Freshwater fungi from southern Australia: Microvesuvius unicellularis gen. et. sp. nov. and Achrochaeta rivulata sp. nov.

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Research Article

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Abstract

During a survey of freshwater fungi in temperate southern Australia, two previously unknown anamorphic ascomycetes were found. The coelomycetous ascomycete was placed in the family Morosphaeriaceae (Pleosporales) as a new genus based on molecular data. We introduce the new genus and species *Microvesuvius unicellularis* with morphological and molecular data. The *Dictyochaeta*-like hyphomycete was placed in *Achrochaeta*based on both morphological characters and phylogenetic analyses using ITS, 28S, and tef1 sequences. *Achrochaeta rivulata* is the second species described within this genus.

Main text

Freshwater fungi are an ecologically important group within freshwater ecosystems around the world. They are found in lakes, rivers, streams, and waterholes as decomposers, endophytes, plant and animal pathogens, and some form mycorrhizae. There are 3,870 species described, mostly within the Sordariomycetes and Dothideomycetes, with many more being found every year (Calabon et al., 2022). It is estimated that there are 20,000 freshwater fungal species worldwide (Gessner & Van Ryckegem, 2003).

Freshwater fungi are any species which, for the whole or part of their life cycle, rely on free freshwater, or which uses any resource of a predominantly aquatic or semi-aquatic nature as a substratum (Thomas, 1996). This excludes spores or fungal DNA from a terrestrial origin found in a freshwater habitat.

During a survey of freshwater fungi on submerged wood in southern Australia, a coelomycete resembling *Hongkongmyces* and a *Dictyochaeta*-like hyphomycete species were found. Coelomycetous fungi are often found in freshwater habitats and occur throughout the ascomycota (Wijayawardene et al., 2020). *Dictyohaeta* and similar genera are found in the Chaetosphaeriaceae.

Réblová (1999) introduced Chaetosphaericaceae to accommodate *Chaetosphaeria* and it is now a genus- and species-rich family, most species of which are only known by their asexual morph (Réblová et al., 2021; Lin et al., 2019). They are commonly found in both terrestrial and freshwater habitats.

In this study, we provide morphological descriptions and phylogenies of these two novel taxa based on multigene analyses.

Materials and methods

Collection details and examination

Submerged wood samples less than 5 cm in diameter were collected from Scott Creek Conservation Park, South Australia from two streams that flow only during winter and are approximately 50cm deep with a muddy base. Samples were sealed in plastic bags for transport to the laboratory. The riparian vegetation is a mixture of native vegetation and invasive weeds.

The wood samples were incubated in sterile plastic containers and regularly examined for fungi using a Leica MZ7s dissecting microscope over 6 months. All fungi observed were photographed, described, then examined using a Nikon Eclipse Ni with differential interference contrast. Imaged were captured using either a Canon 6D or Sony RX-100 camera.

Single spore isolation

Potato dextrose agar (PDA, BD micro) was autoclaved, cooled to 60°C, 100mg/L streptomycin and 70mg/L of penicillin added as filter-sterilised stock solutions and 10 mL poured into each 60mm diameter plate.

Using a sterile needle, spores were transferred to 20 µL of sterile water in an Eppendorf tube and agitated for several seconds to suspend them. The suspension was then pipetted onto a PDA plate. Plates were incubated at room temperature and checked over 5 days for germinating spores using a Leica MZ7s dissecting microscope. Germinated spores were picked off the agar surface using a sterile needle and transferred to individual PDA plates which were incubated at room temperature.

DNA extraction, amplification, and sequencing

Approximately 50 mg of fungal mycelium was scraped from the surface of agar cultures with a sterile scalpel and genomic DNA isolated using a Qiagen DNeasy Plant Mini kit following the manufacturer's protocols. The final DNA extracts were eluted into 100 µL of buffer.

Sequences from translation elongation factor 1-alpha (TEF1) were amplified with primers were EF1-983F and EF1-2218R (Rehner & Buckley 2005). For nuclear ribosomal genes, primers ITS1/ITS 4 (White et al., 1990) were used to amplify ITS1, 5.8S and ITS2 and LROR/LR5 (Vilgalys & Hester, 1990; Rehner & Samuels, 1994) to amplify the sequences from the 28S nrRNA gene

Reaction mixtures contained 1 µL (10 mM each) dNTPs, 1 µL (10µM) of each primer, 0.25 µL hotStart Taq DNA polymerase (New England Biolabs), 1 µL DNA template, 5 µL buffer, and 16.75 µL sterile milliQ water.

PCR amplification was performed in an Applied Biosystems 2720 Thermo Cycler. Cycling conditions for PCR were initial denaturation at 95°C for 3 min; 35 cycles of

denaturation at 95°C for 1 min, annealing at 52°C (ITS) or 54°C (28S) for 50 s, and extension at 72°C for 1 min; and a final extension at 72°C for 10 min. Cycling conditions for TEF1 was 95°C for 1 min; 35 cycles of 95°C for 30s, 57°C for 50 s, and 68°C for 1 min; followed by 68°C for 5 min.

PCR amplicons were visualised on 1.5% agarose electrophoresis gels stained with Gel Red (Gene Target Solutions).

PCR products were purified using a Qiagen QIAquick PCR Purifcation Kit and sequenced in both directions using the respective primers by the Australian Genome Research Facility. Raw sequence reads

were assembled, examined, and edited using Sequencher v. 5.3 (Gene Codes Corporation). Newly generated sequences were submitted to NCBI GenBank under the accession numbers listed in Tables 1 and 2.

Table 1
GenBank accession numbers of strains used for phylogenetic analyses of *Microvesuvius unicellularis*. Newly generated sequences are shown in bold.

