Seven new Neocallimastigomycota genera from fecal samples of wild, zoo-housed, and domesticated herbivores: Description of *Ghazallomyces constrictus* gen. nov., sp. nov., *Aklioshbomyces papillarum* gen. nov., sp. nov., *Agriosomyces longus* gen. nov., sp. nov., *Capellomyces foraminis* gen. nov., sp. nov. and *Capellomyces elongatus* sp. nov., *Joblinomyces apicalis* gen. nov., sp. nov., *Khoyollomyces ramosus* gen. nov., sp. nov., and *Tahromyces munnarensis* gen. nov., sp. nov.

Radwa A. Hanafy[1], Vikram B. Lanjekar[2], Prashant K. Dhakephalkar [2], Tony M. Callaghan[3,] Sumit S. Dagar[2], Gareth W. Griffith[3], Mostafa S. Elshahed[1], and Noha H. Youssef[1*]

[1]Department of Microbiology and Molecular Genetics, Oklahoma State University, Stillwater, OK. [2]Bioenergy group, Agharkar Research Institute, Pune, India. [3]Institute of Biological, Environmental, and Rural Sciences (IBERS) Aberystwyth University, Aberystwyth, Wales, UK.

*__Correspondence:__ Noha H. Youssef: Noha@Okstate.edu.

ABSTRACT

We isolated and characterized sixty-five anaerobic gut fungi (AGF, Neocallimastigomycota) strains from fecal samples of five wild (W), one zoo-housed (Z), and three domesticated (D) herbivores in the US states of Texas (TX) and Oklahoma (OK), Wales (WA), and the Indian states of Kerala (KE) and Haryana (HA). Phylogenetic assessment based on D1-D2 region of the large rRNA subunit (LSU) identified seven distinct lineages, with strains recovered from Axis Deer (W-TX) clustering within the *Orpinomyces-Neocallimastix-Pecoramyces-Feramyces* clade; Boer Goat-domesticated Goat strains (W-TX, D-KE) clustering within the *Oontomyces-Anaeromyces-Liebetanzomyces* clade; and domesticated Goat and Sheep strains (D-HA) as well as Nilgiri Tahr strains (W-KE) forming two distinct clades associated with genus *Buwchfawromyces*. The remaining three lineages, represented by strains recovered from Mouflon-Boer Goat (W-TX), White Tailed Deer (W-OK), and Zebra-Horse (Z-OK, and D-WA), displayed no specific suprageneric affiliation. All strains displayed monocentric thalli and produced mono/uniflagellate zoospores with the exception of Axis Deer strains, which produced polyflagellate zoospores. Isolates displayed multiple interesting microscopic features including sporangia with tightly constricted necks and fine septa at the base (Axis Deer), papillated and pseudo-intercalary sporangia (White-Tailed Deer), swollen sporangiophores and zoospores with long flagella (Mouflon-Boer Goat), zoospore release through an apical pore followed by either sporangial wall collapse (Axis Deer and Boer Goat-domesticated Goat) or sporangial wall remaining intact after discharge (Zebra-Horse), multi-sporangiated thalli with branched sporangiophores (Zebra-Horse), and short sporangiophores with subsporangial swellings (Nilgiri Tahr). Internal transcribed spacer-1 region (ITS-1) sequence analysis indicated that Zebra-Horse strains are representatives of the AL1 lineage, frequently encountered in culture-independent

surveys of the alimentary tract and fecal samples from hindgut fermenters. The other six lineages, five of which were isolated from wild herbivores, have not been previously encountered in such surveys. Our results significantly expand the genus level diversity within the Neocallimastigomycota, and strongly suggest that wild herbivores represent a yet-untapped reservoir of AGF diversity. We propose the creation of seven novel genera and eight novel Neocallimastigomycota species to accommodate these strains, for which we propose the names *Agriosomyces longus* (Mouflon and wild Boer Goat), *Aklioshbomyces papillarum* (White tailed Deer), *Capellomyces foraminis* (wild Boar Goat) and *C. elongatus* (domesticated Goat), *Ghazallomyces constrictus* (Axis Deer), *Joblinomyces apicalis* (domesticated Goat and Sheep), *Khoyollomyces ramosus* (Zebra-Horse), and *Tahromyces munnarensis* (Nilgiri Tahr). The type species are strains Axs-31, WT-2, MS-4, BGB-11, GFKJa1916, GFH683, ZS-33, and TDFKJa193, respectively.

KEY WORDS: Anaerobic gut fungi, Neocallimastigomycota, Herbivores, 8 new taxa.

INTRODUCTION

Members of the anaerobic gut fungi (AGF, Phylum Neocallimastigomycota) colonize the alimentary tract of mammalian and reptilian herbivores (Gruninger and others 2014; Ljungdahl 2008). Interest in the taxonomy, ecology, cell biology, and genomics of the Neocallimastigomycota has been driven by their unique habitat, physiological preferences, and evolutionary history, as well as by their possession of superior plant polymers degradation capacities (Youssef and others 2013). Such characteristics render them a promising platform for biofuel and biogas production from plant biomass (Ranganathan and others 2017; Young and others 2018).

Currently, eleven different AGF genera have been described (Ariyawansa 2015; Barr and others 1989 ; Breton and others 1990; Callaghan and others 2015; Dagar and others 2015; Gold and others 1988; Hanafy and others 2017; Hanafy and others 2018; Heath and others 1983; Joshi and others 2018; Li 2016; Ozkose and others 2001). However, it is reasonable to assume that multiple novel, yet-uncultured AGF lineages remain to be isolated and characterized. The inherent difficulty in isolating and maintaining these strictly anaerobic and senescence-prone organisms severely hampers isolation and characterization efforts, and limits the number of research groups dedicated to uncovering AGF diversity. Further, it is entirely plausible that multiple AGF taxa are extremely fastidious, with complex nutritional requirements that are not satisfied in current isolation protocols. Indeed, culture-independent diversity surveys utilizing the internal transcribed spacer 1 (ITS-1) as a phylogenetic marker demonstrated that multiple novel AGF lineages remain to be isolated and characterized (Kittelmann and others 2012; Liggenstoffer and others 2010; Paul and others 2018; Mura and others 2019).

On the other hand, culturing efforts have occasionally recovered novel AGF strains that bear no clear similarities to clades identified in culture-independent studies (Callaghan and others 2015; Joshi and others 2018). This surprising observation could be attributed to mismatches in the isolates' ITS-1 region to commonly utilized ITS-1 AGF primers (Callaghan and others 2015), extremely narrow host range of some AGF taxa (Callaghan and others 2015), or existence in extremely low relative abundance *in-situ*.

Moreover, it is important to note that while both culture-based and culture-independent surveys have reported the presence of AGF communities in a relatively wide range of animal hosts, such studies by no means represent an exhaustive catalogue of global AGF diversity in nature. For example, due to logistical considerations, the great majority of studies have utilized samples from domesticated herbivores, with efforts to isolate AGF strains from wild herbivores being extremely rare (Nagpal and others 2011; Paul and others 2010; Tuckwell and others 2005; Hanafy and others 2018).

In an effort to broaden the current Neocallimastigomycota global culture collection, we conducted a multi-year isolation effort targeting novel AGF taxa in fecal samples from a wide range of wild, zoo-housed, and domesticated herbivorous mammals. Here, we report on the isolation and characterization of seven novel AGF genera, including the first cultured representative of the hitherto uncultured AGF lineage AL1. The results expand the known AGF genus-level diversity by >50% (from 11–18), and strongly suggest that wild undomesticated herbivores represent a yet-untapped reservoir of novel AGF taxa.

MATERIALS AND METHODS

Samples— Fecal samples were obtained from Axis Deer (*Axis axis*), White Tailed Deer (*Odocoileus virginianus*), Mouflon Sheep (*Ovis orientalis*), and Boer Goat (*Capra aegagrus*) in two separate hunting expeditions in Sutton and Val Verde counties (TX), and Payne county (OK) in October 2017 and April 2018 (TABLE 1). The hunting parties had all appropriate licenses, and the animals were shot either on private land with the owner's consent or on public land during the hunting season. Samples were also obtained from a Grevy's Zebra (*Equus grevyi*) housed in the Oklahoma City Zoo in May 2018, with the sampling protocol approved by the Oklahoma City Zoo and Botanical Garden's Scientific Review Committee. All fecal samples were placed on ice on site, transferred to the laboratory within 24h of collection, where they were immediately used as an inoculum for subsequent enrichment and isolation procedures.

In India, dried fecal samples were obtained from Nilgiri Tahr (*Nilgiritragus hylocrius*), and domesticated but forest grazing Goat (*Capra aegagrus hircus*) in Munnar in the State of Kerala. Fresh fecal samples were also obtained from domesticated Goats and Sheep (*Ovis aries*) in Sonipat in the State of Haryana. Fresh fecal samples were transferred to the laboratory within 24h of collection, while dried fecal samples were transferred within 72 hours of collection. In Wales, samples were obtained from two domesticated Horses in Llanbadarn Fawr, Ceredigion County, and promptly transferred to the laboratory for processing.

Isolation procedures— In the USA, isolation of anaerobic fungal strains was conducted as previously described (Hanafy and others 2017). Feces were suspended in rumen-fluid (RF) media (Calkins and others 2016; Hanafy and others 2018) with either cellobiose, or 0.5% cellobiose and switchgrass (0.5%) used as a substrate. Antibiotics (50 µg/mL kanamycin, 50 µg/mL penicillin, 20 µg/mL streptomycin, and 50 µg/mL chloramphenicol, respectively) were

added to inhibit growth of bacteria. Samples were serially diluted and incubated at 39 C for 24–48 h. Dilutions showing visible signs of growth (clumping or floating plant materials and production of gas bubbles) were then used for the preparation of roll tubes (Hungate 1969) on RF-cellobiose agar media. Single colonies were picked into liquid RF-cellobiose media, and at least three rounds of tube rolling and colony picking were conducted to ensure purity of the obtained colonies. Strains were maintained by bi-weekly subculturing into RF-cellobiose media.

In India, the fecal samples were homogenised in anaerobic diluent (McSweeney and others 2005) using BagMixer (Interscience, France). One ml of homogenate was inoculated into 9 ml fungal culture medium (Joshi and others 2018) containing neutral detergent fibre (NDF) as the sole carbon source, and serially diluted up to 10^{-3} dilution. The antibiotics benzylpenicillin and streptomycin sulfate (final concentration 2 mg/ml) were used to inhibit the bacterial growth. Following incubation at 39±1 C for 5–10 d, the tubes showing visible colonization of NDF were used to isolate pure cultures of anaerobic fungi using serum roll bottle method as described previously (Joshi and others 2018). The colonies differing in morphology were picked, grown in liquid culture medium, and re-roll bottled until single culture was established.

Long-term storage was conducted by surface inoculation of RF-cellobiose agar media as described previously (Calkins and others 2016), or by cryopreservation at -80 °C using 0.64 M ethylene glycol as the cryoprotectant (Callaghan and others 2015). Cultures are available at Oklahoma State University, Department of Microbiology and Molecular Genetics culture collection, and at MACS Collection of Microorganisms (MCM), Agharkar Research Institute, Pune, India. In Wales, isolation procedures were conducted as previously described in (Callaghan and others 2015).

Morphological characterization— The colony morphology of 3d old cultures on roll bottles was measured using a stereomicroscope (Leica M205 FA) equipped with a digital camera (Leica DFC450 C), or directly from the roll tube. Samples for light and scanning electron microscopy were obtained from liquid cultures at various stages of growth. Lactophenol cotton blue stain was used for visualization of fungal structures using an Olympus BX51 microscope (Olympus, Center Valley, Pennsylvania) equipped with a DP71 digital camera (Olympus), or a phase contrast microscope equipped with a Canon DS126191 digital camera. For examination of nuclei localization, samples were stained with 4′, 6 diamidino-2-phenylindole (DAPI, 10 µg/ml), as previously described (Callaghan and others 2015; Hanafy and others 2018; Joshi and others 2018) and examined using a fluorescence Olympus BX51 microscope (Olympus, Center Valley, Pennsylvania) equipped with a Brightline DAPI high-contrast filter set for DAPI fluorescence and a DP71 digital camera (Olympus), or an Olympus BX53 differential interference contrast (DIC) microscope equipped with a DP73 digital camera (Olympus). Scanning electron microscopy was conducted with a FEI quanta scanning electron microscope (Hillsboro, Oregon, USA), or a Carl Zeiss EVO MA15 (Hanafy and others 2017; Joshi and others 2018).

Phylogenetic analysis— Biomass was harvested and crushed in liquid N_2. DNA was extracted from the ground fungal biomass using DNeasy PowerPlant Pro Kit (Qiagen Corp., Germantown, MD, USA) according to the manufacturer's instructions, or using the CTAB DNA extraction protocol (Joshi and others 2018). To assess phylogenetic relationships, the ITS-1 region, and the D1/D2 region of the 28S rRNA (hereafter LSU) were amplified using the MN100 (5′-TCCTACCCTTTGTGAATTTG-3′) / MNGM2 (5′-CTGCGTTCTTCATCGTTGCG-3′) pair, and the NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) / NL4 (5′-GGTCCGTGTTTCAAGACG G-3′) pair, respectively as previously described (Hanafy and

148 others 2017; Joshi and others 2018). The resulting PCR amplicon for the ITS-1 region was
149 cloned into a TOPO-TA cloning vector according to the manufacturer's instructions (Life
150 Technologies®, Carlsbad, CA) and several clones were Sanger sequenced, while the purified
151 LSU PCR amplicons were directly sequenced using the services of the Oklahoma State
152 University DNA core facility or a commercial provider (1st BASE, Singapore). The obtained
153 sequences were aligned to anaerobic fungal reference ITS-1 and LSU sequences downloaded
154 from GenBank using MAFFT aligner (Nakamura and others 2018) and the alignments were used
155 to construct maximum likelihood phylogenetic trees in MEGA7 (Kumar and others 2016), using
156 *Chytriomyces* sp. JEL176 as the outgroup. Bootstrap values were calculated on the basis of 100
157 replicates.
158 *Ecological distribution*— We queried GenBank and ITS-1 datasets (Kittelmann and others 2012;
159 Liggenstoffer and others 2010; Paul and others 2018) using reference ITS-1 sequences from
160 strains recovered in this study. The phylogenetic position of all closely related sequences (> 87%
161 sequence similarity) was evaluated by insertion into maximum likelihood trees. Taxonomy of
162 uncultured taxa followed the schemes outlined in prior publications (Kittelmann and others 2012;
163 Liggenstoffer and others 2010; Paul and others 2018).
164 *Accession numbers*— Sequences generated in this study have been deposited in GenBank under
165 accession numbers MK881965-MK882046, MK775304, MK775310-MK775313, MK775315,
166 MK775321-MK775324, MK755326-MK755327, MK755330, MK755333. Alignments and
167 phylogenetic trees are available through TreeBase under study accession URL
168 http://purl.org/phylo/treebase/phylows/study/TB2:S24394

RESULTS

Isolation summary— Sixty-five different strains were obtained and characterized in this study (TABLE 1). These isolates were obtained from fecal samples of five wild undomesticated herbivores: Axis Deer (W-TX), White tailed Deer (W-OK), Mouflon (W-TX), Boer Goat (W-TX), and Nilgiri Tahr (Munnar, Kerala, India), one zoo-housed Grevy's Zebra (Z-OK), two domesticated Horses (D-WA), two domesticated Goats (D-HA and D-KE) and a domesticated Sheep (D-HA) (TABLE 1). Morphological, microscopic and phylogenetic analysis described below grouped these isolates into seven distinct clades (Labeled clades 1-7 in TABLE 1 according to alphabetical order of suggested genus names). Isolates representing three clades were obtained from one host animal only: White Tailed Deer strains (Clade 2), Axis Deer strains (Clade 4), and Nilgiri Tahr strains (clade 7); while isolates representing four clades were identified in more than sample: Mouflon-Boer Goat strains (clade 1), Boer Goat-domesticated Goat strains (clade 3), domesticated Sheep-Goat strains (clade 5), and Zebra-Horse strains (clade 6). No specific morphological or microscopic decipherable differences were identified between different strains belonging to most of these clades, and one strain from each group was chosen for detailed analysis (TABLE 1). The only two exceptions were: 1. Strains belonging to clade 3 (Boer Goat-domesticated Goat); where strains from wild Boer Goat (W-TX) displayed distinct microscopic and phylogenetic differences from those obtained from the domesticated Goat (D-KE) to warrant the detailed characterization and eventual description of two different strains (TABLE 1 and detailed descriptions below), and 2. Strains belonging to clade 6 (Zebra-Horse) where few microscopic, but negligible phylogenetic differences were observed between the 16 Zebra strains (Z-OK) and the 5 Horse strains (D-WA) identified. These differences are highlighted below, but we do not believe they warrant the description of a new species, given the

192 negligible sequence divergences between these strains. Below, we provide a detailed
193 characterization of these seven novel groups.
194 *Colony morphology and macroscopic growth characteristics*—Clade 1 (Mouflon-Boer Goat)
195 strain MS-2 produced small brown circular colonies (0.2–1 mm diam.) on agar, and a thin
196 biofilm-like growth in liquid media (FIG. 1a). Clade 2 (White tailed Deer) strain WT-2 produced
197 beige circular colonies (0.5–2.5 mm diam.) with a brown central core of dense sporangial
198 structures and an outer ring of light gray hyphal growth. In liquid media, it produced heavy
199 growth of thick biofilms that firmly attached to the tube's glass surface (FIG. 1b). Clade 3 (Boar
200 Goat-domesticated Goat) strain BGB-11 produced small circular brown colonies (0.1–0.5 mm
201 diam.), with dark center of sporangia structures and a thin fungal biofilm in liquid media (FIG.
202 1c), while Boar Goat-domesticated Goat strain GFKJa1916 produced compact cottony off-white
203 colonies of 2–3 mm size, with a fluffy center of thick sporangial structures and surrounded by
204 radiating rhizoids (FIG. 1d). In liquid media, strain GFKJa1916 produced numerous fungal thalli
205 attached to the glass bottles on initial days of growth, which later developed into thin mat-like
206 structures. Clade 4 (Axis Deer) strain Axs-31 produced small circular white colonies (1–4 mm
207 diameter), with a brown central core of dense sporangial structures on agar and a thick fungal
208 biofilm-like growth in liquid media (FIG. 1e). Clade 5 (Domesticated Goat-Sheep) strain
209 GFH683 produced 1–2 mm sized colonies, having a dense dark central core of abundant
210 sporangial growth, surrounded by long and thin radiating rhizoids (FIG. 1g). In liquid media,
211 strain GFH683 produced numerous fungal thalli attached to the glass bottles on initial days of
212 growth, which later developed into thin mat-like structures. Clade 6 (Zebra-Horse) strains Zebra
213 strain ZS-33 produced small yellow to yellowish brown irregularly shaped colonies (FIG. 1f). In
214 liquid media, the fungal thalli were loose and exhibited a sand-like appearance resembling liquid

growth patterns generally observed with isolates belonging to the bulbous genera *Caecomyces* and *Cyllamyces* (personal observation) (FIG. 1f). Finally, clade 7 (Nilgiri Tahr) strain TDFKJa193 were smaller, approximately 1 mm in size, white in color with a compact and fluffy center, surrounded by dotted circles of fungal thalli. In liquid media, the strain produced numerous fungal thalli attached to the glass bottles on initial days of growth, which later developed into thin mat-like structures (FIG. 1h).

