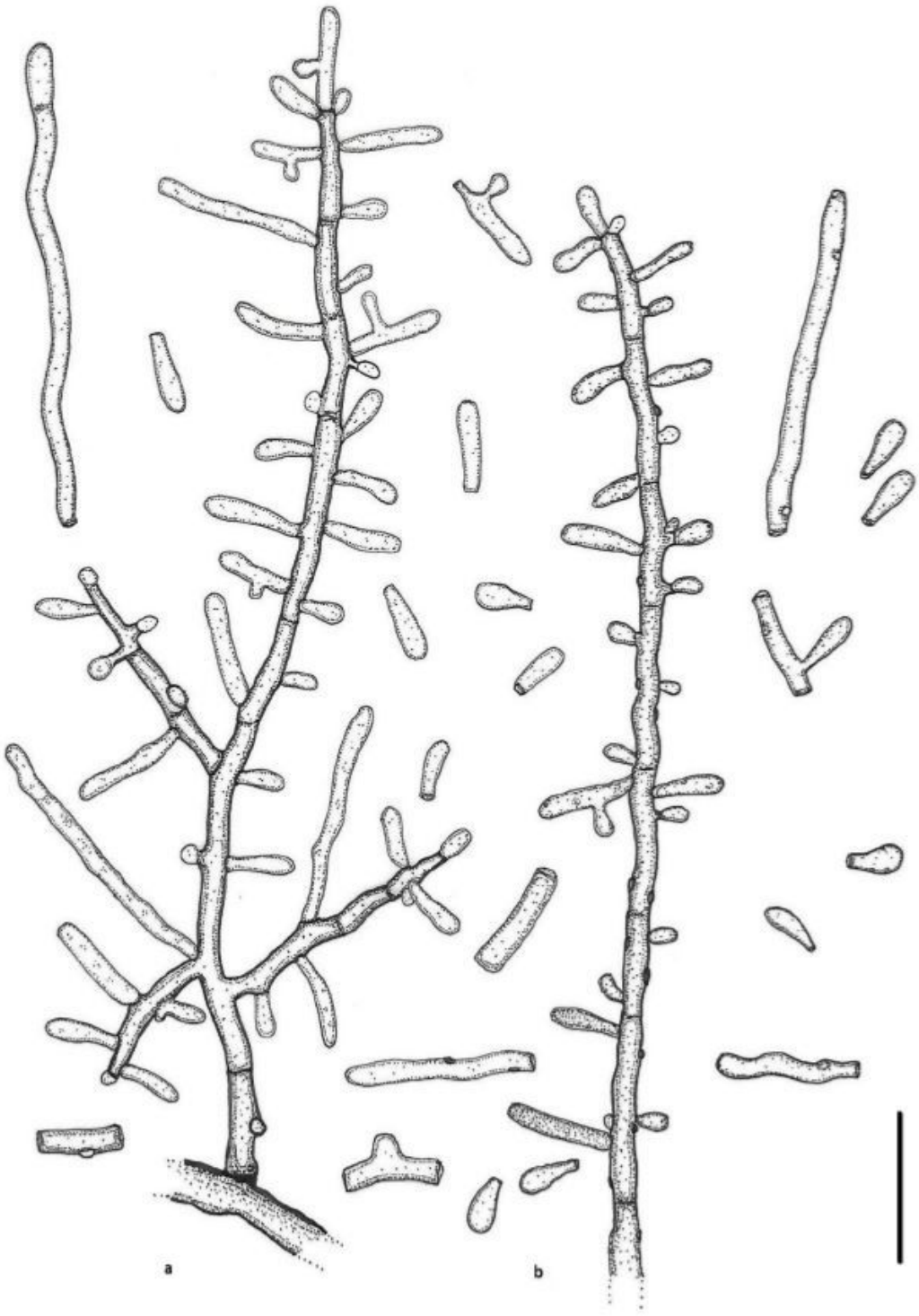

Figure 3

Line drawing of micromorphology of Keratinophyton lemmensii (BiMM-F76). (a, b) conidiophores with young and mature aleurioconidia, including arthroconidia on PDA (14 days). (a) branched conidiophore. (b) unbranched conidiophore with sessile aleurioconidia. Scale bar = 10 µm (a-b).