Species	Strain	ITS	28S
Aliquandostipite khaoyaiensis	ISAN100	MT864350	MT860428
Aquilomyces metrosideri	CBS 146782	NR_173036	NG_076729
Aquilomyces patris	REF101	JN859321	JN859476
Aquilomyces patris	CBS 135760	KP184004	KP184042
Aquliomyces patris	REF099	JN859319	JN859475
Aquliomyces patris	CBS 135662	KP184003	KP184043
Aquliomyces patris	CBS 135661	NR_137961	NG 057057
Aquliomyces rebunensis	KT 732 2	AB809630	AB807542
Aquliomyces rebunensis	HHUF 27556	NR_154664	NG_056937
Clypeoloculus hirosakiensis	HHUF 30144	NR_153866	NG_068961
Clypeoloculus microsporus	HHUF 30143	NR_153867	NG_068960
Clypeoloculus towadaensis	HHUF 30145	NR_153865	NG_058722
Helicascus alatus	MFLUCC 17–0147	MG356480	MG356478
Helicascus mangrovei	MCR355	KX957960	KX639747
Helminthosporium chengduense	CGMCC 3.23575	ON557751	ON557745
Jahnula dianchia	MFLUCC 16-1353	MH793538	MH793544
Leptosphaeria etheridgei	CBS 125980	NR_111620	JF740291
Leptosphaeria irregularis	MFLUCC 15-1118	NR_171725	KX856055
Leptospora macarangae	MFLUCC 18–0553	NR_174831	MW063232
Massarina pandanicola	MFLUCC 17–0596	MG646958	MG646947
Microvesuvius unicellularis	**AD291626**	**OQ799384**	**OQ799383**
Microvesuvius unicellularis	**AD291633**	**OQ799382**	**OQ799391**
Morosphaeria muthuputensis	PUFD87	MF614795	MF614796
Morosphaeria velatispora	PUFD25	MK026766	MK026764
Neohelicascus aquaticus	KUMCC 19–0107	MT627719	MT627662
Neohelicascus submersus	MFLU 20–0436	NR_172425	MT627656

Species	Strain	ITS	28S
Periconia cyperacearum	CPC 32138	NR_160357	NG_064549
Periconia neobritannica	CPC 37903	NR_166344	NG_068342
Phaeosphaeria sinensis	MFLUCC 18-1552	NR_163350	NG_070076

Table 2
GenBank accession numbers of Chaetosphaeriaceae species used for phylogenetic analyses. Newly generated sequences are shown in bold.

Species	Strain	ITS	28S	*TEF1*
Achrochaeta rivulata	**AD291612**	**OQ799381**	**OQ799389**	**OQ866587**
Achrochaeta rivulata	**AD291619**	**OQ799348**	**OQ799390**	**OQ866588**
Achrochaeta talbotii	ICMP 15161	MT454480	MT454495	OL653988
Chaetosphaeria dilabens	CBS 734.83	MH861683	MH873395	-
Chaetosphaeria guttulata	MFLU 18-1617	MK828636	MK835837	MN194087
Chaetosphaeria hebetiseta	MR 938	AF178549	AF178549	-
Chaetosphaeria hebetiseta	SMH 2729	AY906955	AF466069	-
Chaetosphaeria innumera	M.R. 1175	AF178551	AF178551	-
Chaetosphaeria mangrovei	MCD 069	MG813821	MG813820	-
Chaetosphaeria myriocarpa	CBS 143389	MH107883	MH107931	-
Dictyochaeta callimorpha	ICMP 15130	MT454483	MT454498	MT454673
Dictyochaeta detriticola	ICMP 14948	MT454486	MT454501	MT454676
Dictyochaeta fuegiana	ICMP 15153	MT454487	EF063574	MT454677
Dictyochaeta montana	CBS 145342	MT454488	MT454502	MT454678
Dictyochaeta querna	CBS 145503	MT454490	MT454504	MT454680
Dictyochaeta stratosa	CBS 138739	MT454492	MT454506	MT454682
Fuscocatenula submersa	MFLU 18-1616	NR_168802.1	NG_068637.1	MN194085.1
Gongromeriza pygmaea	M.R. 1365	AF178545	AF178545	-
Sporoschisma hemipsilum	S-877	MK828617.1	MK835817.1	MN194070.1
Sporoschisma hemipsilum	S-627	MK828618.1	MK835818.1	MN194071.1
Sporoschisma juvenile	MFLU 18-1608	MK828619.1	MK835819.1	MN194072.1

Phylogenetic analyses

The generated sequences for each gene were subjected to megablast searches (Zhang et al., 2000) to identify closely related sequences in NCBI's GenBank nucleotide database.

Other sequences used in this study were derived from GenBank. Sequences were aligned in Geneious Prime v. 2023.0.4 using MUSCLE. Alignments were imported into Mega X 10.2.6 (Stecher et al., 2020) to find the best substitution models for phylogenetic analyses.

Genes were then concatenated and maximum-likelihood phylogenetic trees were constructed using RAxML v. 8.2.11 (Stamatakis, 2014) within Geneious and branch support values were calculated with 1000 rapid bootstrap inferences. The same alignment was analysed with MrBayes (v 3.2.6, Huelsenbeck & Ronquist, 2001) within Geneious. All resulting trees were formatted in Geneious, then further edited in Adobe Illustrator v. 27.0.

Results

Phylogenetic analyses

Phylogenetic trees based on multi-locus analyses (Figs. 1 & 2) show the relationships between the new species and other related taxa. Branch supports of Maximum Likelihood bootstrap ≥ 70% and Bayesian p value ≥ 0.90 are indicated near the branches.

BLAST searches showed that the undescribed coelomycetous species from our survey was closest to species in the Morosphaeriaceae. In order to classify this species, ITS and 28S sequences were aligned with 24 species within the Pleosporales, including representatives of all accepted genera in the Morosphaeriaceae. The dataset including alignment gaps, comprised 1884 characters: 890 for ITS and 994 for 28S. The tree is rooted to *Aliquandostipite khaoyaiensis* (ISAN100) and *Jahnula dianchia* (MFLUCC 16-1353). The undescribed fungus formed a new clade within the Morosphaeriaceae, separate to previously described genera, therefore representing a new genus (Fig. 1).

