INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY

REVIEW

Hanafy *et al.*, *Int. J. Syst. Evol. Microbiol.* 2022;72:005322
DOI 10.1099/ijsem.0.005322

MICROBIOLOGY SOCIETY

OPEN ACCESS

# Taxonomy of the anaerobic gut fungi (*Neocallimastigomycota*): a review of classification criteria and description of current taxa

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**Abstract**

Members of the anaerobic gut fungi (*Neocallimastigomycota*) reside in the rumen and alimentary tract of larger mammalian and some reptilian, marsupial and avian herbivores. The recent decade has witnessed a significant expansion in the number of described *Neocallimastigomycota* genera and species. However, the difficulties associated with the isolation and maintenance of *Neocallimastigomycota* strains has greatly complicated comparative studies to resolve inter- and intra-genus relationships. Here, we provide an updated outline of *Neocallimastigomycota* taxonomy. We critically evaluate various morphological, microscopic and phylogenetic traits previously and currently utilized in *Neocallimastigomycota* taxonomy, and provide an updated key for quick characterization of all genera. We then synthesize data from taxa description manuscripts, prior comparative efforts and molecular sequence data to present an updated list of *Neocallimastigomycota* genera and species, with an emphasis on resolving relationships and identifying synonymy between recent and historic strains. We supplement data from published manuscripts with information and illustrations from strains in the authors' collections. Twenty genera and 36 species are recognized, but the status of 10 species in the genera *Caecomyces*, *Piromyces*, *Anaeromyces* and *Cyllamyces* remains uncertain due to the unavailability of culture and conferre (*cf.*) strains, lack of sequence data, and/or inadequacy of available microscopic and phenotypic data. Six cases of synonymy are identified in the genera *Neocallimastix* and *Caecomyces*, and two names in the genus *Piromyces* are rejected based on apparent misclassification.

## INTRODUCTION

The kingdom fungi encompasses a bewilderingly diverse array of organisms that evolved from a unicellular flagellated ancestor, and have since diversified to colonize a wide range of terrestrial, freshwater, marine, engineered and host-associated habitats [1]. Most fungi thrive as free-living organisms, but many forge symbiotic, predatory, pathogenic and commensal relationships with algae [2], plants [3, 4] and animals [5, 6]. One of the most peculiar evolutionary trajectories and niche adaptation events within the fungi is their sequestration into the herbivorous gut, an event that spurred the evolution of a strictly anaerobic fungal lineage (the anaerobic gut fungi, phylum *Neocallimastigomycota*) [7, 8]. Since their sequestration ≈66 Mya [9], this fascinating group of fungi has been blazing their own evolutionary trail; with multiple studies describing the impact of fungal sequestration on their genome architecture, metabolic capacities, cellular structure and physiological preferences [10–13].

The history of the discovery and characterization of the anaerobic gut fungi spans more than a century, although documenting their affiliation with the fungal kingdom [7, 14–16] and isolating anaerobic gut fungal strains in pure cultures [17] were achieved relatively recently (Fig. 1). A study in 1843 surveyed 'animalcules' in the stomach and intestine of herbivores and carnivores, and observed small (10 µm) motile propagules (fitting zoospore description) that developed in large number in the horse caecum [18]. Subsequent reports described spherical and ovoid flagellated structures with a single [19–21], or multiple flagella [20, 21] from the rumen and horse caecum. These studies [19–21] have assumed that such structures were part of the protozoan component of the herbivorous gut and proposed the names *Sphaeromonas*, *Piromonas* and *Oikomonas* for the single-flagellated and *Callimastix* for the polyflagellated structures. The widespread occurrence of these flagellates (especially *Callimastix*) in the

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**Keywords:** fungi; *Neocallimastigomycota*; herbivores.
**Abbreviations:** DAPI, 4′,6′ diamidino-2-phenylindol; ITS, internal transcribed spacer; LSU, large ribosomal subunit; RFLP, restriction fragment length polymorphism.
A supplementary figure is available with the online version of this article.