Figure 4

Keratinophyton gollerae (BiMM-F250). (a) Colonies on PDA (14 days old) at 20, 25 and 30 °C; (b) conidiophores with aleurioconidia; (c) aleurioconidia and arthroconidia (on PDA, 14 days old); (d) Scanning electron microscopy (SEM) of aleurioconidia (on PDA, 14 days old). Scale bars = 20 µm (b), 10 µm (c), 2 µm (d).

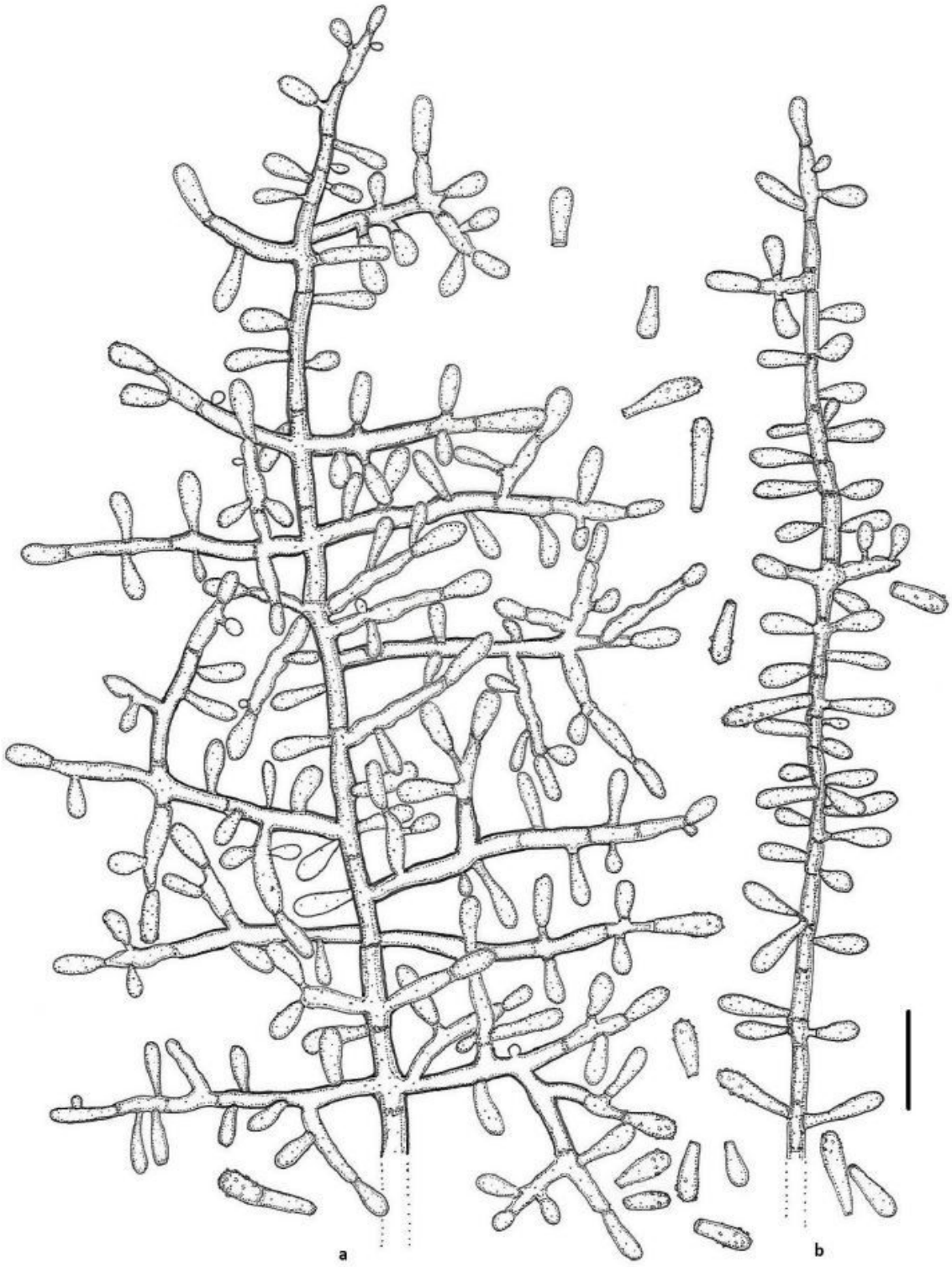

Figure 5

Line drawing of micromorphology of Keratinophyton gollerae (BiMM-F250). (a, b) conidiophores with young and mature aleurioconidia on PDA (14 days old). (a) branched conidiophore. (b) unbranched conidiophore with sessile aleurioconidia. Scale bar = 10 µm (a-b).