BLAST searches of ITS sequences from the *Dictyochaeta*-like species, showed the closest match to be *Achrochaeta talbotti* at 92% similarity. Phylogenetic analyses of 21 of the closest species within Chaetosphaeriaceae included 2433 characters: 573 for ITS, 875 for 28S, and 985 for tef1. The tree is rooted to 3 *Sporoschisma* strains. The undescribed species formed a well-supported clade sister to *Achrochaeta tablotti* (Fig. 2).

Taxonomy

Microvesuvius Fryar & D.E.A. Catches. gen. nov. MB848293

Conidiomata pycnidial, immersed in substrate, semi-immersed, or superficial, globose to ellipsoid, black, ostiolate. *Wall* made of indistinct dark brown cells, irregular thickness and an inner layer of hyaline, ellipsoid, thick-walled cells. *Conidiogenous cells* hyaline, cylindrical, percurrent proliferation. *Conidia* hyaline, appendages absent, sheath present.

Etymology: This genus is named after Mount Vesuvius due to the resemblance of the some of the pycnidia to small volcanoes.

Microvesuvius unicellularis Fryar & D.E.A. Catches. sp. nov. MB848294, Figs. 3 & 4

Typus: **Australia**, South Australia, Scott Creek Conservation Park (S35° 5' 45.90", E138° 40' 59.16) on submerged decaying wood in an ephemeral stream, S. Fryar (holotype AD291633). GenBank numbers: ITS - OQ799382; 28S - OQ799391; TEF1 - OQ866585.

Etymology: The epithet refers to the aseptate conidia.

Saprobic on decaying wood in an ephemeral freshwater stream. **Sexual morph** undetermined. **Asexual morph**: On natural substrate. *Conidiomata* pycnidial, immersed in substrate, semi-immersed or rarely superficial, globose to ellipsoid, lying horizontal to substrate, scattered, black, ostiolate, (134)335–840 · (135)194–480 µm. *Conidiomatal wall* made of indistinct dark brown cells, irregular thickness up to 80 µm and an inner layer of hyaline, ellipsoid, thick-walled cells, irregular thickness up to 50 µm. *Conidiogenous cells* hyaline, cylindrical, percurrent proliferation, (12)22–29(35) · 3.5–4 µm. *Conidia* hyaline, aseptate, 1 large oil globule and multiple smaller oil globules, appendages absent, thin irregular sheath surround most conidia, 11–18 · 10–15 µm.

Culture characteristics: On PDA after 13 days 18 x 14 mm, irregular shape, 1 main colony, several satellite colonies, grey tinged with brown, margin white, 1 mm, slimy appearance, centre fluffy. Agar not discoloured. Reverse grey interior, white margin. Hyphae hyaline, twisted and intertwined, branched, septate, 2.5–3 µm wide. Aerial hyphae hyaline to dark grey, breaking easily, verrucose, 2–4 µm wide.

Additional material examined: **Australia**, South Australia, Scott Creek Conservation Park (S35° 5' 45.90", E138° 40' 59.16) on submerged decaying wood in an ephemeral stream, S. Fryar, AD291626, AD291608, AD291689. GenBank numbers (AD291626): ITS - OQ799384; 28S - OQ799383; TEF1 - OQ866586.

Notes:

Phylogenetic analyses of ITS and 28S sequences place our specimens in a clade representing Morosphaericaeae (Pleosporales) alongside *Aquilomyces* and *Clypeoloculus* as a separate lineage (Fig. 1). There were very few comparison *TEF1* Morosphaeriaceae sequences in GenBank, so those data were not used in our analyses.

Most of the species described in the Morosphaericaeae only have descriptions of the sexual morph, so morphological comparisons are mostly not possible. However, the asexual morph of *Neohelicascus aquaticus*, also within the Morosphaeriaceae, is described and is coelomycetous (Zhang et al., 2013). *Microvesuvius unicellularis* is different to *N. aquaticus* in many ways. The conidia of *M. unicellularis* are globose to subglobose compared with the ellipsoid to obovoid conidia of *N. aquaticus*, the conidiogenous cells of *M. unicellularis* are larger, and percurrent, and the pycnidia are immersed and black, rather than superficial and brown.

Morphologically, *M. unicellularis* shares characters with *Hongkongmyces aquaticus*. They both have globose to subglobose, hyaline, aseptate conidia with large oil globules. However, the conidia of *H. aquaticus* do not have a sheath and the conidiogenous cells of *H. aquaticus* are shorter and not described as percurrent. *Hongkongmyces* is a member of the family Lindgomycetacae, also within the Pleosporales (Tsang et al., 2014).

Achrochaeta rivulata Fryar & D.E.A. Catches. sp. nov. MB848295, Figs. 5 & 6

Etymology: The epithet refers to the habitat where this species was found, a small stream.

Typus: AUSTRALIA, Scott Creek Conservation Park (S35° 5' 46", E138° 40' 59"), on decaying wood submerged in a stream, 26 August 2020, AD291612. GenBank numbers: ITS - OQ799381; OQ799389; *TEF1* - OQ866587

Saprobic on decaying wood in an ephemeral freshwater stream. **Sexual morph** undetermined. **Asexual morph**: On natural substrate. *Conidiophores*, mononematous, macronematous, 77–170 · 2.5–3.6 µm, simple, dark brown, hyaline or subhyaline at the apex, septate, single or branched at the base into 3, straight or slightly flexuous, cylindrical. *Conidiogenous cells* 20–37 · 3 µm, monophialidic, tapering towards the apex, hyaline to subhyaline. *Collarettes* funnel shaped, 1.5–2 · 1.5–2 µm. *Setae* 70–99 · 3–3.5 µm, rounded, slightly bulbous apex, dark brown at base graduating through to subhyaline at the apex, septate. *Conidia* 6–10 · 2.5 µm, hyaline, aseptate, without appendages, straight or curved, ornamentation or sheath, smooth, ellipsoid, one end rounded, one end pointed.