Microscopic features—

Clade 1 (Mouflon-Boer Goat) strains— Strain MS2 produced small globose zoospores, with an average diameter of 4 ± 1.1 µm (average ± standard deviation values from 29 zoospores, range 2.7–7.5 µm). Zoospores were mainly mono-flagellate with a flagellum length of 22 ± 3.8 µm (average ± standard deviation values for 29 zoospores, range 16.6–30 µm), approximately 5–6 times longer than the zoospore body (FIG. 2a). Biflagellate zoospores (FIG. 2b) were rarely encountered. Zoospores germinated into monocentric thalli with filamentous anucleate rhizoidal systems (FIG. 2c-d). Both endogenous and exogenous sporangia were observed, which were very homogenous and displayed no pleomorphism. Endogenous sporangia were globose, with a diameter range of 15–65 µm (FIG. 2e-f). The rhizoid was swollen below the sporangial neck, which was tightly constricted (FIG. 2e-f)**.** Exogenous sporangia were also consistently globose and developed at the end of swollen sporangiophores (30–80 µm L X 5–10 µm W) (FIG. 2g-h). The sporangial neck was constricted with a narrow neck port. Zoospores were released through dissolution and rupturing of the sporangial wall (FIG. 2i).

Clade 2 (White-tailed Deer) strains— Strain WT-2 produced globose zoospores, with an average diameter of 7.4 ± 2.4 µm (average ± standard deviation values for 35 zoospores, range 4.5–13

μm). Zoospores were mostly monoflagellate, with an average flagellum length of 22.8 ± 6.3 μm (average ± standard deviation values for 35 zoospores, range 12–35 μm) (FIG. 3a). Zoospores with two (FIG. 3b) to three (FIG. 3c) flagella were less frequently observed. Fungal thalli were consistently monocentric with filamentous anucleate rhizoids (FIG. 3d). Germination of zoospores produced two types of monocentric thalli, endogenous and exogenous. Endogenous sporangia with single (FIG. 3e) and two adjacent rhizoidal systems (FIG. 3f) were observed. Occasionally, pseudo- intercalary endogenous sporangia (sporangia present in the middle of two main rhizoids) were encountered (FIG. 3g), Similar to what have previously been observed with the genera *Oontomyces* (Dagar and other, 2015) and *Feramyces* (Hanafy and others, 2017). Exogenous sporangia developed at the end of unbranched sporangiophores of varying length from a few microns to 230 μm (FIG. 3h-j). No morphological differences were noticed between endogenous and exogenous sporangia and their shapes ranged from ovoid (FIG. 3e-f), globose (FIG. 3g-h), obpyriform (FIG. 3j-k and 3n-o), and ellipsoidal (FIG. 3i and 3l). Many, but not all, sporangia were papillated with one (FIG. 3m-p) or two (FIG. 3q) papillae. These papillated sporangia are similar to those previously observed in *Piromyces mae* (Li and others 1990). It is believed that these papillae disintegrate to facilitate zoospore release. However, we were unable to observe zoospore discharge through papillae in strain WT-2.

Clade 3 (Boer Goat-domesticated Goat) strains— Boer Goat Strain BGB-11 produced globose zoospores, with an average diameter of 5.5 ± 0.97 μm (average ± standard deviation values for 40 zoospores, range 4–7 μm). The majority of zoospores were mono-flagellate with a flagellum length of 19.6 ± 3.2 μm (average ± standard deviation values for 40 zoospores, range 15–25 μm), (FIG. 4a). Occasionally, biflagellate zoospores were observed (FIG. 4b). Zoospores encystment followed flagellar shedding (FIG. 4c). Zoospore cyst germinated producing germ tube (FIG. 4d)

that subsequently branched (FIG. 4e) into monocentric thalli with filamentous anucleate rhizoidal systems (FIG. 4f-g).

The expansion of the zoospore cysts resulted in the formation of endogenous sporangia that were ellipsoidal (FIG. 4h) and ovoid (FIG. 4i). In addition to endogenous sporangia, exogenous sporangia were also observed at the end of unbranched sporangiophores ranging in length between 20–150 µm (FIG. 4j-p). Some of the sporangiophores ended with sub-sporangial swellings (FIG. 4l-m). Exogenous sporangia varied in shape from ovoid (FIG. 4k-l), ellipsoidal with a single constriction (FIG. 4n), and globose (FIG. 4o-p). Zoospores were liberated through a wide apical pore at the top of the sporangia followed by sporangial wall collapse (FIG. 4m, q-r).

Domesticated Goat strain GFKJa1916 on the other hand produced globose zoospores (FIG. 5a), with an average diameter of 4–5 µm. The majority of zoospores were mono-flagellate with a flagellum length of 15–20 µm. Bi- and tri-flagellate zoospores were also observed. Strain GFKJa1916 zoospores germinated either endogenously or exogenously into a single monocentric thallus, which was also confirmed by the presence of nuclei only in sporangia and their absence in rhizoids (FIG. 5b-c). Endogenous sporangia varied in shape between cylindrical, elongate, globose, sub-globose, ellipsoid & obovoid with sizes ranging between 8–60 µm wide & 10–140 µm long (FIG. 5d-g). Unlike Boer Goat Strain BGB-11, exogenous sporangia in the domesticated Goat strain GFKJa1916 developed at the end of long thick sporangiophores (up to 300 µm in some cases) (FIG. 5h-l), and multisporangiate thalli were commonly observed with two sporangia of either the same (FIG. 5j) or different (FIG. 5K-l) shape, similar to *Piromyces rhizinflatus* (Ho and Barr 1995) and *Neocallimastix frontalis* (Barr and others 1995).

Clade 4 (Axis Deer) strains— Strain Axs-31 produced globose zoospores, with an average diameter of 8.1 ± 1.3 µm (average ± standard deviation values for 35 zoospores, range 6–10.5

284 μm). All zoospores were polyflagellate, with (7–14) flagella and an average flagellum length of
285 23.5 ± 4.9 μm (average ± standard deviation values for 35 zoospores, range 16–31 μm) (FIG.
286 6a). Zoospores germinated into monocentric thalli with highly branched anucleate rhizoidal
287 systems (FIG. 6b-c).

288 Strain Axs-31 exhibited both endogenous and exogenous monocentric thallus
289 development. In endogenous thalli, zoospore cysts enlarged into new sporangia of different
290 shapes, including globose (FIG. 6d), tubular (FIG. 6e), clavate (FIG. 6f) and ellipsoidal (FIG.
291 6g). Endogenous sporangia displayed tightly constricted necks (point between sporangia and
292 rhizoids) with narrow ports (arrows in FIG. 6d-g).

293 During exogenous thallus development, zoospore cysts germinated from both ends.
294 Rhizoids developed on one side while sporangiophores developed on the opposite side. The
295 empty zoospore cyst remained as a persistent swollen structure at the base of sporangiophore
296 (FIG. 6h). Exogenous sporangia developed at the end of unbranched sporangiophores of varied
297 lengths. Short sporangiophores had an average length of 6–20 μm (FIG. 6h-i), while long
298 sporangiophores extended up to 200 μm (FIG. 6j). Some of the short sporangiophores had
299 eggcup-shaped appearance (FIG. 6k). Exogenous sporangia were ellipsoidal (FIG. 6j), ovoid
300 (FIG. 6k), globose (FIG. 6l), constricted ellipsoidal (FIG. 6m), pyriform (FIG. 6n), bowling pin-
301 shaped (FIG. 6o), and rhomboidal (FIG. 6p). Sporangial necks were constricted with narrow port
302 (FIG. 6m-p). At maturity, a fine septum developed at the base of the sporangium (FIG. 6n & p,
303 arrow). Zoospores were released through an apical pore followed by collapse of the sporangial
304 wall (FIG. 6q).
305

306 *Clade 5 (Domesticated Goat and Sheep) strains*— Strain GFH683 produced globose zoospores
307 (FIG. 8a-b), with an average diameter of 5–6 µm. The majority of zoospores were mono-
308 flagellate with 1, or 2 flagella (FIG. 7a-b). Flagellum length ranged between 20–22 µm.
309 Zoospores germinated to produce both endogenous and exogenous monocentric thalli (FIG. 7c-
310 f), as evident by presence of a single sporangium per thallus, nucleated sporangia but anucleate
311 rhizoids. Endogenous sporangia were globose, sub-globose, ovoid, and obovoid (FIG. 7g) with
312 sizes ranging between 8–40 µm wide & 10–40 µm long. Exogenous sporangia were terminal and
313 varied in shape between globose, ovoid, and obovoid with sporangiophores that varied in length
314 from 20–80 µm (FIG. 7h-i). Zoospores discharge occurred through gradual dissolution of a wide
315 apical portion of sporangial wall, resulting in formation of an empty cup-shaped sporangium
316 (FIG. 7j-l). Such zoospore liberation patterns, and empty cup shaped sporangia were earlier
317 documented for *Piromyces minutus* (Ho and Barr 1995).
318 *Clade 6 (Zebra-Horse) strains*— Strain ZS-33 produced spherical zoospores, with an average
319 diameter of 10.8 ± 3 µm (average ± standard deviation of 54 zoospores, range 6–17 µm). All
320 zoospores were uniflagellate, with an average flagellum length of 26 ± 6.5 µm (average ±
321 standard deviation of 54 zoospores, range 18–40 µm) (FIG. 8a). After shedding their flagella,
322 zoospores started to encyst (FIG. 8b) and germinate producing germ tube (FIG. 8c). Germ tube
323 branched and developed a highly branched anucleate rhizoidal system (FIG. 8d-e). Both narrow,
324 0.5–2.5 µm wide, and broad, 3–12.5 µm wide, hyphae were observed; intercalary swellings were
325 frequently encountered in the broad hyphae (arrow in FIG. 8f).
326 Both endogenous and exogenous sporangia were observed. Endogenous sporangia varied
327 in shape and size. Small endogenous sporangia were mainly subglobose (20–60 µm in diameter).
328 (FIG. 8g). Large endogenous (80–160 µm L X 35–65 µm W) sporangia were mainly ellipsoidal

(FIG. 8h). Exogenous sporangia size ranged between (80–270 µm L X 35–85 µm W) and displayed a wider range of morphologies, e.g. heart-shaped (FIG. 8k), ovoid (FIG. 8l) and pyriform (FIG. 8m). Sporangiophores ranged in length between 20–400 µm. Characteristically, strain ZS-33 displayed a multisporangiate thallus: the majority of sporangiophores were branched and bore two to four sporangia (FIG. 8i-j). Similar sporangial morphology has previously been observed in members of the genus *Piromyces* (e.g. *P. rhizinflatus*), and *Caecomyces* (e.g. *C. communis*) (Akin and others 1988; Akin and others 1989; Breton and others 1991). Unbranched sporangiophores with single sporangia were less frequently encountered (approximately 30% of observed sporangiophores n=50, Fig. 8k-m).

Zoospores were liberated through a wide apical pore at the top of the sporangia. The sporangial wall stayed intact after the discharge (FIG. 8n-p). Further, mature sporangia frequently detached from hyphae or sporangiophores, probably serving as an additional mean of fungal dispersal (FIG. 8q).

The type strain ZS-33 was obtained from Zebra fecal samples collected at the Oklahoma City Zoo. No noticeable differences were observed between ZS-33, and all other strains (n=15) obtained from Zebra fecal samples from the Oklahoma City Zoo. On the other hand, two distinct microscopic differences were identified in strains from domesticated Horses in Llanbadarn Fawr, Ceredigion County, Wales. First, multisporangiate thalli, copiously observed in ZS-33, were extremely rare in Welch Horse strains, and second, Distinct-resting stages (FIC. 8r) were often observed in Welch Horse strains, but never in Oklahoma City Zebra strains. Whether these differences are distinct characteristics of each group of strains, or induced by variations in media composition as well as growth and incubation procedures remain to be seen.

Clade 7 (Nilgiri Tahr) strains— Strain TDFKJa193 produced globose zoospores (FIG. 9a), with an average diameter of 3–4 µm. The majority of zoospores were mono-flagellate with 1, 2, or 3 flagella (FIG. 9a). Flagellum length ranged between 12–15 µm. Strain TDFKJa193 exhibited both endogenous and exogenous monocentric thallus development (FIG. 9b-e). Endogenous sporangia were terminal, varied in shape between globose, ovoid & obovoid, and ranged in size between 10–70 µm wide & 12–100 µm long (FIG. 9f-g). Some endogenous sporangia showed sub-sporangial swellings (FIG. 9f). Endogenous sporangia with one or two main rhizoidal systems (FIG. 9f) and with branched rhizoidal system (FIG. 9g) were also observed. Exogenous sporangia, on the other hand, were globose, ovoid & obovoid and were observed at the end of short sporangiophores (12–20 µm) (FIG. 9h-k). Some of the sporangiophores ended with sub-sporangial swellings with (FIG 9 i-j) or without (FIG. 9h&k) constricted neck of 1–8 µm width and 2–10 µm length. The presence of subsporangial swellings and short sporangiophores were previously reported for *Piromyces mae* (Ho and Barr 1995) and *Buwchfawromyces eastonii* (Callaghan and others 2015). Mature exogenous sporangia often showed the formation of a septum at their base (FIG. 9k) similar to *Neocallimastix frontalis* (Ho and Barr 1995). Zoospores liberation happened after irregular dissolution of the sporangial wall (FIG. 9l).

Phylogenetic analysis— Phylogenetic analysis using LSU (FIG. 10a) placed the isolated strains into seven monophyletic and bootstrap-supported lineages that were distinct from all currently described AGF genera. LSU sequence divergence estimates between various strains within a single clade ranged between 0–1%. The closest cultured representative to each of the seven clades is shown in TABLE 1. Within the LSU taxonomic framework, strains recovered from Axis Deer clustered within the *Orpinomyces-Neocallimastix-Pecoramyces-Feramyces* suprageneric clade, while strains

recovered from Boer Goat and domesticated goat clustered within the *Oontomyces-Anaeromyces-Liebetanzomyces* supragenus clade. On the other hand, strains recovered from Nilgiri Tahr, as well as strains recovered from domesticated Goat and Sheep formed two distinct new clades associated with the genus *Buwchfawromyces*. In contrast, strains recovered from Zebra-Horse, and Mouflon-Boer Goat formed two distinct clades associated together but with no specific affiliation to any suprageneric group. The remaining group represented by strains isolated from White Tailed Deer displayed no specific affiliation to any currently characterized genera or supragenus groups within the Neocallimastigomycota.