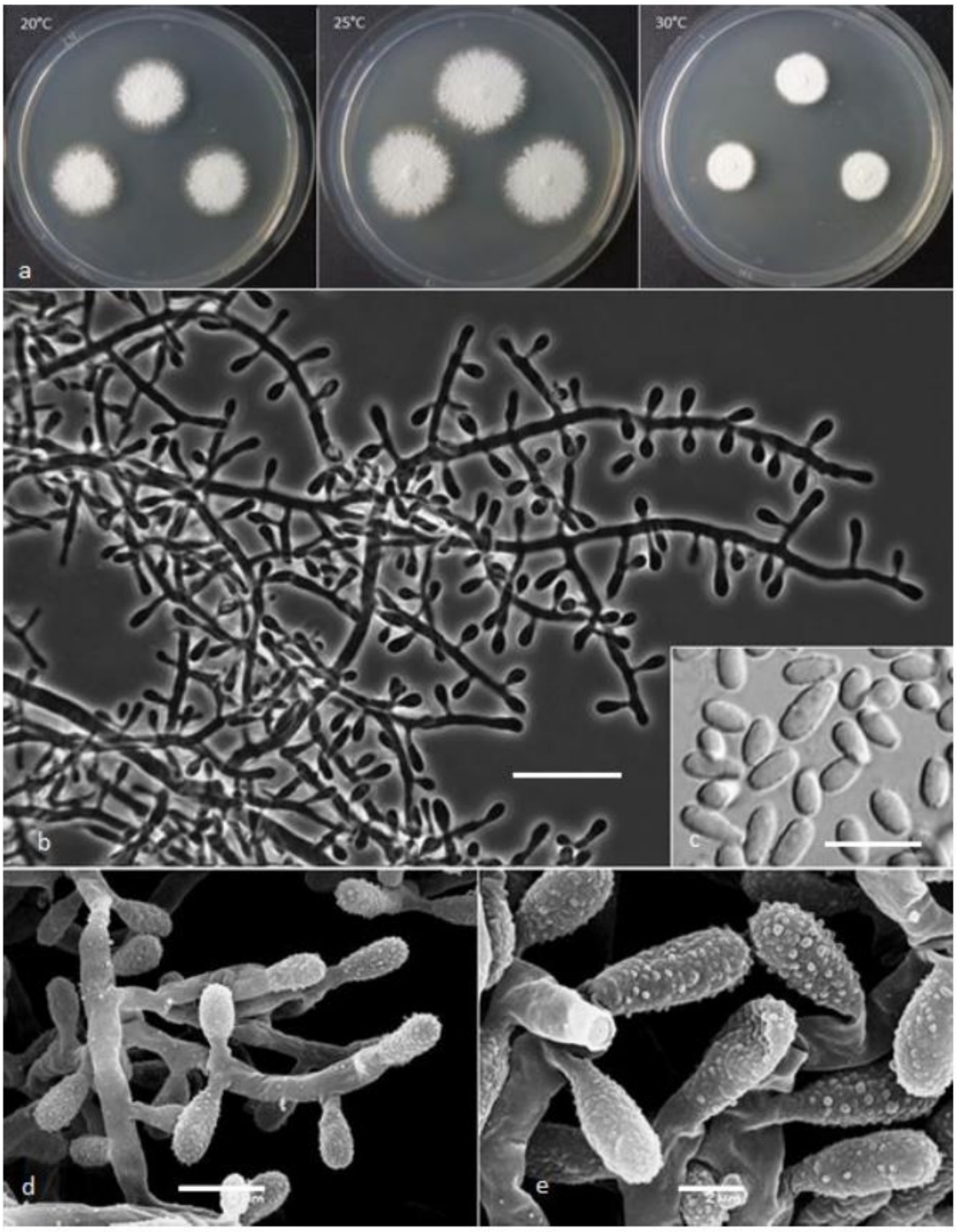

Figure 6

Keratinophyton straussii BiMM-F78. (a) Colonies on PDA (14 days old) at 20, 25 and 30 °C; (b) conidiophores with aleurioconidia; (c) aleurioconidia (on PDA, 14 days old); (d-e) Scanning electron microscopy (SEM) of conidiogenous cells and aleurioconidia (on PDA, 14 days old). Scale bars = 20 µm (b), 10 µm (c), 5 µm (d), 2 µm (e).