On PDA

Colonies on PDA after 4 weeks 15 · 12 mm, margin undulate, finely furrowed, velvety, dark grey in patches, white in patches, white outer zone, slightly convex (indented), reverse dark grey, with white margin. Hyphae hyaline, septate, branching, 2.5–4 µm wide. *Sheath* 8–18 · 7–10 µm, on some of the darker hyphae, often near the base of the conidiophores. *Conidiophores* monophialidic, tapering towards the apex, 12–54 · 2.5–4 µm, mononematous, subhyaline to brown, collarette funnel-shaped 1–2 x 1–2 µm. *Conidia* 5–13 · 2–3 µm, hyaline, mostly straight, some curved, aseptate, without sheaths or appendages, ellipsoid, one end pointed, other end rounded.

Additional material examined: AUSTRALIA, Scott Creek Conservation Park (S35° 5' 46", E138° 40' 59"), on decaying wood submerged in a stream, 26 August 2020, (AD291619, AD291630, AD291604, AD291618, AD219605. GenBank numbers (AD291619): ITS - OQ799348, 28S - OQ799390, *TEF1* - OQ866588

Notes:

The conidiophores of *Achrochaeta rivulata* on PDA appear different to those on the natural substrate, wood. They are shorter and lighter in colour. There is also a sheath present on some hyphae which is not visible on the natural substrate. Also, no setae were observed in culture.

The phylogram of ITS, 28S and *TEF1* sequences (Fig. 2) shows our new species to be in a clade with *Achrochaeta talbotti*, *Chaetosphaeria hebetiseta* and *C. dilabens*, sister to *A. talbotti.* In a megaBLAST search of ITS sequences, *Achrochaeta rivulata* was most similar to *A. talbotii* (MT454480) with 92% similarity. The two specimens share many characters such as unbranched, brown, septate conidiophores with hyaline phialidic conidiogenous cells, and funnel-shaped collarettes. However, *A. talbotti* (ICMP 15161) does not have setae, and the conidia are cylindrical-clavate rather than the ellipsoid. In addition, *A. rivulata* only has an apical opening, whereas *A. talbotti* (ICMP 15161) has either a single apical opening on the conidiogenous cells or also has lateral openings.

Achrocheata talbotti was originally described as *Chaetosphaeria talbotii* S. Hughes, W.B. Kendr. & Shoemaker (Hughes & Kendrick, 1968). The original collection of *C. talbottti* by Hughes and Kendrick was in Kuitpo Forest, South Australia, which is 20 km from the collection site of *A. rivulata*. There are close similarities between the original description of *C. talbotti* and *A rivulata*, but as with the comparison with *A. tablotti* (ICMP 15161), *A. rivulata* is monophialidic, not polyphialidic, has setae, and has ellipsoid conidia rather than cylindrical-clavate conidia.

Discussion

Microvesuvius forms a well-supported clade within the Morosphaeriaceae based on phylogenetic analyses of combined rDNA sequences. Suetrong et al. (2009) introduced the Morosphaeriaceae based on molecular data and sexual morphs, placing *Morosphaeria* and *Helicascus* within this family. The family now has 4 addition genera, *Aquilhelicascus*, *Aquilomyces*, *Clypeoloculus*, and *Neohelicascus,* plus our newly described genus, *Microvesuvius*. The ascomata in the family are subglobose, conical, lenticular, immersed or superficial and are dark coloured. They have clavate to cylindrical, thick-walled, fissitunicate asci, with an ocular chamber and a non-amyloid apical ring. Ascospores are hyaline to brown, septate and can be with or without a sheath or cap (Suetrong et al., 2009).

Microvesuvius is not unusual within Morosphaeriaceae in being from a freshwater habitat. A number of species from Morosphaeriaceae are found in fresh water including *Aquihelicascus thalassiodieus*, *A. yunnanensis*, *A. songkhlaensis* (Dong et al., 2020), *Clypeoloculus akitaensis*, *C. hirosakiensis*, *C. microsporus*, *C. towadaensis* (Tanaka et al., 2015), *Helicascus alatus* (Zeng et al., 2018), *Neohelicascus aquaticus, N. aegyptiacus*, *N. chiangraiensis*, *N. elaterascus*, *N. gallicus*, *N. submersus* (Dong et al., 2020), *N. unilocularis* (Zhang et al., 2015), and *N. uniseptatus* (Luo et al., 2016).

While coelomycetes are rare within Morosphaeriaceae, Pleosporales commonly have both coelomycetous and hyphomycetous asexual morphs (Yu et al., 2022) and are mostly coelomycetous (Zhang et al., 2012). *Phoma* or *Phoma*-like anamorphs are the most common anamorphs of Pleosporales (Zhang et al., 2012) and *Microvesuvius unicellularis* has many similarities to these anamorphs such as pycnidia that are immersed to erumpent, ostiolate, dark-coloured, with annellidic conidiogenous cells and globose to obovoid, unicellular conidia (Kohlmeyer & Kohlmeyer 1979).

Based on morphological differences and multi-gene phylogenies, we introduce the new genus *Microvesuvius* and the two new species *Microvesuvius unicellularis* and *Achrochaeta rivulata*.

Declarations

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The authors declare that there are no competing interests.