To investigate ITS-1 sequence variability often reported within a single AGF strain, the ITS-1 region was amplified, cloned, and sequenced from all type strains. ITS-1 sequence divergence within type strains ranged between 0% for strain TDFKJa193 representative of the Nilgiri Tahr strains clade, and 0–8.4% (average 3.4%) for strain MS-2 representative of the Mouflon-Boer Goat strains clade. ITS-1-based analysis confirmed the monophyletic and distinct nature of all seven lineages, but yielded a different topology (FIG. 10b), as consistently observed in prior studies (Hanafy and others 2017; Wang and others 2017). Of special note, was the surprisingly high ITS-1 sequence similarity of the Boer Goat-domesticated Goat clade represented by strains BGB-11 and GFKJa1916 (FIG. 10B), to an *Anaeromyces* sp. isolate GA-04 (GenBank accession number FJ912851.1, unpublished) and to *Anaeromyces robustus* (GenBank accession number NR_148182.1 (Li and others 2016)). Average ITS-1 sequence divergence between various clones of the Boer Goat-domesticated Goat clade and *Anaeromyces* sp. GA-04 was 1.2%, and between various clones of the Boer Goat-domesticated Goat clade and *A. robustus* and was 4.2%. Unfortunately, the LSU sequence data from both *Anaeromyces* sp. GA-04 and *A. robustus* are not available for further comparison. This may be attributed to the inaccurate identification

and reporting of these two cultures, an issue very well known in the anaerobic fungal taxonomy using ITS-1. We noted that in the paper describing *Anaeromyces robustus*, the type strain seems to deviate from the typical morphology of the genus *Anaeromyces*, e.g. multi-flagellate zoospores, lack of sausage shaped hyphae, and whale-tail like sporangia (see Figure 148 in (Li and others 2016)), casting doubts on the phylogenetic affiliation of *Anaeromyces robustus* to the genus *Anaeromyces*. Regardless, that stark morphological differences exist between Boer Goat-domesticated Goat strains and all other members of the genus *Anaeromyces*, e.g. monocentric thalli as opposed to polycentric thalli, absence of hyphal constrictions as opposed to sausage-shaped hyphae with multiple constrictions, and monoflagellate (2–4 flagella) versus uniflagellate zoospores (TABLE 1, FIG. 4-5). Such differences, in addition to the high ITS-1 sequence divergence values between the Boer Goat-domesticated Goat clade and other members of the genus *Anaeromyces* (7.1–13.1% to *A. mucronatus* and 8.5–18.6% to *A. contortus*), strongly support the distinction between these strains and the genus *Anaeromyces*.

Ecological distribution— We queried the GenBank nr database to determine whether representatives of these seven novel clades were encountered in prior ITS-1-based culture-independent AGF diversity surveys. Multiple sequences with high (95.3-100%) sequence similarity to Zebra-Horse strain ZS-33 ITS-1 sequence were identified. These sequences were recovered from fecal samples obtained from multiple animals housed in the Oklahoma City Zoo (Liggenstoffer and others 2010), as well as in various locations (left and right dorsal colon, caecum and right ventral colon) within the digestive tract of horses (Mura and others 2019). This group has previously been assigned the alphanumeric designation (AL1) (Kittelmann and others 2012; Liggenstoffer and others 2010).

Surprisingly, ITS-1 sequences from the remaining six lineages (Axis Deer, White-Tailed

Deer, Mouflon-Boer Goat, Boer Goat-domesticated Goat, domesticated Goat-domesticated Sheep, and Nilgiri Tahr) bore no close resemblance to all currently available ITS-1 sequence data (TABLE 1), with highest similarity being 83% in White-Tailed Deer to sequences from Bontebok, 84% in Mouflon-Boer Goat to sequences from Bontebok, 88% in domesticated goat-domesticated sheep to sequences from horse, 89% in Nilgiri Tahr strains to sequences from Okapi, 91% in Axis Deer to sequences from Llama, and 91-92% in Boer Goat-domesticated Goat strains to sequences from cow. As such, representatives of these novel lineages, the absolute majority of which (5/6) recovered from fecal samples of wild non-domesticated herbivores, do not appear to correspond to any of the alphanumerically designated uncultured groups previously identified in prior culture-independent efforts.

TAXONOMY

Agriosomyces Hanafy, Vikram B. Lanjekar, Prashant K. Dhakephalkar, T.M. Callaghan, Dagar, G.W. Griff, Elshahed, and N.H. Youssef, gen. nov.

MycoBank ID: MB830737

Typification. *Agriosomyces longus*. Hanafy, Vikram B. Lanjekar, Prashant K. Dhakephalkar, T.M. Callaghan, Dagar, G.W. Griff, Elshahed, and N.H. Youssef,

Etymology: Agrioso= derived from the Greek word for wild; *myces* = the Greek name for fungus.

Obligate anaerobic fungus that produces small spherical monoflagellate zoospores with an extremely long flagellum (22 ± 3.8 μm). Zoospores germinate into monocentric thalli with filamentous anucleate rhizoidal systems. Both endogenous and exogenous globose sporangia are observed, which are very homogenous and display no pleomorphism. Rhizoids are swollen below the sporangial neck, which is tightly constricted. Zoospores are released through

dissolution and rupturing of the sporangial wall. The clade is defined by the sequences MK882010-MK882013 (ITS-1) and MK881996 (D1-D2 28S rDNA).

Agriosomyces longus Hanafy, Vikram B. Lanjekar, Prashant K. Dhakephalkar, T.M. Callaghan, Dagar, G.W. Griff, Elshahed, and N.H. Youssef, sp. nov.

MycoBank ID: MB830738.

Typification: The holotype is FIG. 2g in this manuscript, derived from the following: U.S.A. TEXAS: Val verde county, 29.369′ N and 100.829′ W ~300 m ASL, 3d old culture of isolate MS-2, originally isolated from freshly deposited feces of male Mouflon Sheep (*Ovis orientalis*), April 2018, *Radwa Hanafy*. Ex-type strain: MS2, GenBank: MK881996 (D1-D2 28S rDNA).

Etymology: The species epithet "*longus"* refers to the extremely long flagellum observed in zoospores of strain MS-2 (FIG. 2a).

Obligate anaerobic fungus that produces small globose monoflagellate zoospores with an average diameter of 4 ± 1.1 µm. Zoospores are mainly mono-flagellate with a flagellum length of 22 ± 3.8 µm, approximately 5-6 times longer than the zoospore body. Bi-flagellate zoospores are rarely encountered. Zoospores germinate into monocentric thalli with filamentous anucleate rhizoidal systems. Both endogenous and exogenous sporangia are observed, which display no pleomorphism and both show globose morphology. In endogenous sporangia, the rhizoids are swollen below the sporangial neck, which is tightly constricted. Exogenous sporangia develop at the end of swollen sporangiophores, and the sporangial neck is constricted with a narrow neck port. Zoospores are released through dissolution and rupturing of the sporangial wall. Produces small brown spherical colonies on agar, and a thin biofilm-like growth in liquid media. The clade is defined by the sequences MK882010-MK882013 (ITS-1) and MK881996 (D1-D2 28S rDNA).

Additional specimens examined: Radwa Hanafy strain MS-4 (GenBank accession number of D1-D2 28S rDNA amplicon MK881997) isolated from the same freshly deposited feces of male Mouflon Sheep (*Ovis orientalis*) from which the type strain originated, April 2018, and strain BGS-13 (GenBank accession number of D1-D2 28S rDNA amplicon MK881995) isolated from freshly deposited feces of female wild Boer Goat *(Capra aegagrus),* April, 2018.

Aklioshbomyces Hanafy, Vikram B. Lanjekar, Prashant K. Dhakephalkar, T.M. Callaghan, Dagar, G.W. Griff, Elshahed, and N.H. Youssef, gen. nov.

MycoBank ID: MB830735

Typification: Aklioshbomyces papillarum Hanafy, Vikram B. Lanjekar, Prashant K. Dhakephalkar, T.M. Callaghan, Dagar, G.W. Griff, Elshahed, and N.H. Youssef.

Etymology: Aklioshb= derived from the Arabic word for grass-eaters (herbivores); *myces* = the Greek name for fungus.

Obligate anaerobic fungus that produces globose monoflagellate zoospores. Zoospores germinate into monocentric thalli with filamentous anucleate rhizoids. Exhibits both endogenous and exogenous monocentric thallus development. Exogenous sporangia develop at the end of unbranched sporangiophores of varying length. No morphological differences are observed between endogenous and exogenous sporangia, with ovoid, globose, and obpyriform sporangial shapes noted. The clade is defined by the sequences MK882038-MK882042 (ITS-1) and MK882001 (D1-D2 28S rDNA).

Aklioshbomyces papillarum Hanafy, Vikram B. Lanjekar, Prashant K. Dhakephalkar, T.M. Callaghan, Dagar, G.W. Griff, Elshahed, and N.H. Youssef, sp. nov.

MycoBank ID: MB830736

Typification: The holotype is FIG. 3m in this manuscript, derived from the following: U.S.A.

488 OKLAHOMA: Payne county, 36.145′ N and 97.007′ W ~300 m ASL, 3d old culture of isolate
489 WT-2, originally isolated from freshly deposited feces of female White-Tailed Deer (*Odocoileus*
490 *virginianus*), October 2017, *Radwa Hanafy*. Ex-type strain: WT-2, GenBank: MK882001 (D1-
491 D2 28S rDNA).
492 *Etymology:* The species epithet "*papillarum"* refers to the papillae observed on the majority of
493 strain WT-2 sporangia (FIG. 3m-q).
494 Obligate anaerobic fungus that produces globose monoflagellate zoospores with an average
495 diameter of 7.4 ± 2.4 µm. The majority of zoospores are monoflagellate, with zoospores with two
496 to three flagella less frequently observed. Fungal thalli are consistently monocentric with
497 filamentous anucleate rhizoids. Germination of zoospores produces two types of monocentric
498 thalli, endogenous and exogenous. Endogenous sporangia with single and two adjacent rhizoidal
499 systems are observed. Pseudo-intercalary endogenous sporangia are occasionally observed.
500 Sporangiophores carrying exogenous sporangia exhibit varying length from a few microns to 230
501 µm. Endogenous and exogenous sporangia are ovoid, globose, obpyriform, and ellipsoidal.
502 Sporangia are mostly papillated with one or two papillae. Produces beige circular colonies with a
503 brown central core of dense sporangial structures and an outer ring of light gray hyphal growth
504 on agar, and heavy growth of thick biofilms that firmly attached to the tube's glass surface in
505 liquid media. The clade is defined by the sequences MK882038-MK882042 (ITS-1) and
506 MK882001 (D1-D2 28S rDNA).
507 *Additional specimens examined:* Radwa Hanafy strains WT-1 (MK882000), WT-3 (MK881998),
508 WT-4 (MK881999), WT-41(MK882002), WTS-51 (MK882006), WTS-52 (MK882003), WTS-
509 53 (MK882004), and WTS-54 (MK882005), (GenBank accession number of D1-D2 28S rDNA
510 amplicon in parenthesis) isolated from the same freshly deposited feces of female White-Tailed

Deer from which the type strain originated, October 2017.

Capellomyces Hanafy, Vikram B. Lanjekar, Prashant K. Dhakephalkar, T.M. Callaghan, Dagar, G.W. Griff, Elshahed, and N.H. Youssef, gen. nov.

MycoBank ID: MB830739.

Typification. Capellomyces foraminis. Hanafy, Vikram B. Lanjekar, Prashant K. Dhakephalkar, T.M. Callaghan, Dagar, G.W. Griff, Elshahed, and N.H. Youssef.

Etymology: Capella= derived from the Latin word for Goat; *myces* = the Greek name for fungus.

Obligate anaerobic fungus that produces monoflagellate (1–3 flagella) zoospores. Zoospores germinate into monocentric thalli with filamentous anucleate rhizoidal systems. Both endogenous and exogenous sporangia are observed, with varying shapes and sizes. The clade is defined by the sequences MK882007-MK882009 (ITS-1), and MK881975 (D1-D2 28S rDNA).

Capellomyces foraminis Hanafy, Vikram B. Lanjekar, Prashant K. Dhakephalkar, T.M. Callaghan, Dagar, G.W. Griff, Elshahed, and N.H. Youssef, sp. nov.

MycoBank ID: MB830740

Typification: The holotype is FIG. 4n in this manuscript, derived from the following: U.S.A. U.S.A. TEXAS: Val verde county, 29.369′ N and 100.829′ W ~300 m ASL, 3d old culture of isolate BGB-11, originally isolated from freshly deposited feces of a female Boer Goat (*Capra aegagrus*) April 2018, *Radwa Hanafy.* Ex-type strain: BGB-11, GenBank: MK881975 (D1-D2 28S rDNA).

Etymology: The species epithet "*foraminis"* refers to the wide apical pore at the top of the sporangia through which zoospores are discharged.

Obligate anaerobic fungus that produces spherical monoflagellate zoospores. Zoospores start to encyst after shedding their flagella. Zoospore cyst germinates, producing germ tube that

subsequently branches into monocentric thalli with filamentous anucleate rhizoidal systems. Endogenous and exogenous sporangia are produced. Endogenous sporangia are ellipsoidal or ovoid. Exogenous sporangia are formed at the end of un-branched sporangiophores (20–150 µm). Some of the sporangiophores exhibit sub-sporangial swellings. Exogenous sporangia are ovoid, ellipsoidal with a single constriction, and globose. Zoospores are liberated through a wide apical pore at the top of the sporangia followed by sporangial wall collapse. Colonies are small (0.1–0.5 mm diameter) circular and brown, with dark center of sporangia structures on agar. Produces thin fungal biofilm in liquid media. The clade is defined by the sequences MK882007-MK882009 (ITS-1), and MK881975 (D1-D2 28S rDNA).

Additional species examined. Radwa Hanafy, strains BGB-2 (MK881974), BGC-12 (MK881976), BGS-11 (MK881977), and BGS-12 (MK881978) isolated from the same freshly deposited feces of a female Boer Goat (*Capra aegagrus*), April 2018, from which the type strain originated, April 2018 (D1-D2 28S rDNA amplicon GenBank accession number in parenthesis).

***Capellomyces elongatus*.** Hanafy, Vikram B. Lanjekar, Prashant K. Dhakephalkar, T.M. Callaghan, Dagar, G.W. Griff, Elshahed, and N.H. Youssef.

MycoBank: MB830869.

Typification: The holotype is FIG. 5k in this manuscript, derived from the following: India, KERALA, town of Munnar, 10.219′ N and 77.106′ E ~2100 m ASL, 3d old culture of isolate GFKJa1916, originally isolated from freshly deposited feces of a domesticated but forest grazing Goat (*Capra aegagrus*), *Sumit S. Daggar.* Ex-type strain: GFKJa1916, GenBank: ITS-1 (MK775315), D1-D2 28S rDNA (MK775304).

Etymology: The species epithet "*elongatus"* refers to the characteristic long sporangiophore of exogenous sporangia.

Obligate anaerobic fungus that produces globose monoflagellate (1, 2, or 3 flagella) zoospores. Zoospore cyst germinate both endogenously and exogenously to produce monocentric thalli with filamentous anucleate rhizoidal systems. Endogenous sporangia are cylindrical, elongate, globose, sub-globose, ellipsoid & obovoid with sizes ranging between 8–60 µm wide & 10–140 µm long. Exogenous sporangia are formed at the end of developed at the end of long thick sporangiophores (up to 300 µm). Multisporangiate thalli are commonly observed with two sporangia of either the same or different shapes. Colonies are compact of 2–3 mm size, cottony and off-white in color with a compact and fluffy center made up of thick sporangia type structures, and surrounded by radiating rhizoids. Produces numerous fungal thalli that attach to the glass bottles on initial days of growth, which later develop into thin mat-like structures in liquid media. The clade is defined by the sequence MK775304 (D1-D2 28S rDNA).

Additional species examined. None

Ghazallomyces Hanafy, Vikram B. Lanjekar, Prashant K. Dhakephalkar, T.M. Callaghan, Dagar, G.W. Griff, Elshahed, and N.H. Youssef, gen. nov.

MycoBank ID: MB830733

Typification: Ghazallomyces constrictus Hanafy, Vikram B. Lanjekar, Prashant K. Dhakephalkar, T.M. Callaghan, Dagar, G.W. Griff, Elshahed, and N.H. Youssef.

Etymology: Ghazallo = derived from the Arabic word for Deer (Ghazalla); *myces* = the Greek name for fungus.

Obligate anaerobic fungus that produces polyflagellate zoospores. Zoospores germinate into monocentric thalli with highly branched anucleate rhizoidal systems. Exhibits both endogenous and exogenous monocentric thallus development. Sporangia produced from endogenous and exogenous thalli development are pleomorphic, exhibiting a wide range of sporangial shapes.

During exogenous thallus development, zoospore cysts germinate from both ends, with rhizoids developing from one side and sporangiophore developing from the opposite side. The empty zoospore cyst remains as a persistent swollen structure at the base of unbranched sporangiophore that exhibit wide variations in lengths. Zoospores are released through an apical pore followed by collapse of the sporangial wall. The clade is defined by the sequences MK882043 (ITS-1) and MK881971 (D1-D2 28S rDNA).

Ghazallomyces constrictus Hanafy, Vikram B. Lanjekar, Prashant K. Dhakephalkar, T.M. Callaghan, Dagar, G.W. Griff, Elshahed, and N.H. Youssef, sp. nov.