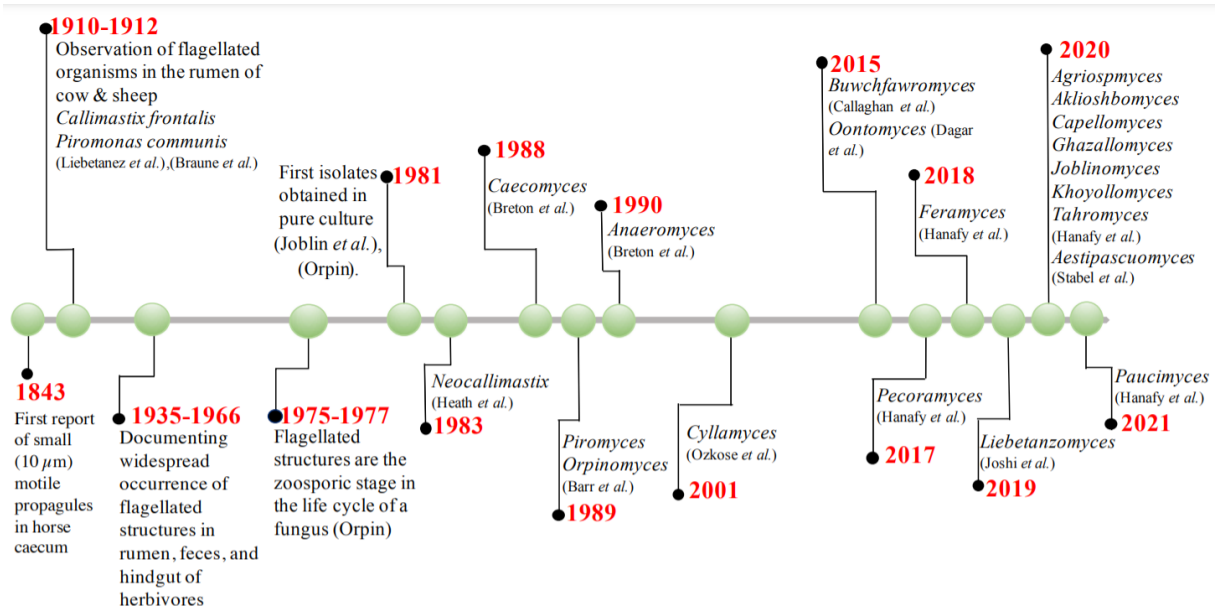



**Fig. 1.** Timeline highlighting salient events in *Neocallimastigomycota* discovery and progress in description of novel genera. References reporting a specific contribution are in parenthesis.

rumen, faeces and hindgut of herbivores was subsequently confirmed in later publications [22, 23]. Warner [24, 25] and Orpin [26] were baffled by the observed huge fluctuation in numbers of these flagellated structures pre and post feeding in sheep, a behaviour unbecoming of organisms thought to divide by binary fission, and suggestive of a pattern of growth involving spore formation and release (although partitioning between the rumen lumen and wall, robust chemotaxis, rapid lysis, and passive distribution of organisms attached to food material were put forth as possible mechanisms to explain such fluctuations [24–26]). Finally, in a series of seminal papers, Orpin unequivocally demonstrated through microscopic observation of sheep rumen fluid and rumen material incubated with aqueous oat extracts under anaerobic conditions that the polyflagellate *Neocallimastix* [7] and the monoflagellates *Sphaermonas* [16] and *Piromonas* [27] are the zoosporic stages in the life cycle of a fungus, and that these zoospores are induced to produce rhizoidal structure with reproductive bodies (sporangia) that, in turn, release zoospores to colonize plant materials. Subsequent studies not only confirmed such observations, but also greatly expanded on the global diversity of the *Neocallimastigomycota* using culture-based and culture-independent approaches [8, 28–32].

Taxonomic monographs serve to synthesize and analyse available data from disparate sources spanning decades of research to provide a detailed, up-to-date description of a lineage of interest. Ho and Barr [33] provided the last detailed monograph on the taxonomy of the anaerobic gut fungi, covering the five genera described at the time of its publication (1995). This landmark contribution remains an extremely valuable resource for *Neocallimastigomycota* researchers. However, after more than a quarter century since its publication, we reason that an updated detailed monograph on anaerobic gut fungal taxonomy is warranted for the following reasons: 1, The need to account for the rapid progress in isolation and characterization of new *Neocallimastigomycota* taxa reported in the last two decades; progress that led to the expansion of reported *Neocallimastigomycota* genera from five (in 1995) to 20. 2, The fact that many *Neocallimastigomycota* type strains have been lost due to the difficulty in maintenance and lack of institutional continuity; a situation that renders preservation and synthesis of currently published data (and yet-unpublished observations) extremely valuable. 3, The shift in traits deemed taxonomically informative in *Neocallimastigomycota* taxonomy: with the strong emphasis on zoospores ultrastructure in earlier manuscripts [34] replaced by an increased dependence on thallus and zoospores morphology [33] and in particular molecular phylogenetic analysis [28, 29]. Such a shift often requires revisiting historic strains, their isotypes or *cf.* strains to fill gaps in their microscopic description and molecular sequence data. 4, The utilization of disparate vehicles for naming new taxa with various levels of characterization provided in such vehicles: from detailed characterization manuscripts [35–37], taxonomic notes [38, 39], naming strains as part of a broader genomic, systems biology or biochemical studies [40, 41], or even proposing names to accompany amplicon data in GenBank.