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Figures

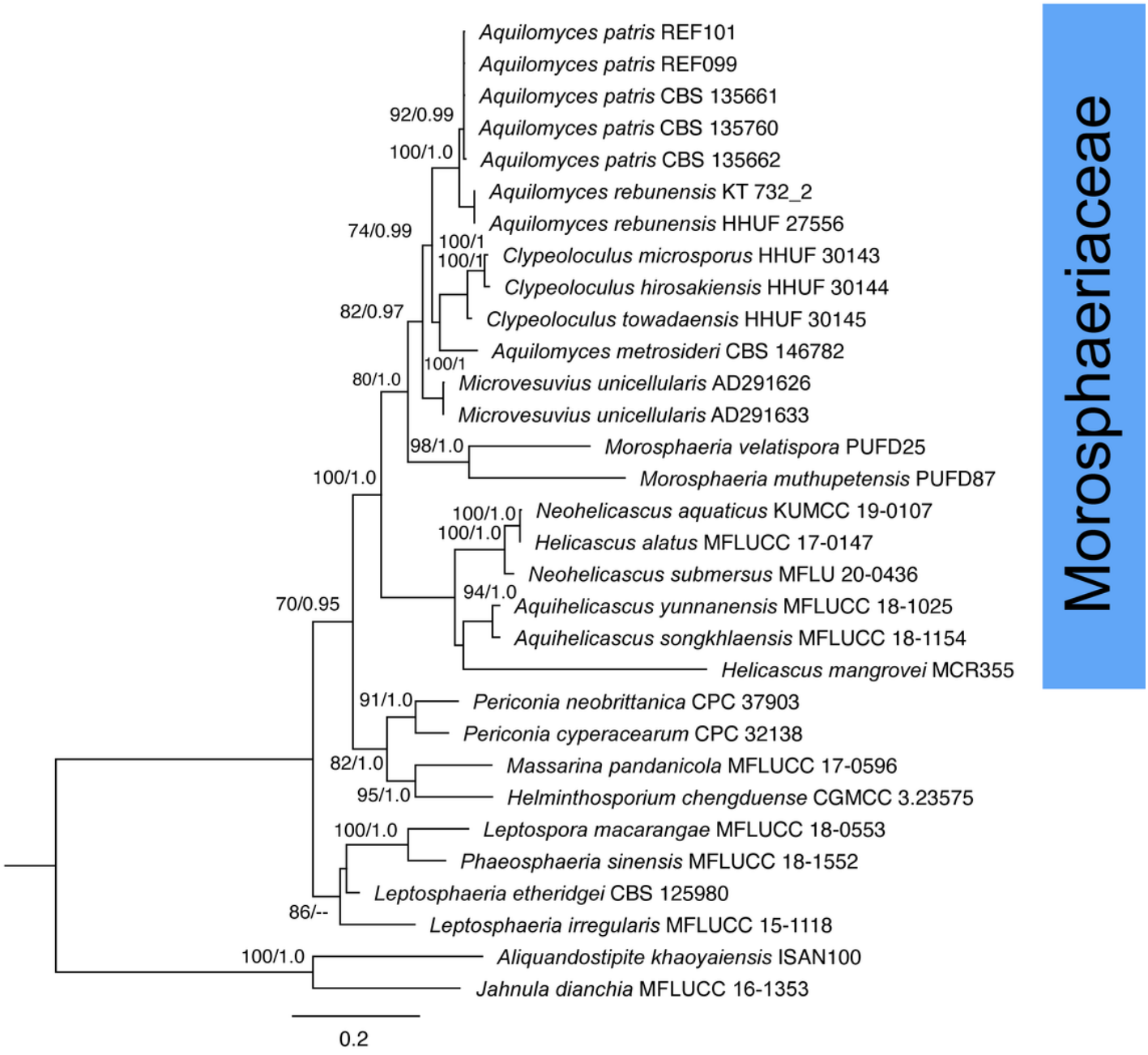

Figure 1

Phylogram generated from maximum likelihood analysis based on combined ITS and 28S sequence data of *Microvesuvius unicellularis* and closely related taxa within the Pleosporales. *Jahnula dianchia* (MFLUCC 16=1353) *and Aliquandostipite khaoyaiensis* (ISAN100) were used as outgroup taxa. Bootstrap values equal to or great that 70% and Bayesian posterior probabilities equal to or greater than 0.95 are given next to the nodes.