MycoBank ID: MB830734

Typification: The holotype is FIG. 6h in this manuscript, derived from the following: U.S.A. TEXAS: Sutton county, 30.591′ N and 100.138′ W ~300 m ASL, 3d old culture of isolate Axs-31, originally isolated from freshly deposited feces content of female Axis Deer (*Axis axis)*, Apr. 2018, *Radwa Hanafy.* Ex-type strain: Axs-31, GenBank: MK881971 (D1-D2 28S rDNA).

Etymology: The species epithet "*constrictus"* refers to the observed constricted necks (point between sporangia and rhizoids) in the species endogenous sporangia (FIG. 6d-g).

Obligate anaerobic fungus that produces globose polyflagellate zoospores with 7–14 flagella. Zoospores germinate into monocentric thalli with highly branched anucleate rhizoidal systems. Exhibits both endogenous and exogenous monocentric thallus development. Endogenous sporangia produced from zoospore cyst enlargement develop into different shapes including globose, tubular, clavate, and ellipsoidal. Endogenous sporangia display tightly constricted necks (point between sporangia and rhizoids) with narrow ports. Exogenous sporangia develop at the end of unbranched sporangiophores of varied lengths. Both short (6–20µm) and long (up to 200µm) sporangiophores are observed. The exogenous sporangia display ellipsoidal, ovoid,

603 globose, constricted ellipsoidal, pyriform, bowling pin-like, and rhomboidal shapes. Sporangial
604 necks are constricted with narrow port. A fine septum develops at the base of the sporangium at
605 maturity. Zoospores are released through an apical pore followed by collapse of the sporangial
606 wall. Produces small circular white colonies (1–4 mm diameter) with a brown central core of
607 dense sporangial structures on agar, and a thick fungal biofilm growth in liquid media. The clade
608 is defined by the sequences MK882043 (ITS-1) and MK881971 (D1-D2 28S rDNA).
609 *Additional specimens examined:* Radwa Hanafy strains ADC-2 (MK881965), ADS-14
610 (MK881966), AXS-33 (MK881967), AXS-34 (MK1881968), ADS-12 (MK881969), AXS-32
611 (MK881970), ADS-11 (MK881972), and ADS-21 (MK881973) (GenBank accession number of
612 D1-D2 28S rDNA amplicon in parenthesis) isolated from the same freshly deposited feces of
613 female Axis Deer (*Axis axis)* from which the type strain originated, April 2018.
614 ***Joblinomyces*** Hanafy, Vikram B. Lanjekar, Prashant K. Dhakephalkar, T.M. Callaghan, Dagar,
615 G.W. Griff, Elshahed, and N.H. Youssef, gen. nov.
616 MycoBank ID: MB830867
617 *Typification. Joblinomyces apicalis*. Hanafy, Vikram B. Lanjekar, Prashant K. Dhakephalkar,
618 T.M. Callaghan, Dagar, G.W. Griff, Elshahed, and N.H. Youssef.
619 *Etymology: Joblino*= honoring Keith N. Joblin for his contributions to the field of anaerobic
620 fungi; *myces* = the Greek name for fungus.
621 Obligate anaerobic fungus that produces globose monoflagellate zoospores. Both endogenous
622 and exogenous sporangia are observed with varying shapes and sizes. Sporangiophores of
623 exogenous sporangia vary in length. Exogenous sporangia have short and frequently swollen
624 sporangiophores. Zoospores discharge occurs through gradual dissolution of a wide apical

625 portion of sporangial wall, resulting in formation of an empty cup-shaped sporangium. The clade
626 is defined by the sequences MK910278 (ITS-1) and MK910268 (D1-D2 28S rDNA).
627 ***Joblinomyces apicalis.*** Hanafy, Vikram B. Lanjekar, Prashant K. Dhakephalkar, T.M.
628 Callaghan, Dagar, G.W. Griff, Elshahed, and N.H. Youssef.
629 MycoBank ID: MB830868
630 *Typification*: The holotype is FIG. 7c in this manuscript, derived from the following: India,
631 HARYANA, city of Sonipat, 28.988′ N and 76.941′ E ~220 m ASL, 3d old culture of isolate
632 GFH683, originally isolated from freshly deposited feces of a domesticated goat (*Capra*
633 *aegagrus hircus*), *Sumit Dagar*. Ex-type strain: GFH683, GenBank: MK910268 (D1-D2 28S
634 rRNA).
635 *Etymology:* The species epithet "*apicalis*" refers to the zoospore discharge through the
636 dissolution of a wide apical portion of the sporangial wall.
637 Obligate anaerobic fungus that produces globose monoflagellate zoospores with 1, or 2 flagella.
638 Zoospores germinate to produce both endogenous and exogenous monocentric thalli.
639 Endogenous sporangia vary in shape between globose, sub-globose, ovoid, and obovoid with
640 sizes ranging between 8–40 µm wide & 10–40 µm long. Exogenous sporangia are terminal and
641 vary in shape between globose, ovoid, obovoid. Sporangiophores vary in length from 20–80 µm.
642 Zoospores discharge occur through gradual dissolution of a wide apical portion of sporangial
643 wall, resulting in formation of an empty cup-shaped sporangium. Produces 1–2 mm sized
644 colonies with a dense dark central core of abundant sporangial growth, surrounded by long and
645 thin radiating rhizoids. In liquid media, it produces numerous fungal thalli that attach to the glass
646 bottles on initial days of growth, and later develop into thin mat-like structures. The clade is

defined by the sequences MK910278-MK910282 (ITS-1) and MK910268-MK910272 (D1-D2 28S rDNA).

Additional specimens examined: Sumit Dagar strains GFH681 (MK910263-MK910267) and GFH682 (MK775330) (GenBank accession number of D1-D2 28S rDNA amplicon in parenthesis) isolated from the same freshly deposited domesticated goat feces from which the type strain originated, and SFH683 (MK775333) isolated from freshly deposited domesticated sheep feces in the city of Sonipat, Haryana, India.

Khoyollomyces Hanafy, Vikram B. Lanjekar, Prashant K. Dhakephalkar, T.M. Callaghan, Dagar, G.W. Griff, Elshahed, and N.H. Youssef, gen. nov.

MycoBank ID: MB830741

Typification. Khoyollomyces ramosus. Hanafy, Vikram B. Lanjekar, Prashant K. Dhakephalkar, T.M. Callaghan, Dagar, G.W. Griff, Elshahed, and N.H. Youssef.

Etymology: Khyollo= derived from the Arabic word for horses; *myces* = the Greek name for fungus.

Obligate anaerobic fungus that produces spherical uniflagellate zoospores. Zoospores encyst and develop a highly branched anucleate rhizoidal system. Both endogenous and exogenous sporangia are observed. Small endogenous sporangia are subglobose and large endogenous sporangia are ellipsoidal. Exogenous sporangia displayed a wider range of shapes. The majority of sporangiophores are branched and bear two to four sporangia. Unbranched sporangiophores bearing a single sporangium are less frequently encountered. Zoospores are liberated through a wide apical pore at the top of the sporangia. Mainly found in the digestive tracts of equids. The clade is defined by the sequences MK882019 (ITS-1), and MK881981 (D1-D2 28S rDNA).

669 ***Khoyollomyces ramosus.*** Hanafy, Vikram B. Lanjekar, Prashant K. Dhakephalkar, T.M.
670 Callaghan, Dagar, G.W. Griff, Elshahed, and N.H. Youssef, sp. nov.
671 MycoBank ID: MB830742.
672 *Typification*: The holotype is FIG. 8j in this manuscript, derived from the following: U.S.A.
673 OKLAHOMA: Oklahoma City, 35.524′ N and 97.472′ W ~300 m ASL, 3d old culture of isolate
674 ZS-33, originally isolated from freshly deposited feces of a Grevy's Zebra *(Equus grevyi)*, May,
675 2018, *Radwa Hanafy*. Ex-type strain: ZS-33, GenBank: MK881981 (D1-D2 28S rDNA).
676 *Etymology:* The species epithet "*ramosus"* (Latin for branched) refers to the observed branched
677 sporangiophores bearing two to four sporangia in *K. ramosus* type strain ZS-33 (FIG. 8i-k).
678 Obligate anaerobic fungus that produces spherical uniflagellate zoospores. Zoospores encyst and
679 germinate producing germ tube that develops into a highly branched anucleate rhizoidal system.
680 Both narrow, 0.5–2.5µm wide, and broad hyphae, 3–12.5µm wide, are produced; intercalary
681 swellings are frequently encountered in the broad hyphae. Both endogenous and exogenous
682 sporangia were observed. Endogenous sporangia vary in shape and size, with small endogenous
683 sporangia mainly subglobose (20–60 µm in diameter) while large endogenous (80–160µm L X
684 35–65µm W) sporangia mainly ellipsoidal. Exogenous sporangia ranged in size between (80–
685 270µm L X 35–85µm W)) and display a wide range of morphologies, e.g. heart-shaped, ovoid,
686 and pyriform. Displays a multisporangiate thallus, with the majority of sporangiophores being
687 branched and bearing two to four sporangia. Unbranched sporangiophores with single sporangia
688 are less frequently encountered (approximately 30% of observed sporangiophores). Zoospores
689 are liberated through a wide apical pore at the top of the sporangia. The sporangia stay intact
690 after the discharge. Mature sporangia frequently detach from hyphae or sporangiophores.
691 Produces small yellow to yellowish brown irregularly shaped colonies on agar. In liquid media,

692 the fungal growth is loose and exhibited a sand-like appearance. The clade is defined by the
693 sequences MK882019 (ITS-1), and MK881981 (D1-D2 28S rDNA).
694 *Additional specimens examined:* Radwa Hanafy strains ZC-31 (MK881979), ZC-32
695 (MK881980), ZC-33 (MK881981), ZC-41 (MK881982), ZC-42 (MK881983), ZC-43
696 (MK881984), ZC-51 (MK881985), ZC-53 (MK881986), ZS-21 (MK881987), ZS-22
697 (MK881988), ZS-31 (MK881989), ZS-32 (MK881990), ZS-41 (MK881992), ZS-42
698 (MK881993), and ZS-43 (MK881994) (GenBank accession number of D1-D2 28S rDNA
699 amplicon in parenthesis) isolated from the same freshly deposited Zebra feces from which the
700 type strain originated, May 2018. Tony Callaghan strains: HoCal4.A2, HoCal4.A2.2, HoCal4.A4
701 isolated from fresh horse feces (Llanbadarn, nr. Aberystwyth; 52.4156,-3.8878), August 2013.
702 Two further cultures (Tmc003.6a, TMC3.6b) were isolated from a different horse at the same
703 site, November 2013.
704 ***Tahromyces*** Hanafy, Vikram B. Lanjekar, Prashant K. Dhakephalkar, T.M. Callaghan, Dagar,
705 G.W. Griff, Elshahed, and N.H. Youssef, gen. nov.
706 MycoBank ID: MB830865
707 *Typification. Tahromyces munnarensis*. Hanafy, Vikram B. Lanjekar, Prashant K.
708 Dhakephalkar, T.M. Callaghan, Dagar, G.W. Griff, Elshahed, and N.H. Youssef.
709 *Etymology: Tahro*= referring to the Nilgiri Tahr from which the species was isolated; *myces* =
710 the Greek name for fungus.
711 Obligate anaerobic fungus that produces globose monoflagellate zoospores. Both endogenous
712 and exogenous sporangia are observed with varying shapes and sizes. Endogenous sporangia
713 with one or two main rhizoidal systems and with branched rhizoidal system are frequently
714 observed. Exogenous sporangia have short and frequently swollen sporangiophores. Sporangial

necks are frequently constricted. Septa often form at the base of mature exogenous sporangia. Zoospores liberation happens after irregular dissolution of the sporangial wall. The clade is defined by the sequences MK775321 (ITS-1), and MK775310 (D1-D2 28S rDNA).

Tahromyces munnarensis Hanafy, Vikram B. Lanjekar, Prashant K. Dhakephalkar, T.M. Callaghan, Dagar, G.W. Griff, Elshahed, and N.H. Youssef.

MycoBank ID: MB830866

Typification: The holotype is FIG. 9h in this manuscript, derived from the following: India, KERALA, town of Munnar, 10.219′ N and 77.106′ E ~2100 m ASL, 3d old culture of isolate TDFKJa193, originally isolated from freshly deposited feces of a Nilgiri Tahr *(Nilgiritragus hylocrius), Sumit Dagar*. Ex-type strain: TDFKJa193, GenBank: ITS-1 accession number (MK775321), D1-D2 28S rDNA (MK775310).

Etymology: The species epithet "*munnarensis"* refers to the town that the type species was isolated from.

Obligate anaerobic fungus that produces globose monoflagellate zoospores with 1, 2, or 3 flagella. Both endogenous and exogenous sporangia were observed. The sporangia vary in size between 12–100 µm in length and 10–70 µm in width, and display a wide range of morphologies like globose, ovoid, and obovoid. Sporangiophores are short (12–20 µm) with frequent subsporangial swellings. Sporangial necks (1–8 µm width and 2–10 µm length) are frequently constricted. Septa often form at the base of mature exogenous sporangia. Zoospores liberation happens after irregular dissolution of the sporangial wall. Produces colonies that are small (1 mm), white in color with a compact and fluffy center, surrounded by dotted circles of fungal thalli. In liquid media, it produces numerous fungal thalli attaching to the glass bottles on initial

737 days of growth, and later developing into thin mat-like structures. The clade is defined by the
738 sequence MK775310 (D1-D2 28S rDNA).
739 *Additional specimens examined:* Sumit Dagar strains TDFKJa1924 (MK775323), TDFKJa1926
740 (MK775322), and TDFKJa1927 (MK775324) (GenBank accession number of D1-D2 28S rDNA
741 amplicon in parenthesis) isolated from the same Nilgirir Tahr feces from which the type strain
742 originated.
743

DISCUSSION

Here, we report on the isolation and characterization of multiple novel AGF strains from a concerted sampling effort of domesticated, zoo-housed, and wild animals from North America, Europe, and Asia. We propose seven new AGF genera to accommodate these novel strains, hence expanding the AGF genus-level diversity by more than 50% (From 11 to 18). All newly described taxa produced filamentous, monocentric thalli, similar to seven of the eleven currently described genera. Six of the seven novel genera described here produce mono/uniflagellate zoospores, similar to eight of the eleven currently described taxa. As such, filamentous taxa with moncentric thalli and monflagellate zoospore appear to be the most common thallus morphology and zoospore flagellation patterns in the Neocallimastigomycota predominant within currently described AGF genera. It is interesting to note that for decades, microscopic-based identification of AGF strains typically assigned isolates with such morphology to the genus *Piromyces* (Ho and others 1993). We note broad similarities between the microscopic features of *Aklioshbomyces papillarum and P. mae* (papillated sporangia), and *Joblinomyces* and *P. Minutus* (zoospores release through a wide apical portion of sporangial wall, resulting in formation of an empty cup-shaped sporangium). Unfortunately, the absnece of sequence data and extant cultures of these previously described "*Piromyces*" taxa prevents futher investigation into this issue (Ho and others 1993).

The current isolates were obtained in a multi-year effort to describe novel AGF strains from a wide range of animal hosts in the United States, India, Wales. The majority of novel taxa described here (5/7 genera, 6/8 species) originated from wild undomesticated animals (Axis Deer, White Tailed Deer, Mouflon, Boer Goat, and Nilgiri Tahr), underscoring their potential as novel, yet-untapped reservoir of AGF diversity. Such novelty, which has recently been

767 postulated (Hanafy and others 2018) could be attributed to higher variability in the quality and
768 quantity of ingested plant material, and the significant daily and seasonal fluctuations in feeding
769 frequencies.

770 Culture-independent surveys utilizing ITS-1 as a phylogenetic marker (Kittelmann and others
771 2012; Liggenstoffer and others 2010), and subsequent meta-analysis (Kittelmann and others 2012;
772 Kittelmann and others 2013) have identified multiple novel yet-uncultured AGF genus-level lineages.
773 This study has been successful in isolating the first representatives of novel group AL1 from Zebra
774 and Horse fecal samples. In a prior survey of AGF in zoo-housed animals (Liggenstoffer and others
775 2010), members of this lineage were encountered in approximately half of the animal hosts examined
776 (18/35). AL1-affiliated sequences were more predominant in hindgut fermenters (7/9 hosts), and
777 comprised a relatively high proportion of the AGF community in multiple hosts, e.g. 99.9% in three
778 different Zebra individuals, 56.7 and 68.3% in two horses, and 29.6% in a Grant's Gazelle). By
779 comparison, they were only encountered in 11/26 foregut fermenters, where they constituted 0.01%
780 to 38% of the AGF community in these animals. Further a recent seminal spatial analysis that
781 analyzed AGF community in samples directly obtained from various locations along horses digestive
782 tracts (Mura and others 2019) identified AL1 group as a prominent component of the AGF
783 community in the right ventral (88%), and left dorsal (98%) colons in horses. As such, this novel
784 genus appears to exhibit a preference for hindgut fermenters of the family Equidae. The reason for
785 such preferences, and the general preference of some fungal taxa to specific hosts remains unclear
786 (Callaghan and others 2015; Dagar and others 2015).