Here, we provide an updated monograph on the taxonomy of the *Neocallimastigomycota*. We start with a detailed description of criteria (morphological, microscopic and phylogenetic) utilized in *Neocallimastigomycota* taxonomy. We then provide an updated key for quick characterization of all *Neocallimastigomycota* genera. Finally, we provide a detailed description of all taxa, with an emphasis on history, nomenclature, occurrence, characteristics, and relationships between recent and historic strains. We

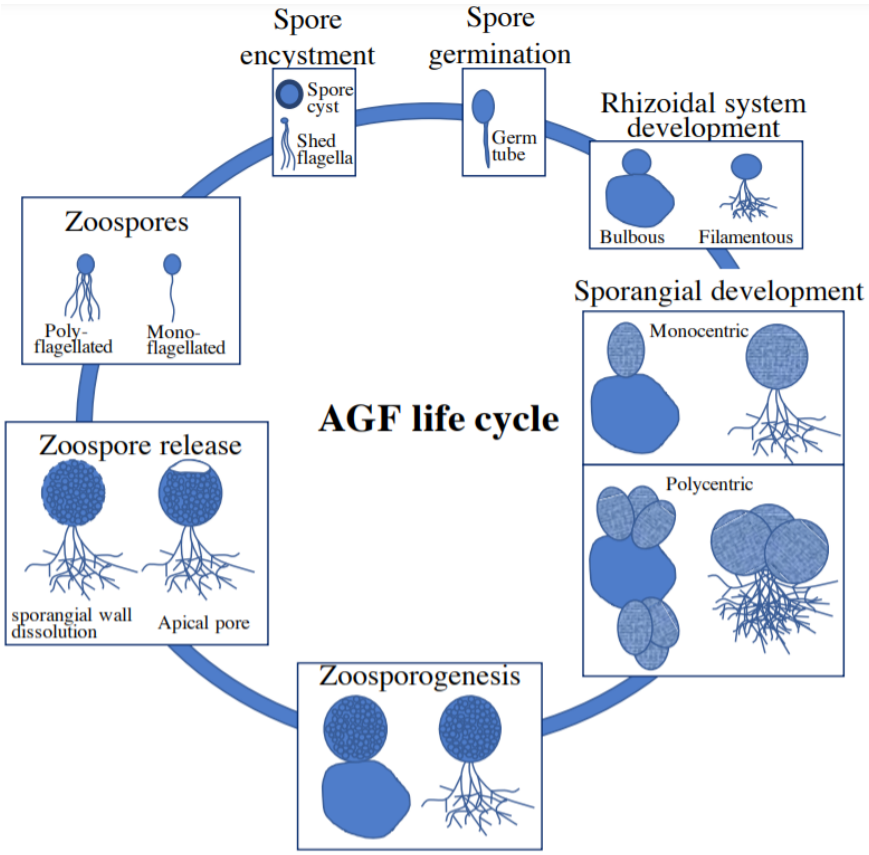



**Fig. 2.** *Neocallimastigomycota* Life cycle.

supplement data from published manuscripts by information and figures from *cf*. strains of historic taxa and of taxa of limited availability that were obtained in the last decade as part of sustained isolation efforts by the authors' laboratories.