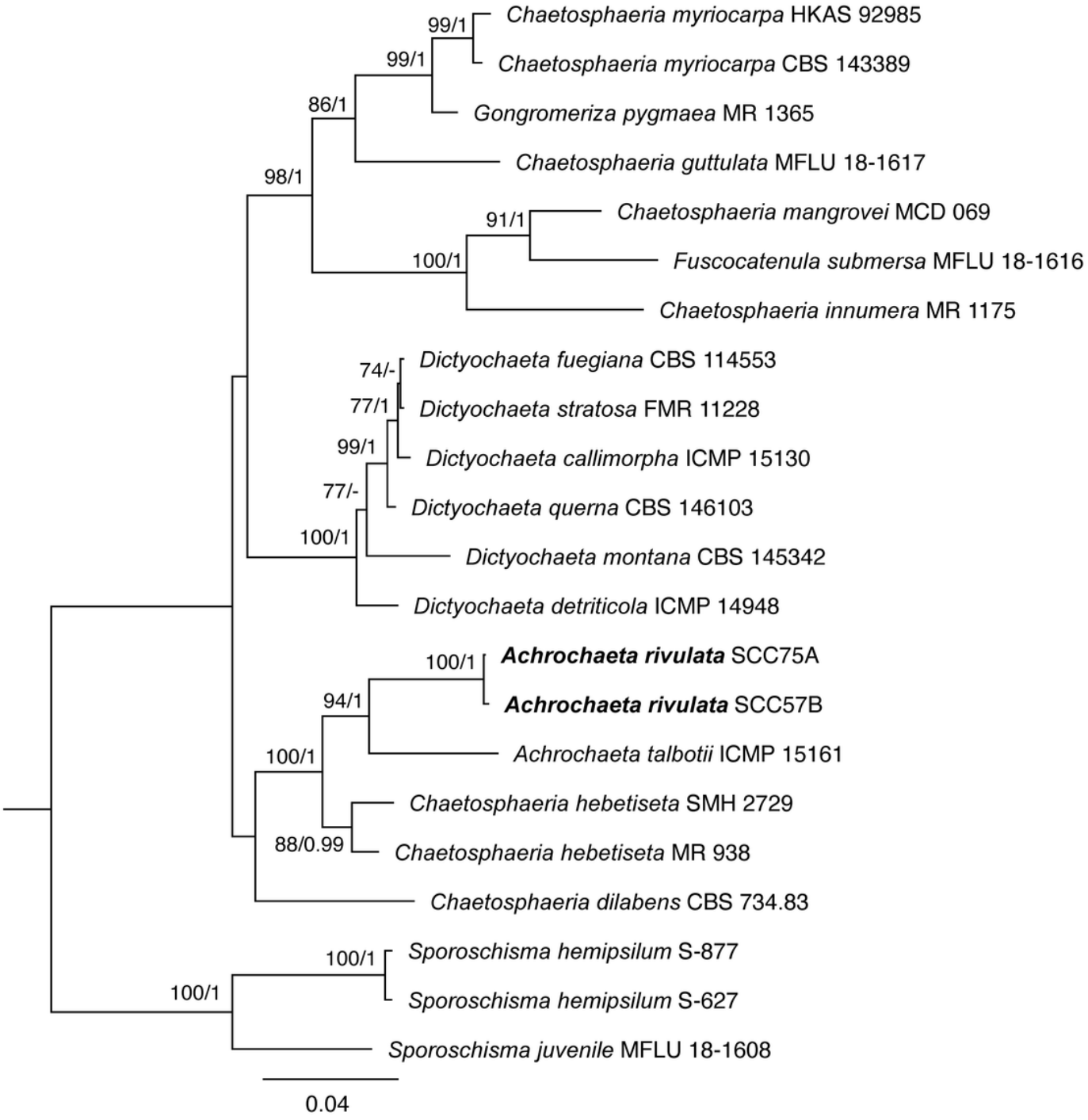

Figure 2

Phylogram generated from maximum likelihood analysis based on combined ITS, 28S, and *TEF1* sequence data of *Achrochaeta rivulata*and closely related taxa within the Chaetosphaeriaceae. *Sporoschisma* species were used as the outgroup taxa. Bootstrap values equal to or great that 70% and Bayesian posterior probabilities equal to or greater than 0.95 are given next to the nodes.

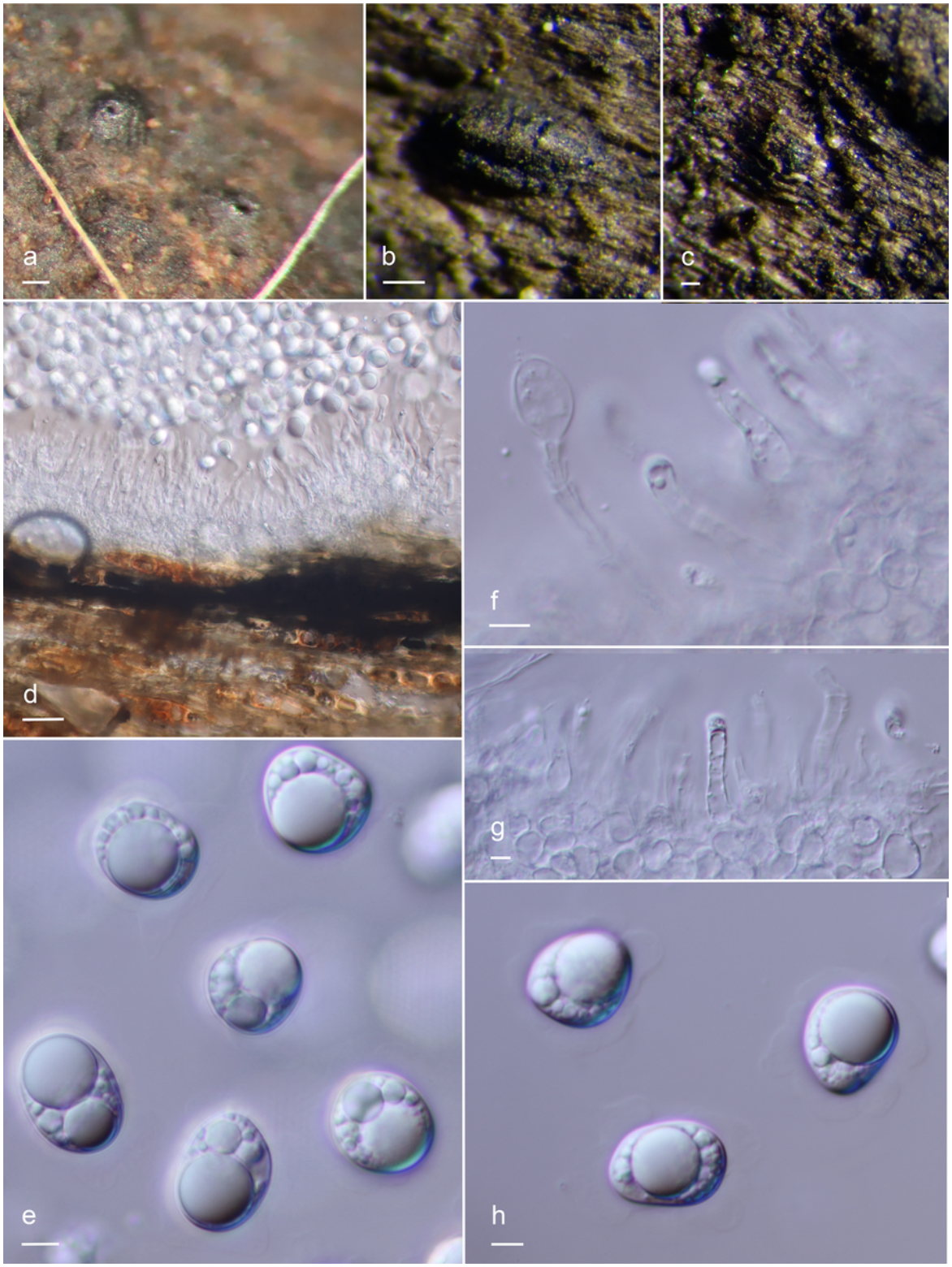

Figure 3

Microvesuvius unicellularis. (holotype AD AD291633). **a-c** Pycnidia on host surface. **d** Cross section through a pycnidium showing pycnidial wall, conidiogenous cells and conidia. **e** Conidia. **f-g** Conidiogenous cells. **h** conidia. Scale bars: **a-c** = 100 mm, **d** = 10 mm, **e-h**= 5 mm