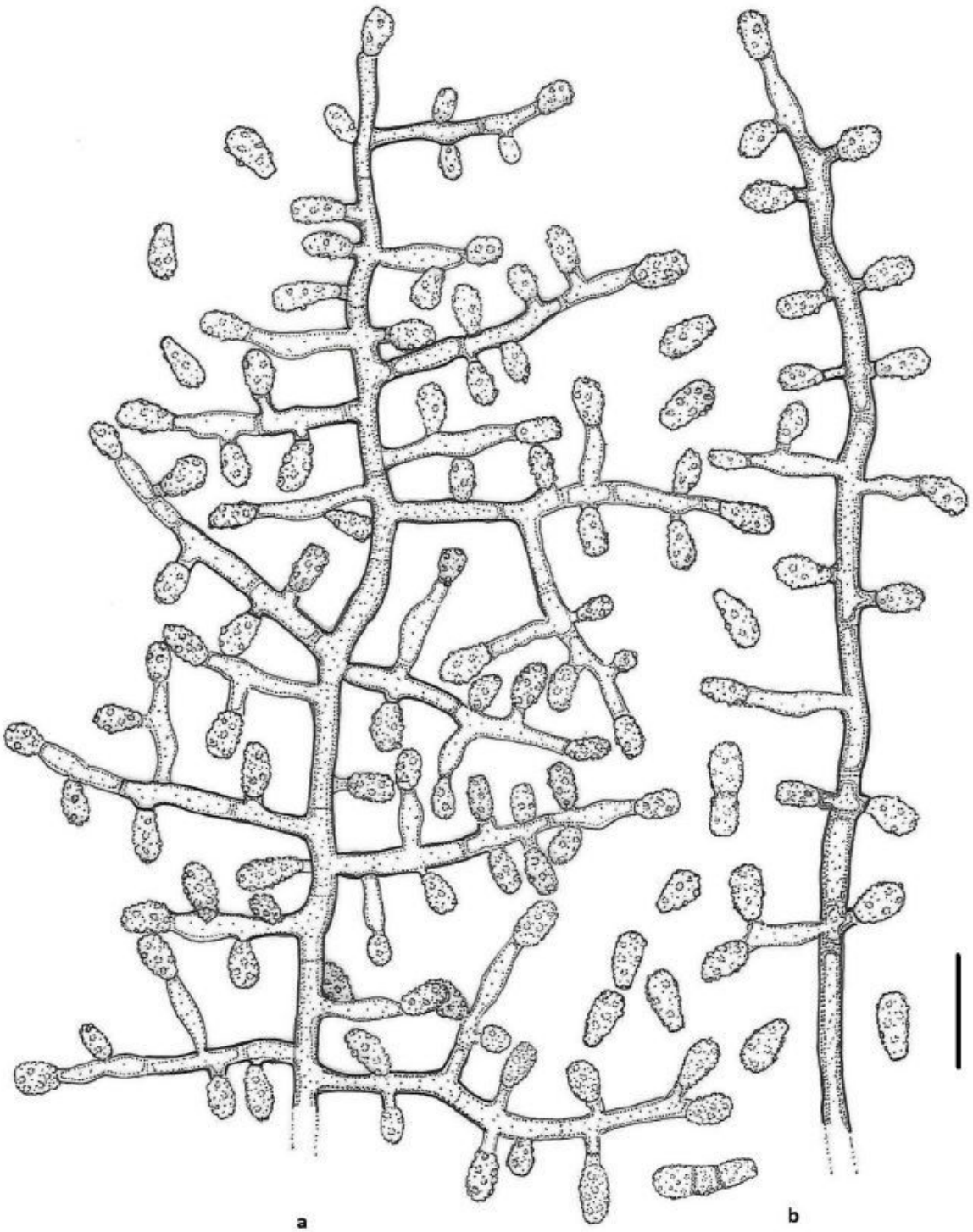

Figure 7

Line drawing of micromorphology of Keratinophyton straussii (BiMM-F78). (a, b) conidiophores with young and mature aleurioconidia on PDA (14 days old). (a) branched conidiophore. (b) unbranched conidiophore with sessile aleurioconidia. Scale bar = 10 µm (a-b).