787 Surprisingly, comparative analysis of ITS-1 sequences indicated that the Axis Deer, White
788 tailed Deer, Mouflon-Boer Goat, Boer Goat-domesticated Goat, Nilgiri Tahr, and domesticated Goat-
789 sheep groups appear to be completely novel, and previously unencountered in prior culture-based or

790 culture-independent studies. ITS-1 sequences from these four isolates did not display mismatches to
791 common ITS-1 primers, did not have an atypical length that could hinder its amplification or
792 detection via PCR, and were readily amplified from pure-cultures' genomic DNA. As such, we posit
793 that the lack of prior observation of these taxa is biologically relevant, and is indicative of their
794 relatively specific host preference and/or predominance in wild, rather than domesticated herbivores.
795 Indeed, although 30 animals were screened in the current study, three of these seven novel genera
796 were isolated only from a single host (White tailed Deer, Axis Deer, and Nilgiri Tahr), while the
797 other four were isolated from only two hosts (Mouflon and Boer Goat, Boer Goat and domesticated
798 Goat, domesticated Goat and Sheep, and Zebra-domesticated Horse) (TABLE 1).
799 Collectively, the steady identification of novel taxa in culture-based and culture-independent
800 surveys, as well as the sparse overlap between these studies strongly suggests that the scope of AGF
801 diversity in nature is much broader than currently estimated (Kittelmann and others 2012; Paul and
802 others 2018). Compared to the prokaryotic component of the rumen and herbivorous gut, the diversity
803 of the rumen mycobiome remains woefully understudied. To provide a more thorough understanding
804 of the AGF diversity in nature, concerted efforts that systematically assess the AGF diversity and
805 community structure in various spatial (e.g. across various compartments of the herbivorous gut),
806 temporal (e.g. across the lifespan of an animal), and geographic dimensions in a wide range of
807 domesticated and wild herbivores is needed. Much remains to be understood regarding the diversity
808 and community structure of AGF within various locations of the gastrointestinal tract of an animal
809 host, interspecies stochastic differences between AGF communities in animal subjects, temporal age-
810 related progression of AGF in animal hosts, and the response of the AGF community to various
811 factors e.g. feeding patterns, antibiotic administration, animal disease, and co-housing arrangements
812 and combinations thereof.

ACKNOWLEDGMENTS We thank Jim and Tammy Austin for providing fecal samples, Britny Johnson for technical assistance, Dr. Karthick Balasubramanian for his valuable help, Drs. Rebecca Snyder and Jennifer D'Agostino for providing fecal samples from the Oklahoma City Zoo, and to the Kerala Forest Department, Munnar for sampling permission (WL10-51713/2017). This work has been funded by the NSF-DEB Grant number 1557102 to NHY and MSE, Department of Biotechnology (DBT) project no. BT/PR15694/PBD/26/506/2015 to SSD.

819 **LITERATURE CITED**

820 Akin D, Borneman W, Windham W. 1988. Rumen fungi: morphological types from Georgia
821 cattle and the attack on forage cell walls. Biosystems 21 385–391.
822
823 Akin D, Lyon C, Windham W, Rigsby L. 1989. Physical degradation of lignified stem tissues by
824 ruminal fungi. Applied and Environmental Microbiology 55:611–616.
825
826 Ariyawansa HA, Hyde KD, Jayasiri SC, Buyck B, Thilini Chethana KW, Dai DQ, Dai YC,
827 Daranaama DA, Jayawardena RS, Lücking R, Ghobad-Nejhad M, Niskanen T, Thambugala KM,
828 Voigt K, Zhao RL, Li G-J, Doilom M, Boonmee S, Yang, ZL, Cai Q, Cui Y-Y, Bahkali AH,
829 Chen J, Cui BK, Chen JJ, Dayarathne MC, Dissanayake AJ, Ekanayaka AH, Hashimoto A,
830 Hongsanan S, Jones EBG, Larsson E, Li WJ, Li Q-R, Liu JK, Luo ZL, Maharachchikumbura
831 SSN, Mapook A, McKenzie EHC, Norphanphoun C, Konta S, Pang KL, Perera RH,
832 Phookamsak R, Phukhamsakda C, Pinruan U, Randrianjohany E, Singtripop C, Tanaka K, Tian
833 CM, Tibpromma S, Abdel-Wahab MA, Wanasinghe DN, Wijayawardene NN, Zhang JF, Zhang
834 H, Abdel-Aziz FA, Wedin M, Westberg M, Ammirati JF, Bulgakov T, Lima DX, Callaghan TM,
835 Callac P, Chang CH, Coca LF, Dal-Forno M, Dollhofer V, Fliegerová K, Greiner K, Griffith
836 GW, Ho H-M, Hofstetter V, Jeewon R, Kang JC, Kirk PM, Kytövuori I, Lawrey JD, Xing J, Li
837 H, Liu ZY, Liu XZ, Liimatainen K, Lumbsch HT, Matsumura M, Moncada B, Nuankaew S,
838 Parnmen S, de Azevedo Santiago ALCM, Sommai S, de Souza CAF, de Souza-Motta CM, Su
839 HY, Suetrong S, Wang Y, Wei S-F, Wen T-C, Yuan HS, Zhou LW, Réblová M, Fournier J,
840 Camporesi E, Luangsa-ard JJ, Tasanathai K, Khonsanit A, Thanakitpipattana D, Somrithipol S,
841 Diederich P, Millanes AM, Common RS, Stadler M, Yan JY, Li X, Lee HW, Nguyen TTT, Lee

842 HB, Battistin B, Marsico O, Vizzini A, Vila J, Ercole E, Eberhardt U, Simonini G, Wen H-A,
843 Chen X-H, Miettinen O, Spirin V, Hernawati. 2015. Fungal diversity notes 111–252 - taxonomic
844 and phylogenetic contributions to fungal taxa. Fungal Diversity 75:27– 274.
845
846 Barr D, Yanke L, Bae H, McAllister T, Cheng K. 1995. Contributions on the morphology and
847 taxonomy of some rumen fungi from Canada. Mycotaxon 54:203–214.
848
849 Barr DJ, Kudo H, Jakober KD, Cheng KJ. 1989. Morphology and development of rumen fungi:
850 *Neocallimastix* sp., *Piromyces communis*, and *Orpinomyces bovis* gen.nov., sp.nov. Canadian
851 Journal of Botany 67 2815–2824.
852
853 Breton A, Bernalier A, Dusser M, Fonty G, Gaillard-Martinie B, Guitlot J. 1990. *Anaeromyces*
854 *mucronatus* nov. gen., nov. sp. A new strictly anaerobic rumen fungus with polycentric thallus.
855 FEMS Microbiology Letters 70:177–182.
856
857 Breton A, Dusser M, Gaillard-Martine B, Guillot J, Millet L, Prensier G. 1991. *Piromyces*
858 *rhizinflata* nov. sp., a strictly anaerobic fungus from faeces of the Saharian ass: a morphological,
859 metabolic and ultrastructural study. FEMS Microbiology Letters 82:1–8.
860
861 Calkins S, Elledge NC, Hanafy RA, Elshahed MS, Youssef NH. 2016. A fast and reliable
862 procedure for spore collection from anaerobic fungi: Application for RNA uptake and long-term
863 storage of isolates. Journal of Microbiological Methods 127:206–213.
864
865 Callaghan TM, Podmirseg SM, Hohlweck D, Edwards JE, Puniya AK, Dagar SS, Griffith GW.

2015. *Buwchfawromyces eastonii* gen. nov., sp. nov.: a new anaerobic fungus (Neocallimastigomycota) isolated from buffalo faeces. Mycokeys 9:11–28.

Dagar SS, Kumar S, Griffith GW, Edwards JE, Callaghan TM, Singh R, Nagpal AK, Puniya AK. 2015. A new anaerobic fungus (*Oontomyces anksri* gen. nov., sp. nov.) from the digestive tract of the Indian camel (*Camelus dromedarius*). Fungal Biology 19:731–737.

Gold JJ, Heath IB, Bauchop T. 1988. Ultrastructural description of a new chytrid genus of caecum anaerobe, *Caecomyces equi* gen. nov., sp. nov., assigned to the Neocallimasticaceae BioSystems 21:403–415.

Gruninger RJ, Puniyab AK, Callaghanc TM, Edwardsc JE, Youssef N, Dagare SS, Fliegerova K, Griffith GW, Forster R, Tsang A et al. 2014. Anaerobic Fungi (Phylum Neocallimastigomycota): Advances in understanding of their taxonomy, life cycle, ecology, role, and biotechnological potential. FEMS Microbiology Ecology. 90:1–17.

Hanafy RA, Elshahed MS, Liggenstoffer AS, Griffith GW, Youssef NH. 2017. *Pecoramyces ruminantium*, gen. nov., sp. nov., an anaerobic gut fungus from the feces of cattle and sheep. Mycologia 109:231– 243.

Hanafy RA, Elshahed MS, Youssef NH. 2018. *Feramyces austinii*, gen. nov., sp. nov., an anaerobic gut fungus from rumen and fecal samples of wild Barbary sheep and fallow deer. Mycologia 110:513–525.

890 Heath BI, Bauchop T, Skipp RA. 1983. Assignment of the rumen anaerobe *Neocallimastix*
891 *frontalis* to the Spizellomycetales (Chytridiomycetes) on the basis of its polyflagellate zoospore
892 ultrastructure. Canadian Journal of Botany 61:295–307.
893
894 Ho YW, Bar DJS, Abdullah N, Jalaludin S, Kudo H. 1993a. A new species of *Piromyces* from
895 the rumen of deer in Malaysia. Mycotaxon 47:285–293.
896
897 Ho YW, Barr DJS. 1995. Classification of anaerobic gut fungi from herbivores with emphasis on
898 rumen fungi from malaysia. Mycologia 87:655–677.
899
900 Hungate RE. 1969. A roll tube method for cultivation of strict anaerobes. Methods in
901 Microbiology 3:117–132.
902
903 Joshi A, Lanjekar VB, Dhakephalkar PK, Callaghan TM, Griffith GW, Dagar SS. 2018.
904 *Liebetanzomyces polymorphus* gen. et sp. nov., a new anaerobic fungus (Neocallimastigomycota)
905 isolated from the rumen of a goat. Mycokeys 40:89–110.
906
907 Kittelmann S, G. E. Naylor, J. P. Koolaard, Janssen PH. 2012. A Proposed Taxonomy of
908 Anaerobic Fungi (Class Neocallimastigomycetes) Suitable for Large-Scale Sequence-Based
909 Community Structure Analysis. PLoS ONE 7:e36866.
910
911 Kittelmann S, H. Seedorf, W. A. Walters, J. C. Clemente, R. Knight, J. I. Gordon, Janssen PH.
912 2013. Simultaneous Amplicon Sequencing to Explore Co-Occurrence Patterns of Bacterial,

913 Archaeal and Eukaryotic Microorganisms in Rumen Microbial Communities. PLoS ONE
914 8:e47879.
915
916 Kumar S, Stecher G, Tamura K. 2016. MEGA7: Molecular evolutionary genetics analysis
917 version 7.0 for bigger datasets. Molecular Biology and Evolution 33:1870–1874.
918
919 Li GJ, Hyde KD, Zhao RL, Hongsanan S, Abdel-Aziz FA, Abdel-Wahab MA, Alvarado P,
920 Alves-Silva G, Ammirati JF, Ariyawansa HA, Baghela A, Bahkali AH, Beug M, Bhat DJ,
921 Bojantchev D, Boonpratuang T, Bulgakov TS, Camporesi E, Boro MC, Ceska O, Chakraborty D,
922 Chen JJ, Chethana KWT, Chomnunti P, Consiglio G, Cui BK, Dai DQ, Dai YC, Daranagama
923 DA, Das K, Dayarathne MC, De Crop E, De Oliveira RJV, de Souza CAF, de Souza JI,
924 Dentinger BTM, Dissanayake AJ, Doilom M, Drechsler-Santos ER, Ghobad-Nejhad M, Gilmore
925 SP, Góes-Neto A, Gorczak M, Haitjema CH, Hapuarachchi KK, Hashimoto A, He MQ, Henske
926 JK, Hirayama K, Iribarren MJ, Jayasiri SC, Jayawardena RS, Jeon SJ, Jerônimo GH, Jesus AL,
927 Jones EBG, Kang JC, Karunarathna SC, Kirk PM, Konta S, Kuhnert E, Langer E, Lee HS, Lee
928 HB, Li WJ, Li XH, Liimatainen K, Lima FX, Lin CG, Liu JK, Liu XZ, Liu ZY, Luangsa-ard JJ,
929 Lücking R, Lumbsch HT, Lumyong S, Leaño EM, Marano AV, Matsumura M, McKenzie EHC,
930 Mongkolsamrit S, Mortimer PE, Nguyen TTT, Niskanen T, Norphanphoun C, O'Malley MA,
931 Parnmen S, Pawłowska J, Perera RH, Phookamsak R, Phukhamsakda C, Pires-Zottarelli CLA,
932 Raspé O, Reck MA, Rocha SCO, de Santiago ALCMA, Senanayake IC, Setti L, Shang QJ,
933 Singh SK, Sir EB, Solomon KV, Song J, Srikitikulchai P, Stadler M, Suetrong S, Takahashi H,
934 Takahashi T, Tanaka K, Tang LP, Thambugala KM, Thanakitpipattana D, Theodorou MK,
935 Thongbai B, Thummarukcharoen T, Tian Q, Tibpromma S, Verbeken A, Vizzini A, Vlasák J,

936 Voigt K, Wanasinghe DN, Wang Y, Weerakoon G, Wen HA, Wen TC, Wijayawardene NN,
937 Wongkanoun S, Wrzosek M, Xiao YP, Xu JC, Yan JY, Yang J, Da Yang S, Hu Y, Zhang JF,
938 Zhao J, Zhou LW, Peršoh D, Phillips AJL, Maharachchikumbura SSN. 2016. Fungal diversity
939 notes 253–366: taxonomic and phylogenetic contributions to fungal taxa. Fungal Diversity 78:1–
940 237.
941
942 Li J, Health B, Bauchop T. 1990. *Piromyces mae* and *Piromyces dumbonica*, two new species of
943 uniflagellate anaerobic chytridiomycete fungi from the hindgut of the horse and elephant.
944 Canadian Journal of Botany 68:1021–1033.
945
946 Liggenstoffer AS, Youssef NH, Couger MB, Elshahed MS. 2010 Phylogenetic diversity and
947 community structure of anaerobic fungi (Phylum Neocallimastigomycota) in ruminant and non-
948 ruminant herbivores. The ISME Journal 4:1225–1235.
949
950 Ljungdahl LG. 2008 The cellulase/hemicellulase system of the anaerobic fungus *Orpinomyces*
951 PC-2 and aspects of its use. Annals of the New York Academy of Sciences 1125 308–321.
952
953 McSweeney CS, Denman SE, Mackie RI. 2005. Rumen bacteria. In: Makkar HPS, McSweeney
954 CS, eds. Methods in Gut Microbial Ecology for Ruminants. Dordrecht, Netherlands: Springer. p.
955 23–37.
956

957 Mura E, Edwards J, Kittelmann S, Kaerger K, Voigt K, Mrázek J, Moniello G, Fliegerova K.
958 2019. Anaerobic fungal communities differ along the horse digestive tract. Fungal Biology
959 123:240–246.
960
961 Nagpal R, Puniya AK, Sehgal JP, Singh K. 2011. In vitro fibrolytic potential of anaerobic rumen
962 fungi from ruminants and non-ruminant herbivores. Mycoscience 52:31–38.
963
964 Nakamura T, Yamada KD, Tomii K, Katoh K. 2018. Parallelization of MAFFT for large-scale
965 multiple sequence alignments. Bioinformatics 34:2490–2492.
966
967 Ozkose E, Thomas BJ, Davies DR, Griffith GW, Theodorou MK. 2001. *Cyllamyces aberensis*
968 gen. nov. sp.nov., a new anaerobic gut fungus with branched sporangiophores isolated from
969 cattle. Canadian Journal of Botany 79:666–673.
970
971 Paul SS, Bu D, Xu J, Hyde KD, Yu Z. 2018. A phylogenetic census of global diversity of gut
972 anaerobic fungi and a new taxonomic framework. Fungal Diversity 89:253–266.
973
974 Paul SS, Deb SM, Punia BS, Singh D, Kumar R. 2010. Fibrolytic potential of anaerobic fungi
975 (*Piromyces* sp.) isolated from wild cattle and blue bulls in pure culture and effect of their
976 addition on in vitro fermentation of wheat straw and methane emission by rumen fluid of
977 buffaloes. Journal of the Science of Food and Agriculture 90:1218–1226.
978
979 Ranganathan A, Smith OP, Youssef NH, Struchtemeyer CG, Atiyeh HK, Elshahed MS. 2017.

Utilizing anaerobic fungi for two-stage sugar extraction and biofuel production from lignocellulosic biomass. Frontiers in Microbiology 8:635.

Tuckwell DS, Nicholson MJ, McSweeney CS, Theodorou MK, Brookman JL. 2005. The rapid assignment of ruminal fungi to presumptive genera using ITS-1 and ITS2 RNA secondary structures to produce group-specific fingerprints. Microbiology 151:1557–1567.