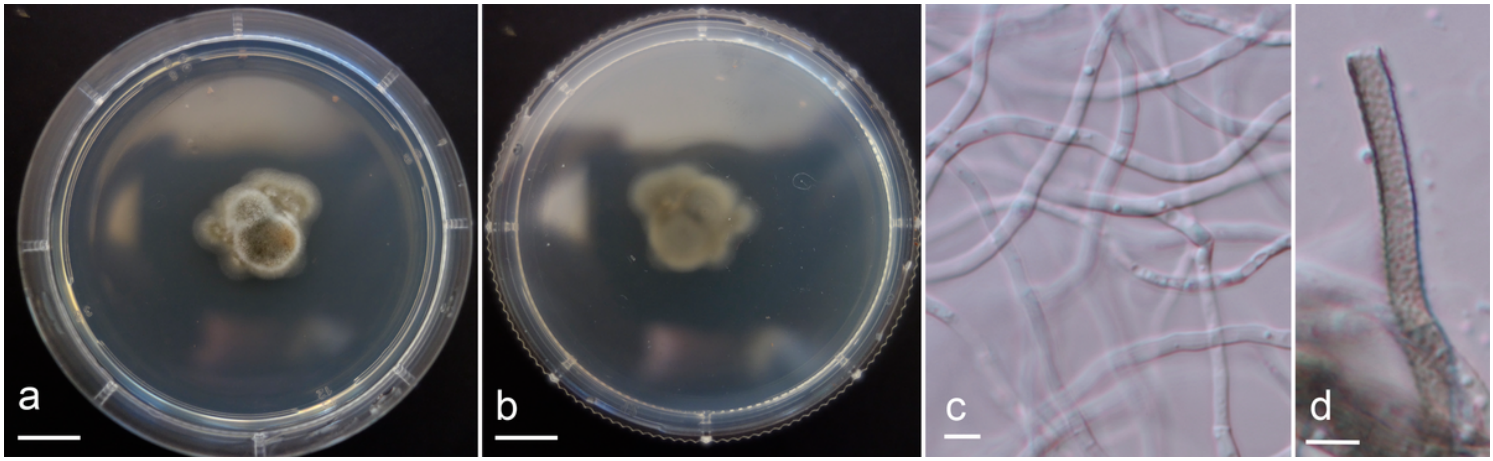

Figure 4

Culture of *Microvesuvius unicellularis* (holotype, AD291633) on 60mm PDA plate after 13 days. **a** Culture. **b**reverse side of culture. **c-d** Hyphae. Scale bars: **a-b** = 1 cm, c-**d**= 5 mm