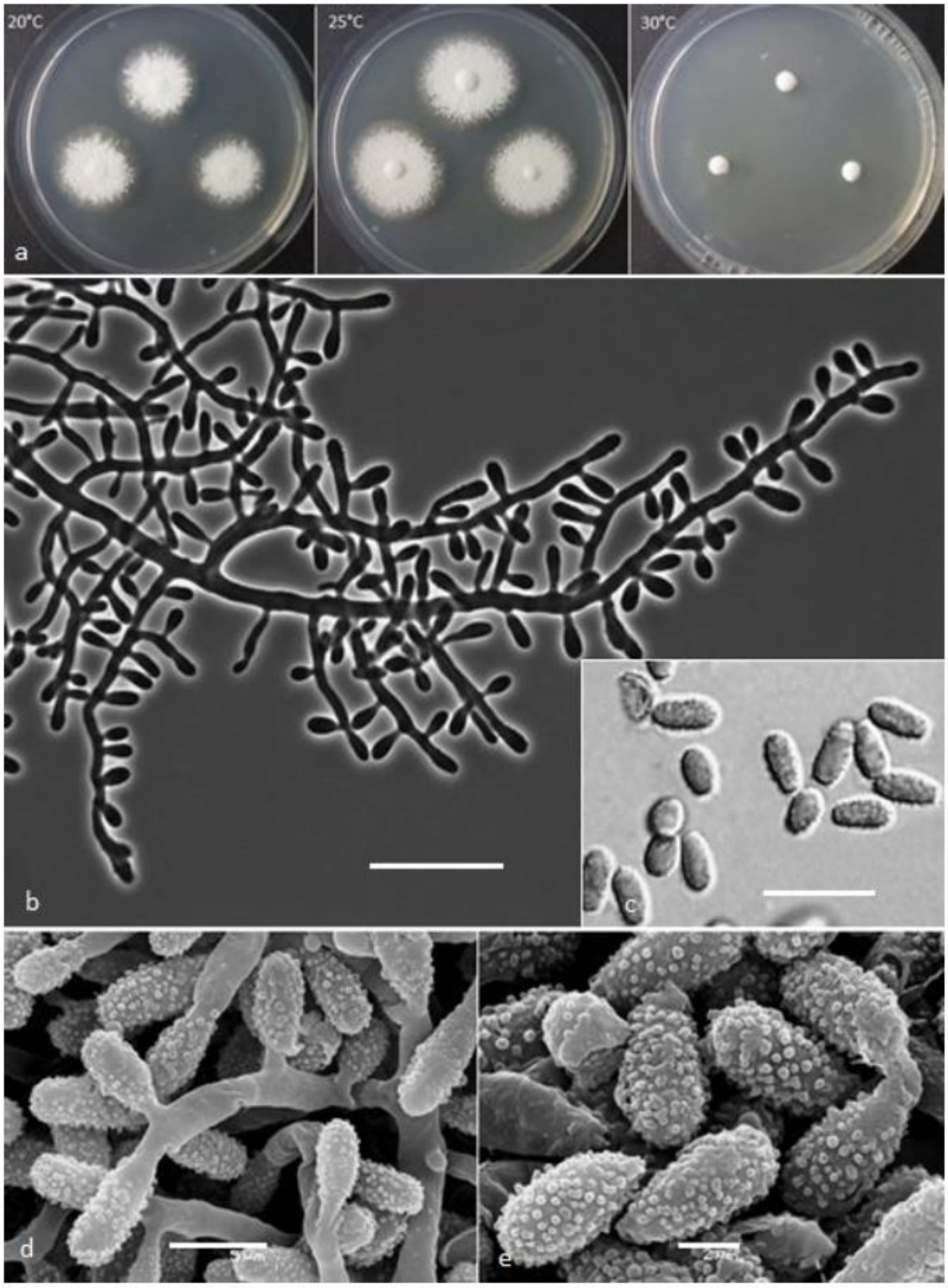

Figure 8

Keratinophyton wagneri (BiMM-F77). (a) Colonies on PDA (14 days old) at 20, 25 and 30 °C; (b) conidiophores with aleurioconidia; (c) aleurioconidia (on PDA, 14 days old); (d-e) Scanning electron microscopy (SEM) of conidiogenous cells and aleurioconidia (on PDA, 14 days old). Scale bars = 20 µm (b), 10 µm (c), 5 µm (d), 2 µm (e).