Wang X, Liu X, Groenewald JZ. 2017. Phylogeny of anaerobic fungi (phylum Neocallimastigomycota), with contributions from yak in China. Antonie Van Leeuwenhoek 110:87–103.

Young D, Dollhofer V, Callaghan TM, Reitberger S, Lebuhn M, Benz JP. 2018. Isolation, identification and characterization of lignocellulolytic aerobic and anaerobic fungi in one- and two-phase biogas plants. Bioresoure Technology 268:470–479.

Youssef NH, Couger MB, Struchtemeyer CG, Liggenstoffer AS, Prade RA, Najar FZ, Atiyeh HK, Wilkins MR, Elshahed MS. 2013. Genome of the anaerobic fungus *Orpinomyces* sp. C1A reveals the unique evolutionary history of a remarkable plant biomass degrader. Applied and Environmental Microbiology 79:4620–4634.

1004 **LEGENDS AND FOOTNOTES.**

1005 **FIG. 1.** Macroscopic features of colony morphology on agar media and fungal biomass in liquid
1006 media for (a) *Agriosomyces longus* strain MS2, brown circular colonies and thin fungal biofilm
1007 in liquid medium. (b) *Aklioshbomyces papillarum* strain WT-2, beige circular colonies with a
1008 brown central core of sporangia and thick fungal biofilm in liquid medium that firmly attaches to
1009 the tube's glass surface, note the inverted tube shows how the fungus thalli attached to the tube.
1010 (c**)** *Capellomyces foraminis* strain BGB-11, small brown circular colonies with dark center and
1011 thin fungal biofilm in liquid medium. (d) *Capellomyces elongatus* strain GFKJa1916, compact
1012 cottony off-white circular colonies with a fluffy central core of sporangia surrounded by
1013 radiating sporangia and thin fungal biofilm in liquid medium that attach to the tube's glass. (e)
1014 *Ghazallomyces constrictus* strain Axs-31**,** white circular colonies on roll tubes and thick fungal
1015 biofilm in liquid medium. (f**)** *Joblinomyces apicalis* strain GFH683**,** beige circular colonies with
1016 a brown central core of sporangia and thin fungal biofilm in liquid medium. (g) *Khyollomyces*
1017 *ramosus* strain ZS-33, yellowish brown colonies of irregular shape on agar medium and loose
1018 fungal thalli with sand-like appearance. (h) *Tahromyces munnarensis* strain TDFKJa193 white
1019 circular colonies with fluffy center, surrounded by dotted circles of fungal thalli and thick fungal
1020 biofilm in liquid medium that attach to the tube's glass.

1021 **FIG. 2.** Microscopic features of *Agriosomyces longus* (Clade 1, Mouflon-Boer Goat) strain
1022 MS2. Light (e-h) and scanning electron (a and i) micrographs are shown. DAPI staining for
1023 nuclei visualizing using a fluorescence microscope equipped with a Brightline DAPI high-
1024 contrast filter set (c). Overlay image is shown in (d). (a) A monoflagellate zoospore. (b) A
1025 biflagellate zoospore. (c-d) Monocentric thalli, with nuclei occurring in sporangia, not in rhizoids
1026 or sporangiophores. (e-f) Endogenous globose sporangia with tightly constricted necks and

subsporangial swellings (arrows). (g-h) Exogenous globose sporangia, note the swollen sporangiophores. (i) An empty sporangium after zoospore release and rupturing of the sporangial wall. Bar: a=5 µm, b=20 µm c-i=50 µm.

FIG. 3. Microscopic features of *Aklioshbomyces papillarum* (Clade 2, White-Tailed Deer) strain WT-2. Light (a-e, and g-q) and scanning electron (f) micrographs are shown. Light microscopy pictures were examined after staining with lactophenol cotton blue (a-c, e, and g-q), as well as following nuclei staining with DAPI (d). (a) A monoflagellate zoospore. (b) A biflagellate zoospore. (c) A triflagellate zoospore. (d) Monocentric thalli, with nuclei occurring in sporangia, not in rhizoids or sporangiophores. (e-g) Endogenous sporangial development: (e) Ovoid sporangium with single rhizoidal system, (f) Ovoid sporangium with two main rhizoidal systems, (g) Globose pseudo-intercalary sporangium, between two main rhizoidal systems. (h-k) Exogenous sporangial development: (h) Globose sporangium on a very short sporangiophore, (i) Ellipsoidal sporangium, (j) Obpyriform sporangium on a long sporangiophore, (k) Obpyriform sporangium. (l) Ellipsoidal sporangium. (m) Sporangia with lateral single papilla, (n-p) Sporangia with terminal single papilla. (q) Sporangium with two papillae. Bar: a-c, e, f, n & o =20 µm, g-k, p & q =50 µm, d, l & m =100 µm.

FIG. 4. Microscopic features of *Capellomyces foraminis* (Clade 3, Boer Goat) strain BGB-11. Light (a-i, l, o & p) and scanning electron (j, k, m, n, q &r) micrographs are shown. Light microscopy pictures were examined after staining with lactophenol cotton blue (a-e, h, i, & o), as well as following nuclei staining with DAPI (f). Overlay image is shown in (g). (a) A monoflagellate zoospore. (b) A biflagellate zoospore. (c) Zoospore cyst, arrow points to the shed flagellum. (d) Germinating zoospore cyst producing a germ tube (arrow). (e) Rhizoidal system development. (f-g) Monocentric thalli with nuclei occurring in sporangia, not in rhizoids or

sporangiophores. (h-i) Endogenous sporangial development: (h) Ellipsoidal sporangium with two main rhizoidal systems (arrows), (i) Ovoid sporangium with single rhizoidal system. (j-q) Exogenous sporangial development: (j) Exogeous sporangium with a short sporangiophore (Sp), note the empty zoospore cyst (Zc). (k) Ovoid sporangium with a long sporangiophore, (l) Ovoid sporangium on a long sporangiophore ends with sub-sporangial swelling (arrow), (m) Collapsed empty sporangium on a long sporangiophore ends with sub-sporangial swelling (arrow), (n) Constricted ellipsoidal sporangium, (o-p) Globose Sporangia. (q) Zoospores ae released through apical pore. (r) An empty sporangium following zoospores release. Abbreviations: (Sp), sporangiophore; (Zc), zoospore cyst. Bar: a-j, l, o & p =20 µm, k, m, n, q & r =50 µm.

Fig 5. Microscopic features of *Capellomyces elongatus* (Clade 3, domesticated Goat) strain GFKJa1916**.** Differential interference contrast (a, d-f and i-l), scanning (g-h), phase contrast (b) and fluorescence (c) micrographs. (a) A monoflagellate zoospores. (b-c) Monocentric thalli; nuclei were observed in sporangia, not in rhizoids or sporangiophores. (d-g) Endogenous sporangia. (d) Globose endogenous sporangium with one main rhizoidal systems. (e-f) Endogenous sporangia with multiple rhizoidal systems. (g) Endogenous sporangium on wheat straw fibers. (h-l) Exogenous sporangia: (h) ovoid-shaped sporangium with long sporangiophore. (i) Multiple ovoid, and globose exogenous sporangia with long sporangiophores. (j) Multisporangiate thallus with two sporangia (same shape). (k-l) Multisporangiate thallus with two sporangia (different shape).

Scale bar = 20 µM.

FIG. 6. Microscopic features of *Ghazallomyces constrictus* (Clade 4, Axis Deer) strain Axs-31. Light (a-h, f, k and m-p) and scanning electron (i, j, l and q) micrographs are shown. Light microscopy pictures were examined after staining with lactophenol cotton blue (a, d–h, k, and m-

p), as well as following nuclei staining with DAPI (b). Overlay image is shown in (c). (a) A polyflagellate zoospore. (b-c) Monocentric thalli, with nuclei occurring in sporangia, not in rhizoids or sporangiophores. (d-g) Endogenous sporangia with tightly constricted necks (arrows): (d) Young globose sporangium, (e) Young tubular sporangium, (f) Mature clavate sporangium, (g) and Mature ellipsoidal sporangium. (h-p) Exogenous sporangia: (h) Young sporangium on a short flattened sporangiophore (Sp), note the persistent empty zoospore cyst (Zc) and the rhizoidal system (R), (i) Ovoid sporangium on short sporangiophore, (j) Ellipsoidal sporangium on long sporangiophore, (k) ovoid sporangium on an eggcup-shaped sporangiophore (arrow), (l) Globose sporangium, (m) Constricted ellipsoidal sporangium with tightly constricted neck (arrow) on long sporangiophore, (n) Pyriform sporangium, note the fine septum at the base of sporangium (arrow), (o) Bowling pin-shaped sporangium, (p) Rhomboidal sporangium with constricted neck and fine septum (arrows), note the persistent empty zoospore cyst (Zc). (q) Zoospores are released through apical pore followed by collapse of the sporangial wall. Abbreviations: (Sp), sporangiophore; (Zc), zoospore cyst; (R), rhizoid. Bar: a=20µm, b-q=50 µm.

FIG. 7. Microscopic features of *Joblinomyces apicalis* (Clade 5, domesticated Goat and Sheep) strain GFH683. Phase contrast (b, c and e), fluorescence (d, f), scanning electron (g-h, and m) differential interference (k-l) micrographs. (a) A monoflagellate zoospore. (b) A biflagellate spherical zoospore. (c-f) Monocentric thalli; nuclei were observed in sporangia, not in rhizoids or sporangiophores, note the empty cup-shaped sporangium after zoospore release (arrow). (g) Ovoid endogenous sporangium. (h-i) Exogenous sporangia: (h) Ovoid sporangium with short sporangiophore. (i) Subglobose sporangium with long sporangiophore. (j-l) Zoospore release: (j) Dissolution of the apical portion of sporangial wall (arrow). (k-l) Cup-shaped sporangia with

wide apical pores and intact sporangial walls (arrow). (g) Colonization of rice straw fibers by fungal rhizoids and emanating.

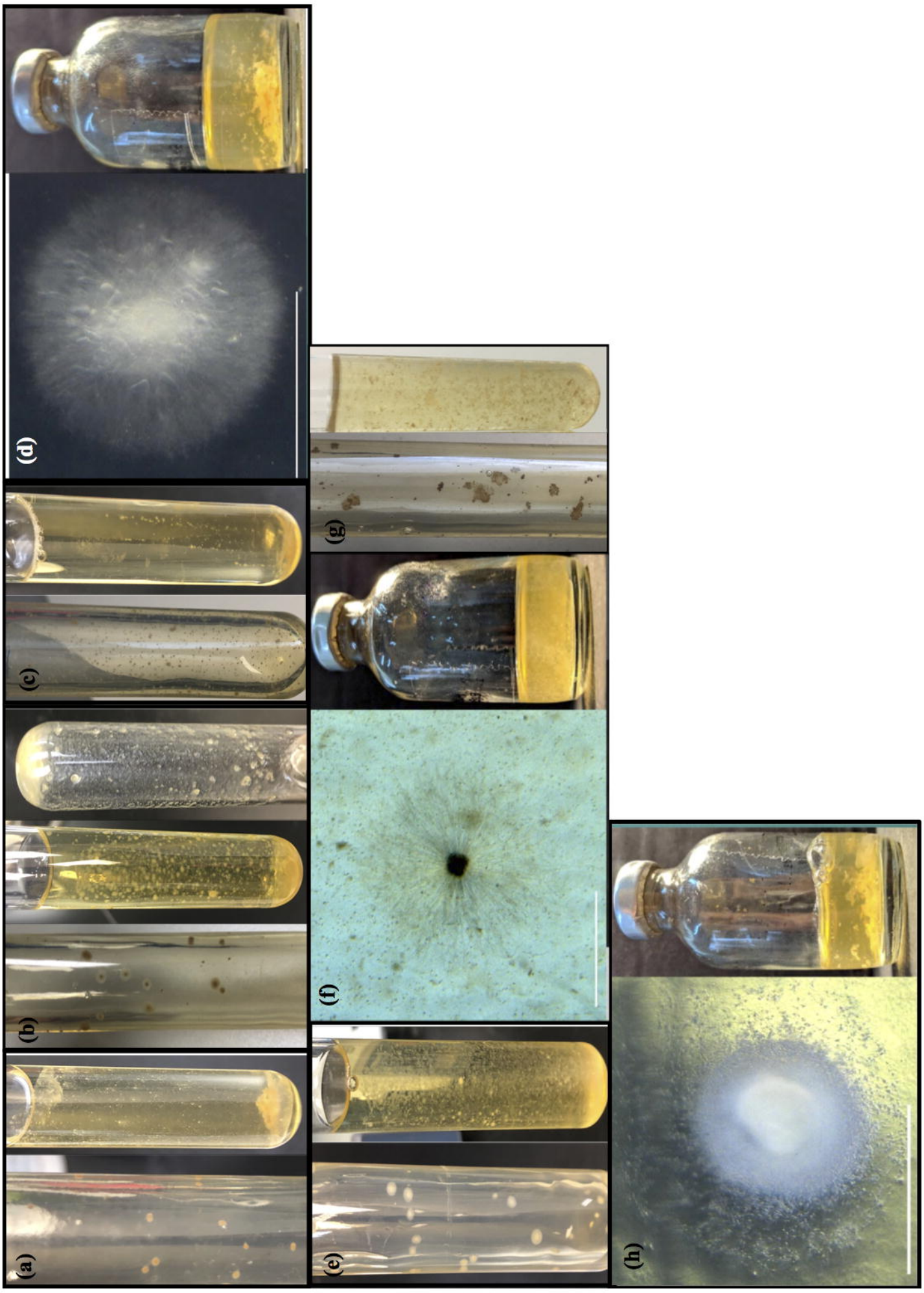
Scale bar = 20 µM.

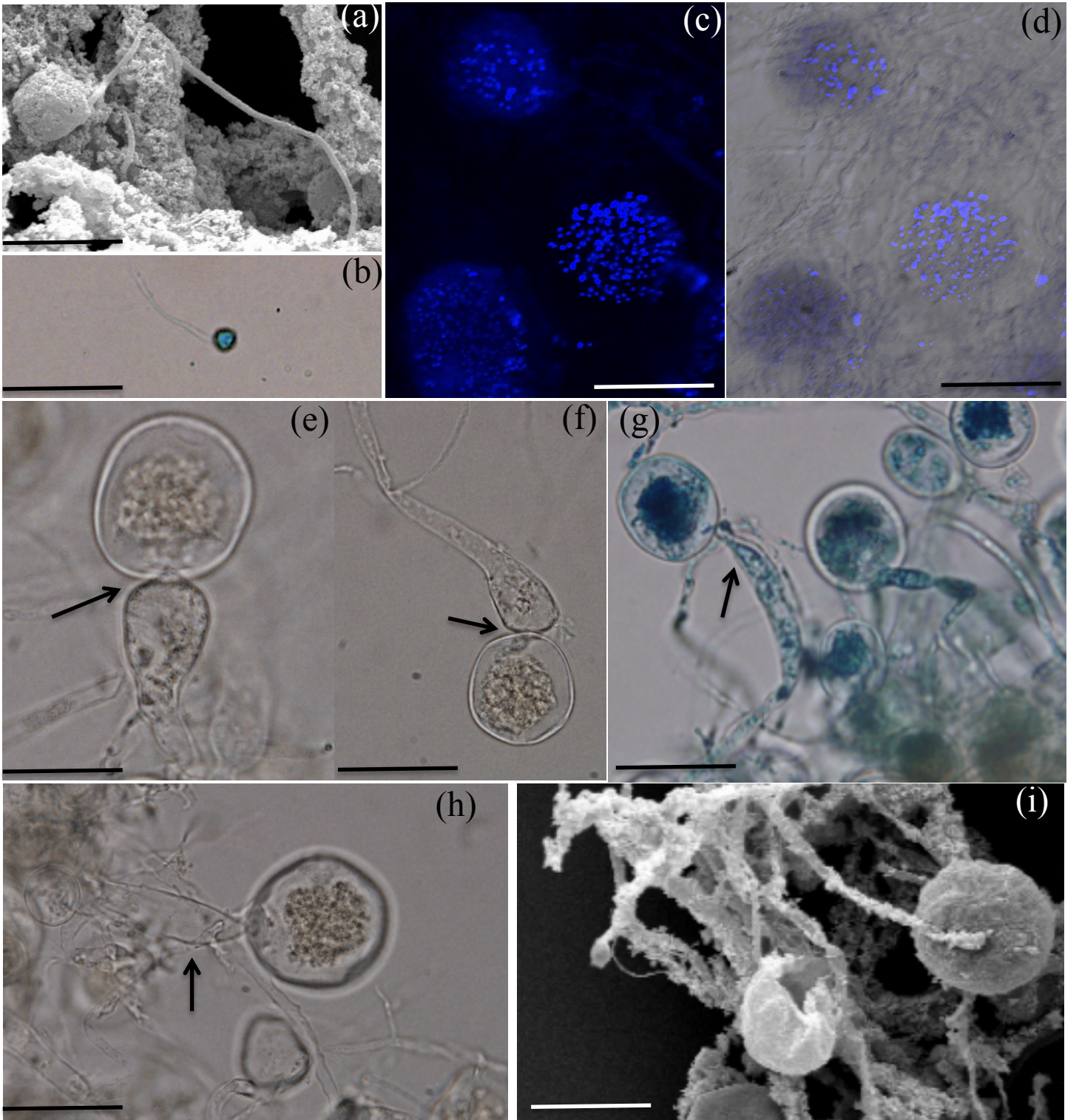
FIG. 8. Microscopic features of *Khyollomyces ramosus* (Clade 6, Zebra-Horse) strain ZS-33 (a-q) and distinct resting stage structure from strain HoCal4.A2.2 (r). Light (a-e, g, i-j, l & q) and scanning electron (f, h, k, m &n-p) micrographs are shown. DAPI staining for nuclei visualizing using a fluorescence microscope equipped with a Brightline DAPI high-contrast filter set (d). Overlay image is shown in (e). (a) A uniflagellate zoospore. (b) Zoospore cyst after shedding of the flagellum. (c) Germinating zoospore cyst producing a germ tube (arrow). (d-e) Monocentric thalli, with nuclei occurring in sporangia, not in rhizoids or sporangiophores. (f) hyphal structures with intercalary swellings in wide hyphae (arrows). (g-h) Endogenous sporangial development: (g) Young subglobose sporangium with single rhizoidal system, (i) Mature ellipsoidal sporangium with two main rhizoidal systems. (i-m) Exogenous sporangial development: (i) Multisporangiate thallus with two sporangia, (j) Multisporangiate thallus with four sporangia, (k) Heart-shaped sporangium. (l) Ovoid sporangium (labeled S) on a wide flattened sporangiophore (labeled Sp), (m) Pyriform sporangium. (n) Zoospores are released through apical pore. (o-p) Empty sporangia with intact sporangial walls after zoospores discharge. (q) Mature sporangia detached from hyphae or sporangiophores. (r) Resting stages from strain HoCal4.A2.2. Bar: a-c & f-g =20 µm, d-e, k &m-q =50 µm and h-j &l =100 µm.