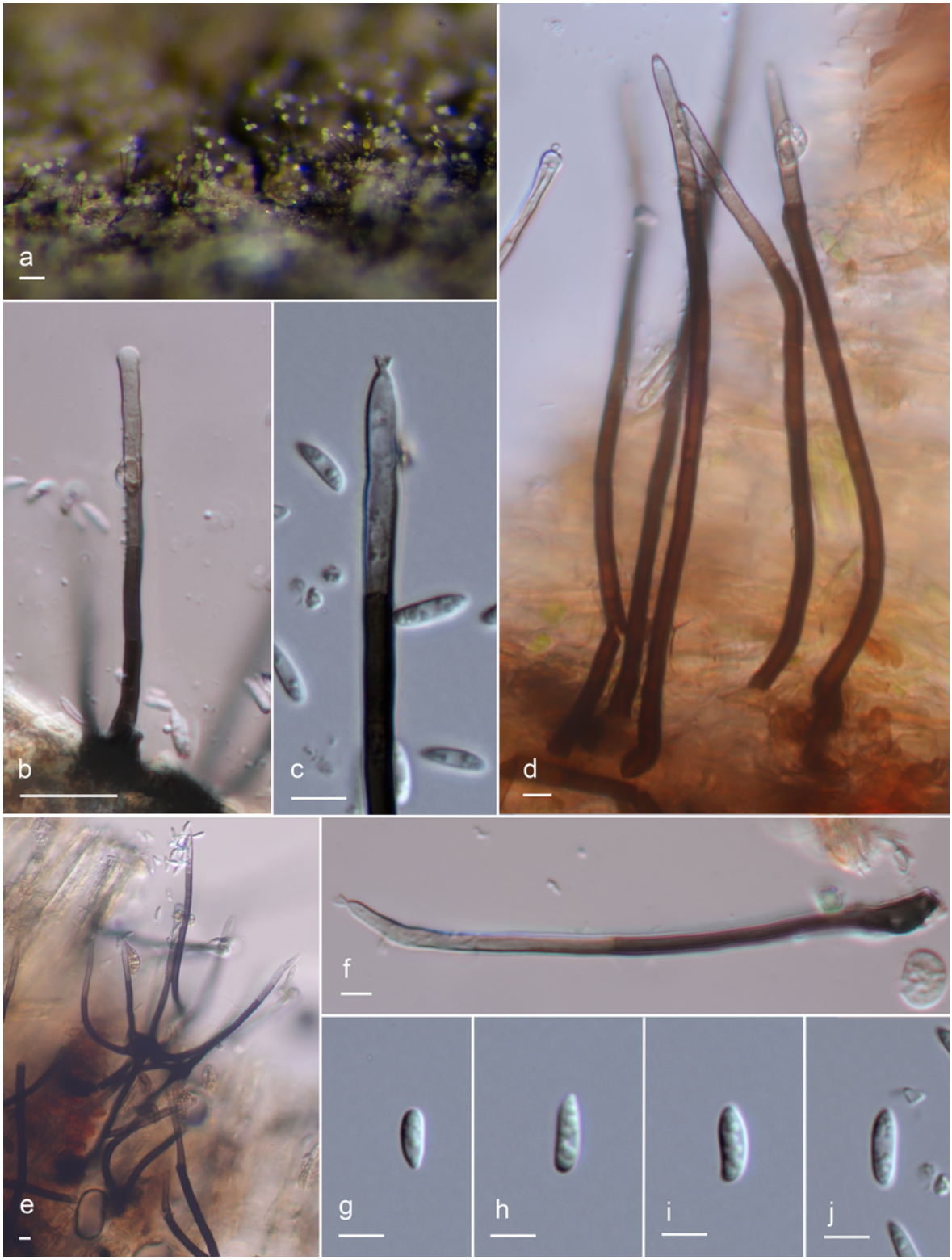

Figure 5

Achrochaeta rivulata (holotype AD291612). **a** Conidiophores on natural substrate. **b** seta. **c** Conidiogenous cell. **d-f** Conidiophores. **g-j** Conidia. Scale bars: **a** = 500 mm, **b** = 20 mm, **c-j**= 5 mm

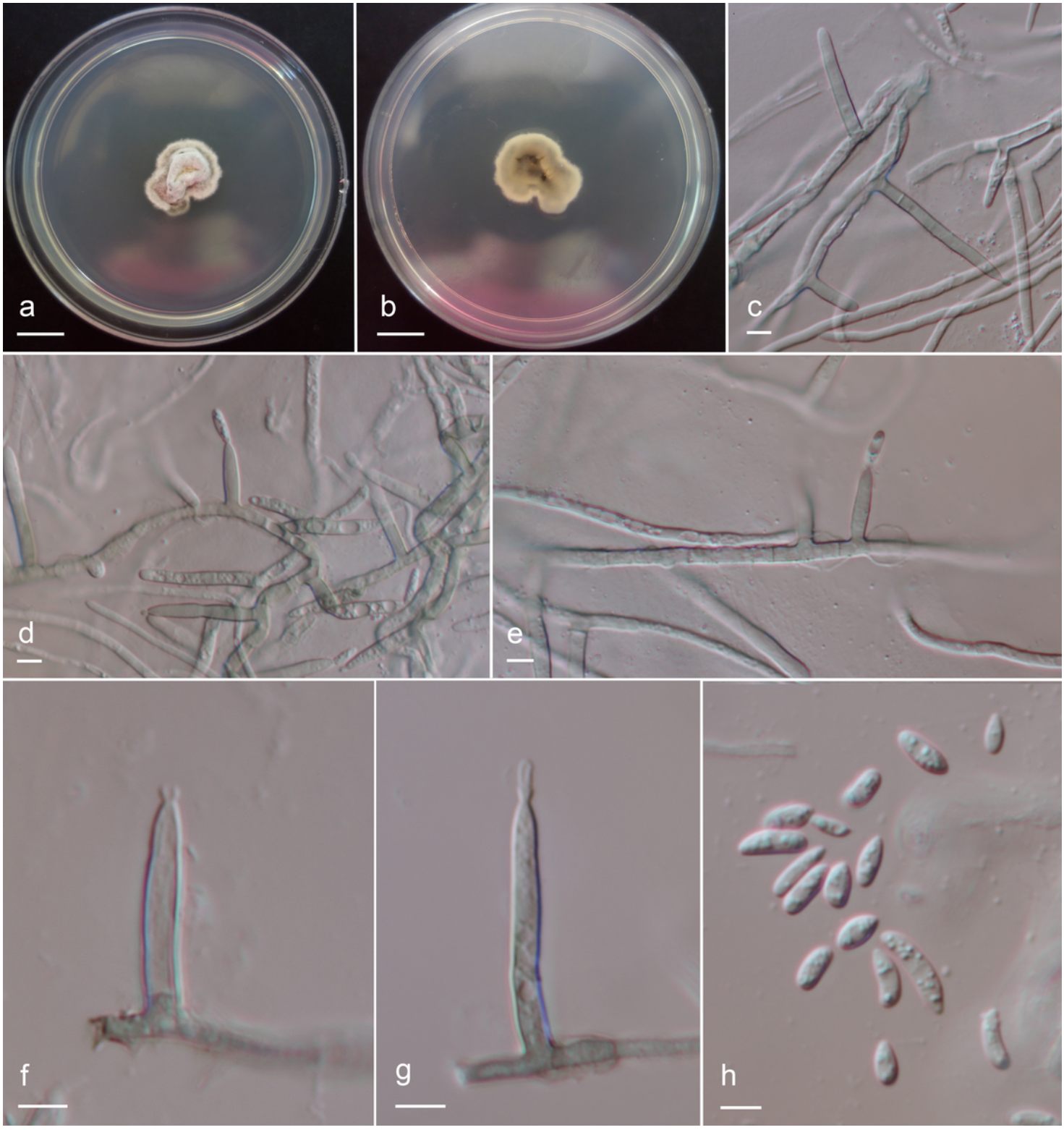

Figure 6

Culture of *Achrochaeta rivulata* (holotype AD291612). **a** Culture on 60mm PDA plate after 4 weeks. **b**Reverse of culture on 60mm PDA plate after 4 weeks. **c-g** Conidiophores. **e**Conidiophore and sheaths on hyphae. **h** Conidia. Scale bars: a = 1 cm, c-h = 5 mm