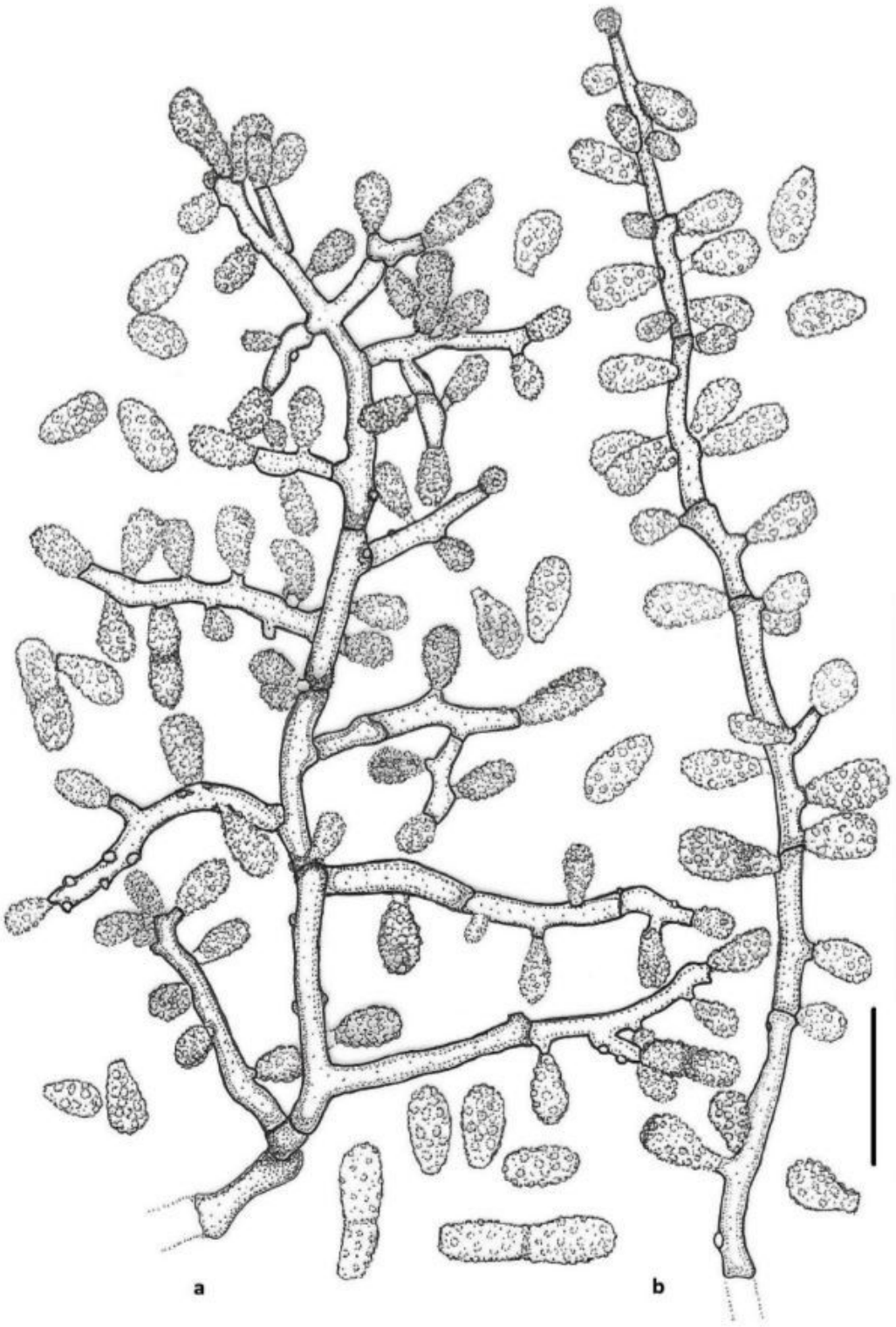

Figure 9

Line drawing of micromorphology of Keratinophyton wagneri (BiMM-F77). (a, b) conidiophores with young and mature aleurioconidia on PDA (14 days old). (a) branched conidiophore. (b) unbranched conidiophore with sessile aleurioconidia. Scale bar = 10 µm (a-b).