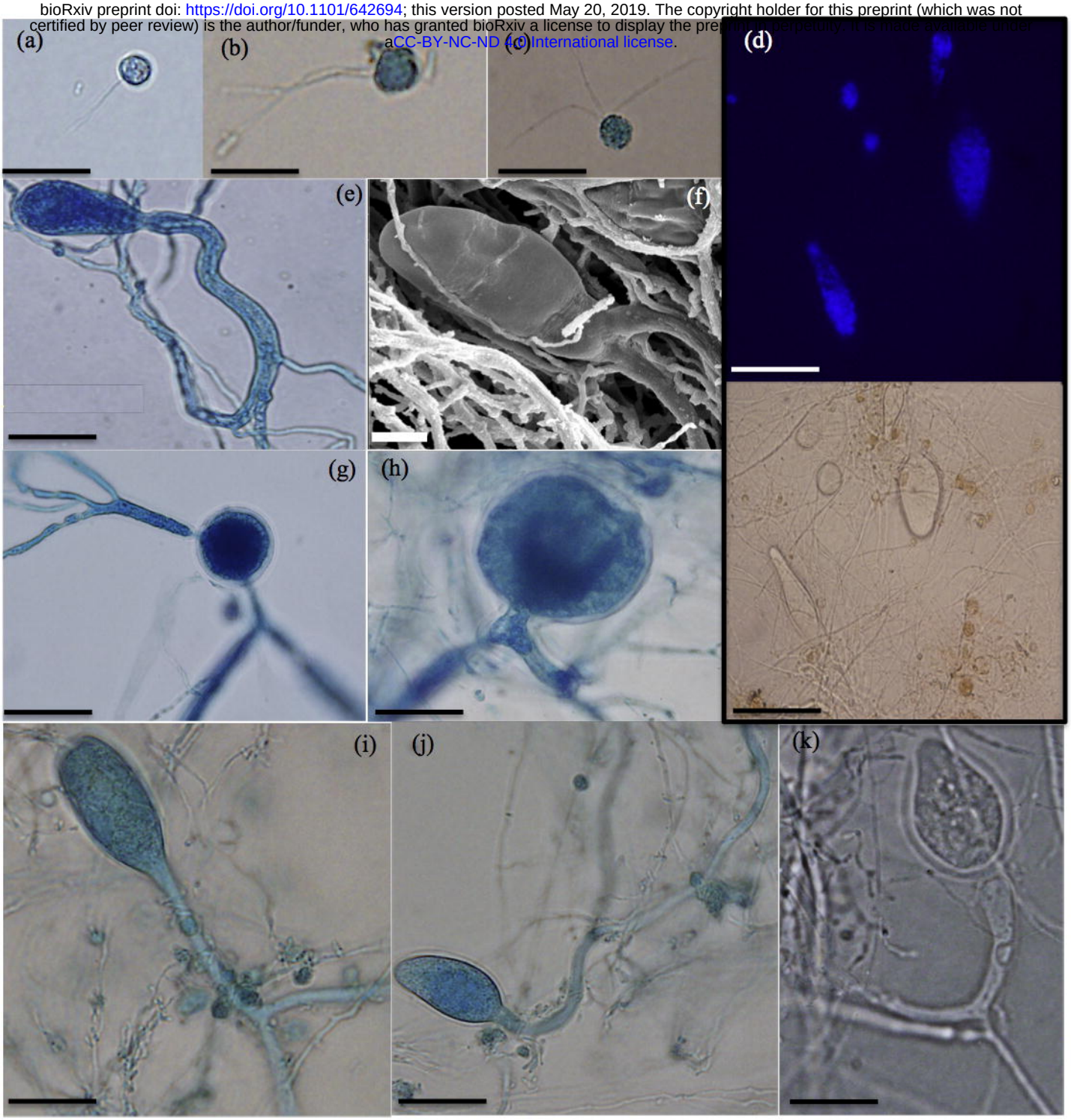
FIG. 9. Microscopic features of *Tahromyces munnarensis* (Clade 7, Nilgiri Tahr) strain TDFKJa193. Differential interference contrast (a, f and i-l), phase contrast (b, d, g and h), fluorescence (c and e) micrographs. (a) A monoflagellate and triflagellate zoospores. (b-e) Monocentric thalli; nuclei were observed in sporangia, not in rhizoids or sporangiophores. (f-g)

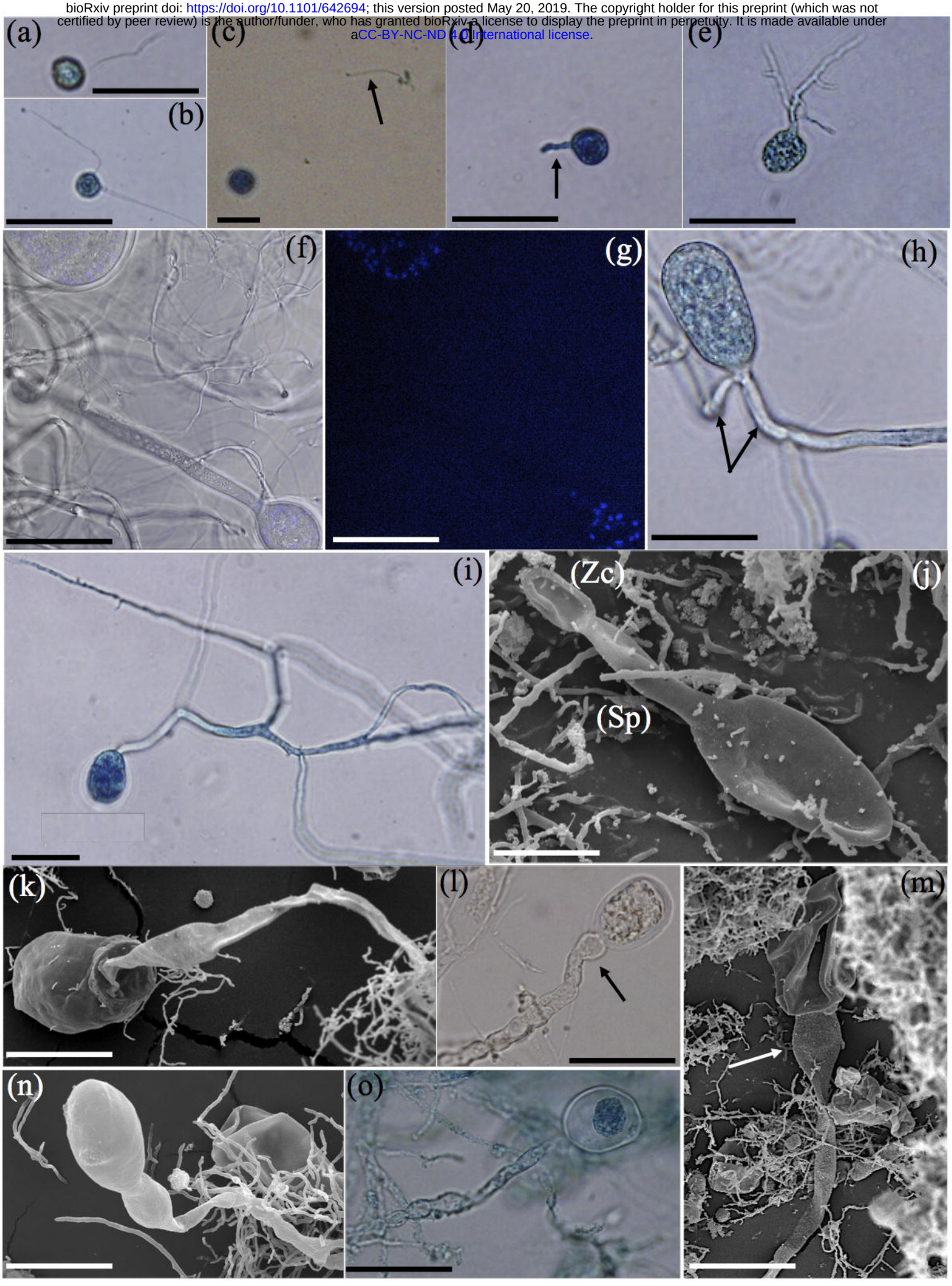
Endogenous sporangia. (f) Ovoid endogenous sporangium with two rhizoidal systems, note the sub-sporangial swelling (arrow). (g) Endogenous sporangium with branched rhizoids. (h-l) Exogenous sporangia: (h) Globose sporangium with short swollen sporangiophore (arrow). (i-j) Exogenous sporangia with sub-sporangial swellings and constricted necks (arrow). (k) Ovoid sporangium with septum at the sporangial base (arrow). (l) Zoospore release through dissolution of a wide apical pore. Scale bar = 20 µM.

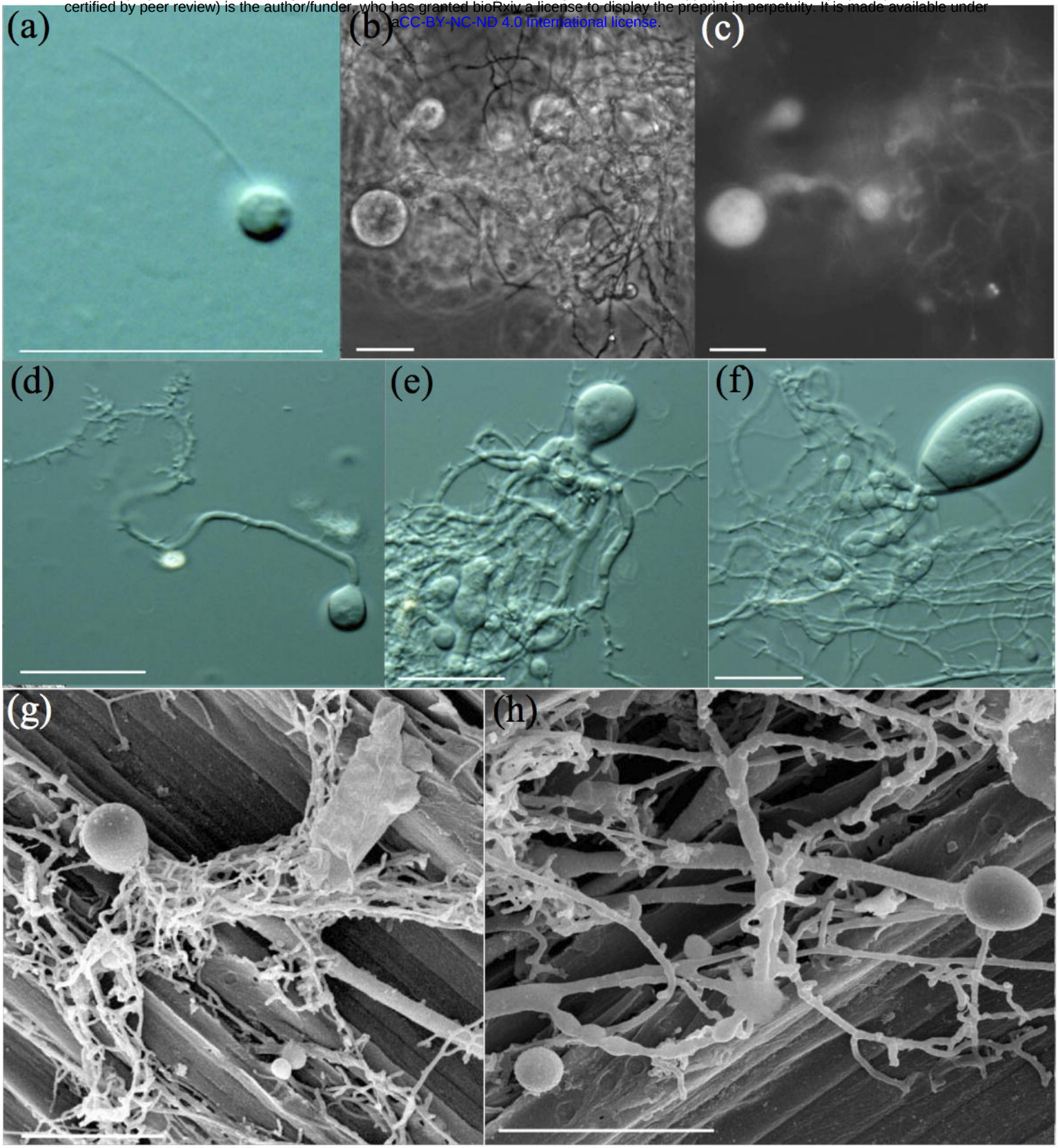
FIG. 10. Phylogenetic affiliation of the 7 newly described genera to other AGF genera based on the sequences of the D1–D2 domains of nuc 28S rDNA gene (a), and partial ITS-1 sequences (b). Sequences were aligned in MAFFT (Nakamura and others 2018) and the alignment was used to construct phylogenetic trees in MEGA7 (Kumar and others 2016) using a maximum likelihood approach. Bootstrap values from 100 replicates are shown for nodes with more than 70% bootstrap support