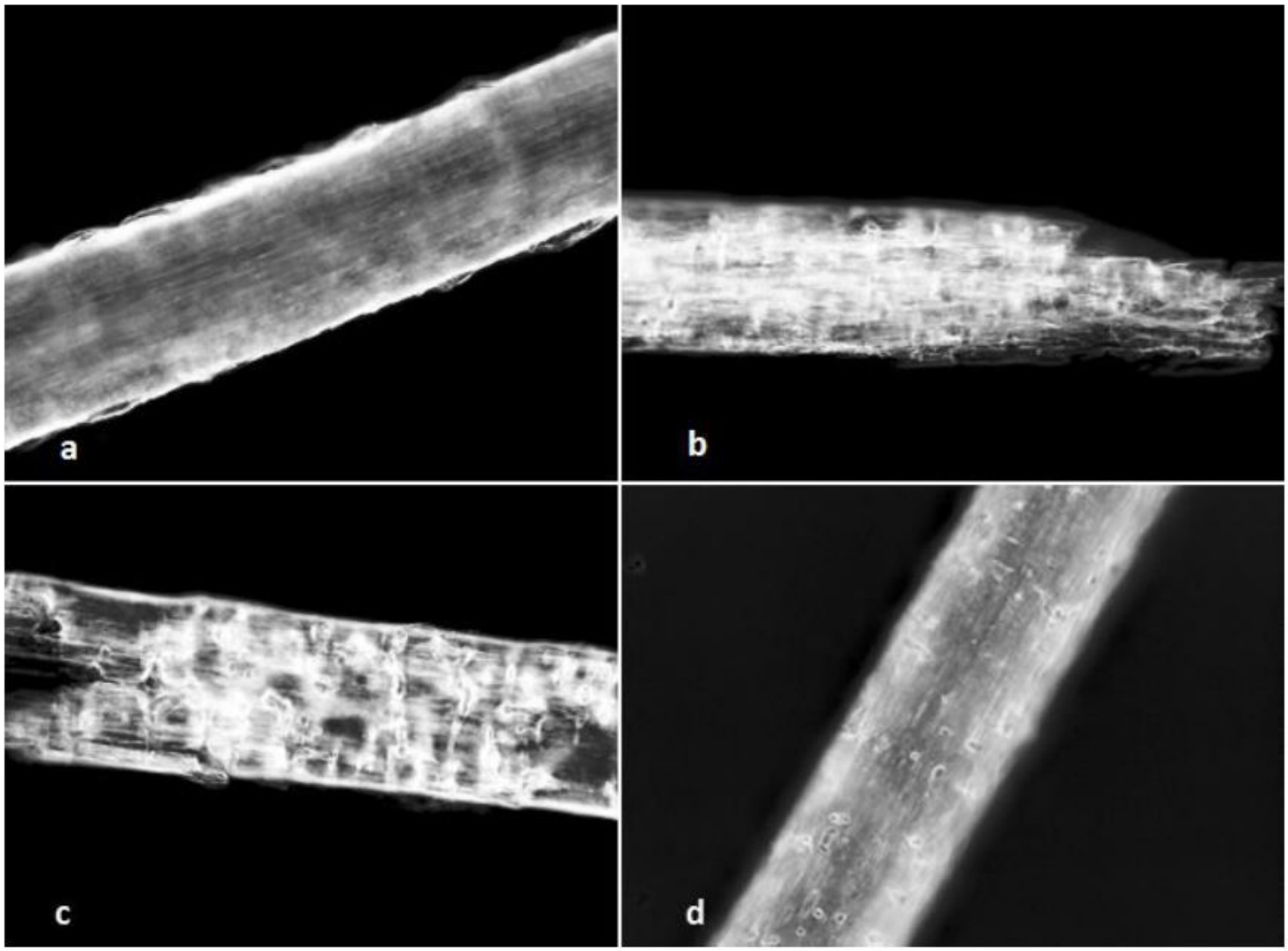

Figure 10

Hair perforation in vitro - keratinolysis. A detail view on a child hair after colonization by the fungus on PDA (21 days old) at 25 °C. (a) Keratinophyton lemmensii (BiMM-F76). (b) Keratinophyton gollerae (BiMM-F250). (c) Keratinophyton straussii (BiMM-F78). (d) Keratinophyton wagneri (BiMM-F77). Intensity of attack on the hair was estimated on a scale of 0 to 4 (Marchisio et al., 1994). (a) 0-1 = light attack to cuticle, (b-c) 4 = cuticle and cortex attack with about 80% destruction, (d) 2 = cuticle and cortex attack with about 20% destruction.