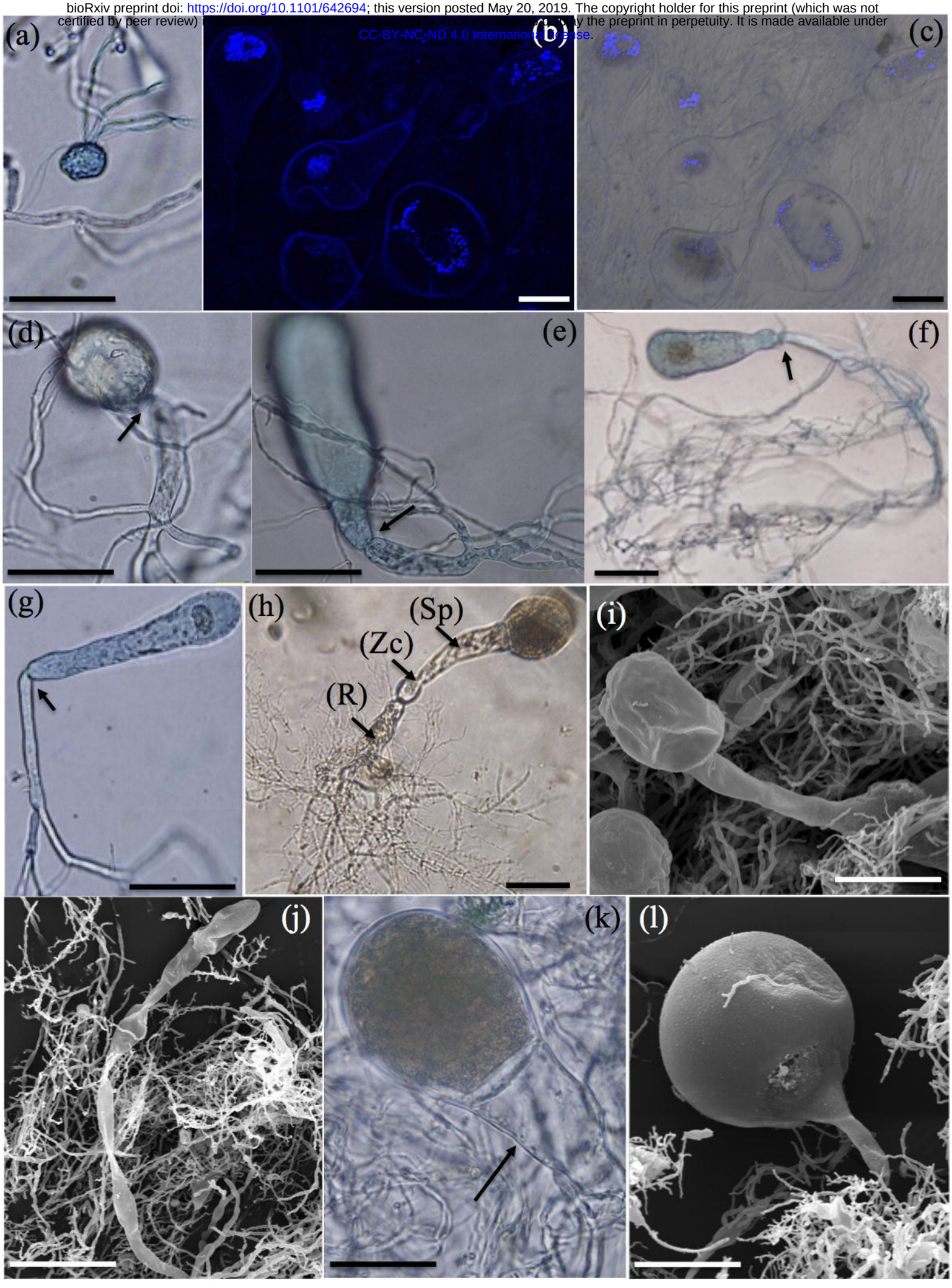


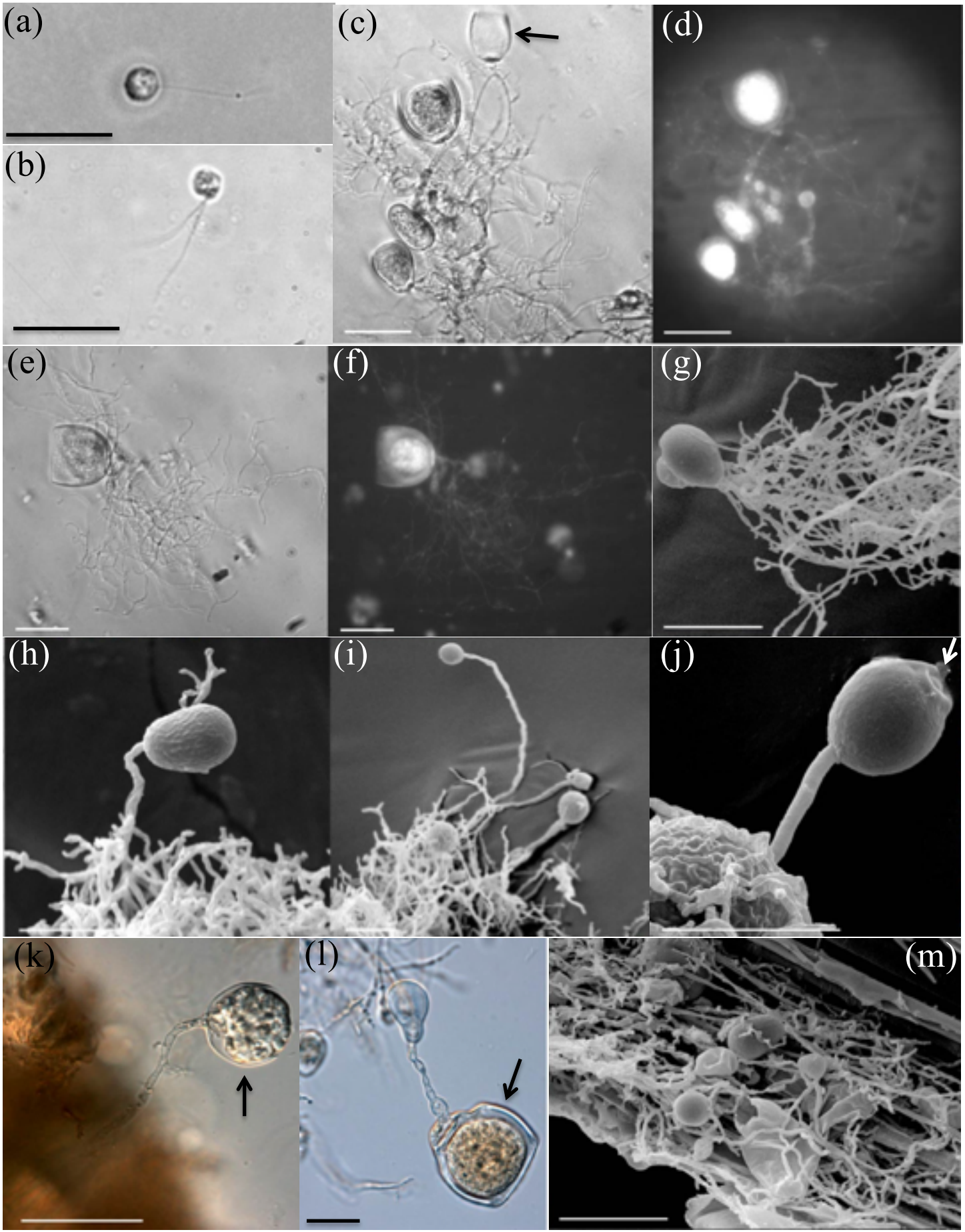

(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)

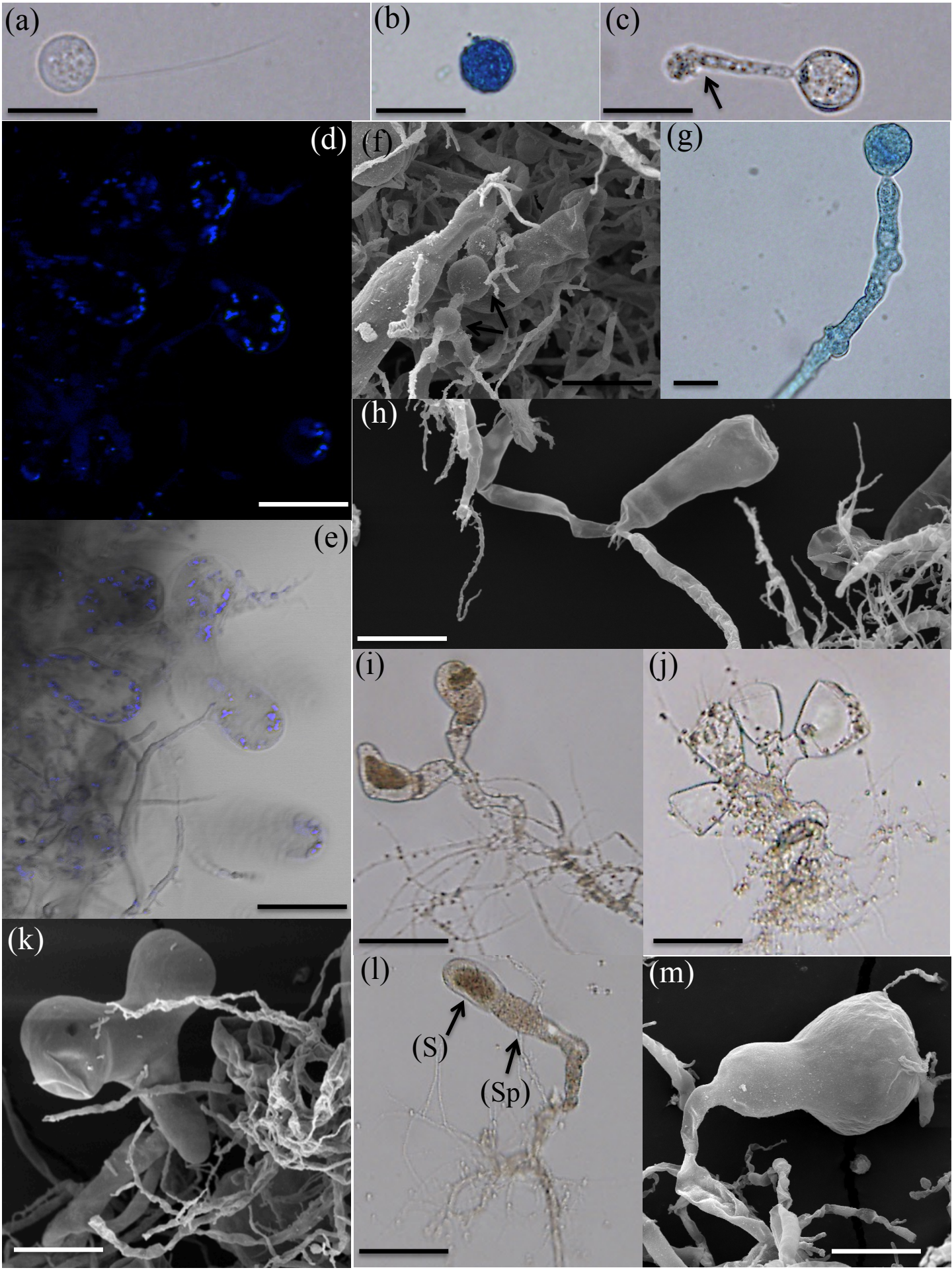


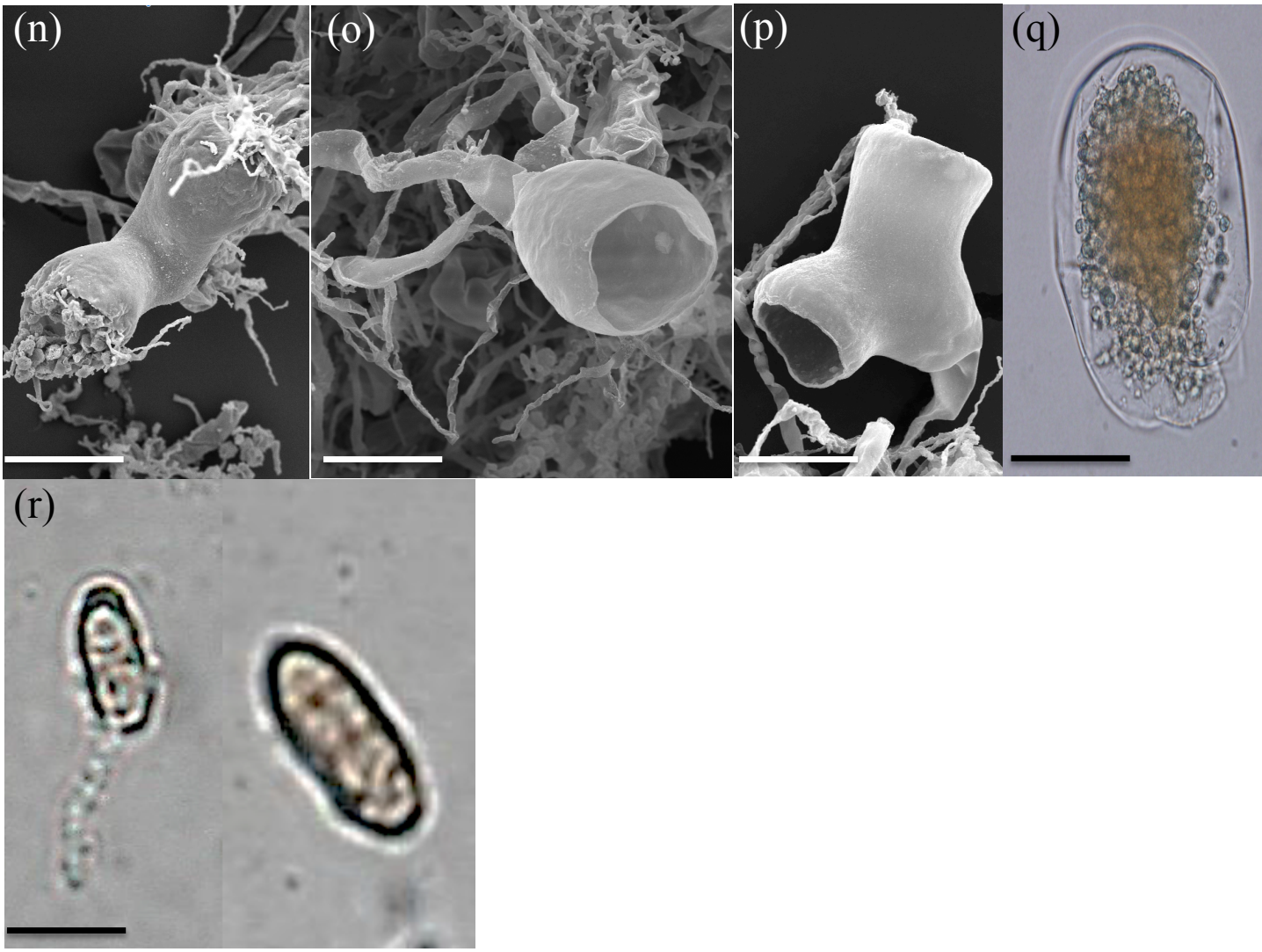


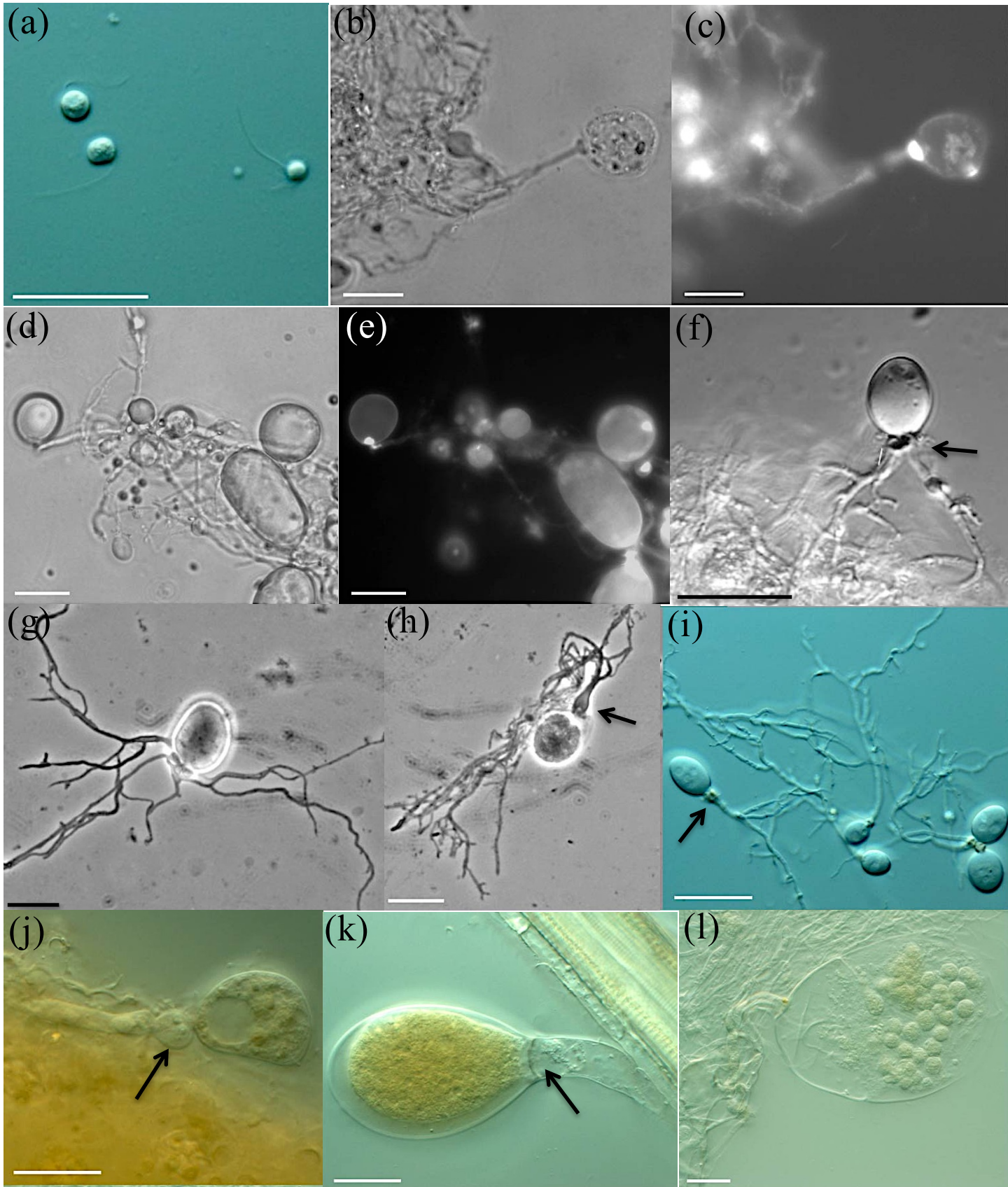





(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
(k)
(l)
(m)


(a)
(b)
(c)
(d)
(f)
(g)
(h)
(e)
(i)
(j)
(k)
(l)
(S)
(Sp)
(m)


(n)
(o)
(p)
(q)
(r)


(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
(k)
(l)