## TAXONOMICALLY INFORMATIVE CRITERIA IN *NEOCALLIMASTIGOMYCOTA* TAXONOMY

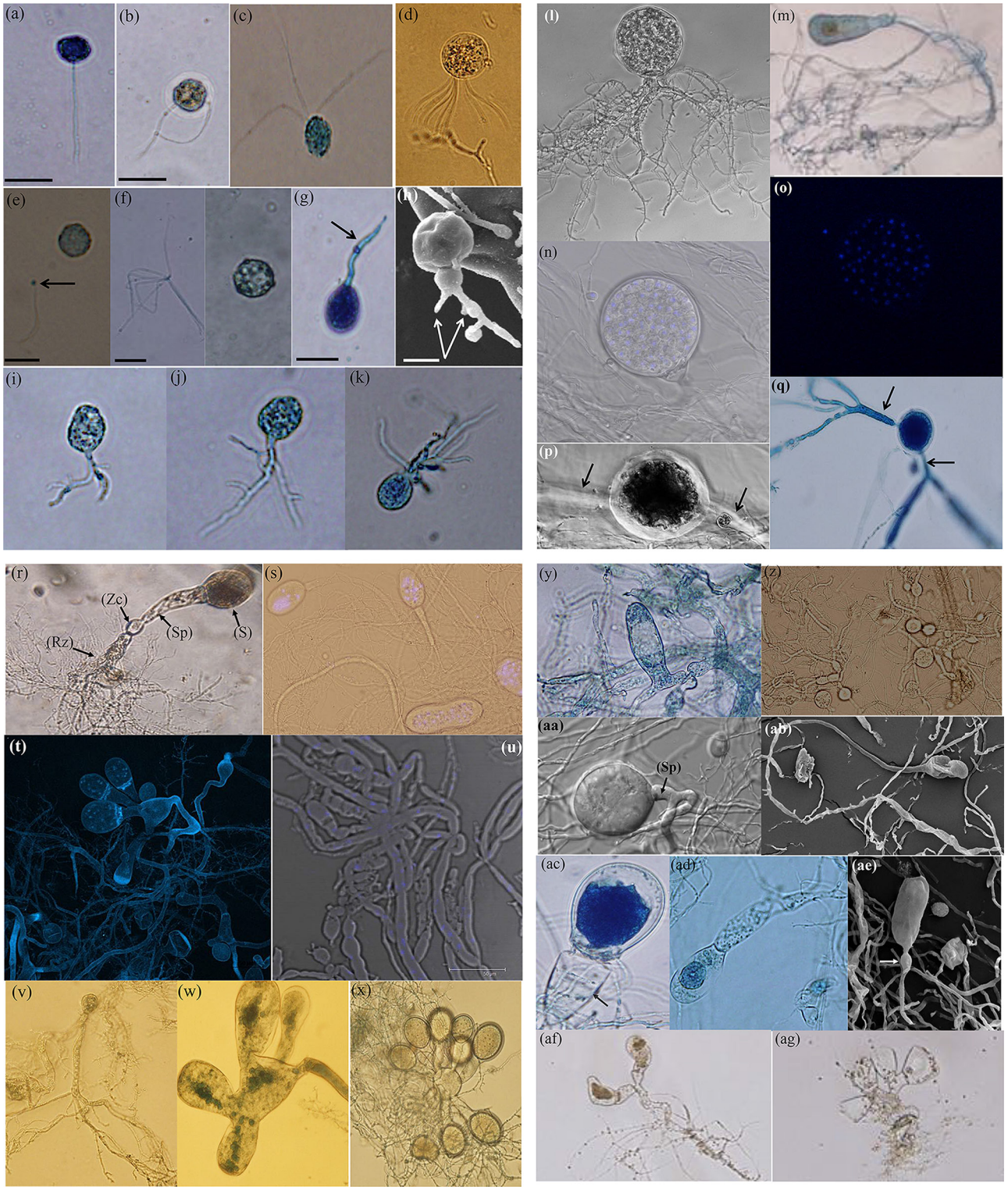
Identification and classification of anaerobic gut fungi currently rely on a combination of macroscopic (colony and liquid growth patterns), phenotypic (substrate utilization patterns and enzymatic activities), microscopic and sequence-based approaches. Microscopic characterization involves identification and documentation of the wide patterns of shapes, sizes and overall developmental processes associated with all structures produced within the complex life cycle of the *Neocallimastigomycota*. Molecular approaches involve utilizing various loci for phylogenetic analysis.

### Microscopic criteria: The life cycle of anaerobic gut fungi

The *Neocallimastigomycota* undergo a life cycle that involves the production and release of motile flagellated spores (zoospores) from sporangia (Fig. 2). These zoospores encyst, germinate and develop into a thallus structure that anchors the formation of new sporangia. Multiple structures within the life cycle display considerable variability between taxa and could hence be utilized for genus or species level delineation.

#### Zoospores

Anaerobic gut fungal spores range in size between 2.5 and 20 µm (Fig. 3a). However, the size of spores can vary depending on the culturing conditions and stage of development. *Neocallimastigomycota* spores are motile (i.e. zoospores) using flagellar movements. Emphasis on flagellar ultrastructure in earlier taxa description manuscripts [34, 42, 43] was a reflection of the importance of such trait in *Chytridiomycota* taxonomy [44]. Such emphasis has been waning, due to its complexity and the recognition that such feature is not absolutely necessary for differentiation of anaerobic fungal taxa. On the other hand, zoospore flagellation pattern is an extremely important trait for genus-level delineation. Most genera are monoflagellated, a term applied to taxa where the majority of zoospores have a single flagellum (Fig. 3a). In most 'monoflagellated' taxa, some zoospores with two, three

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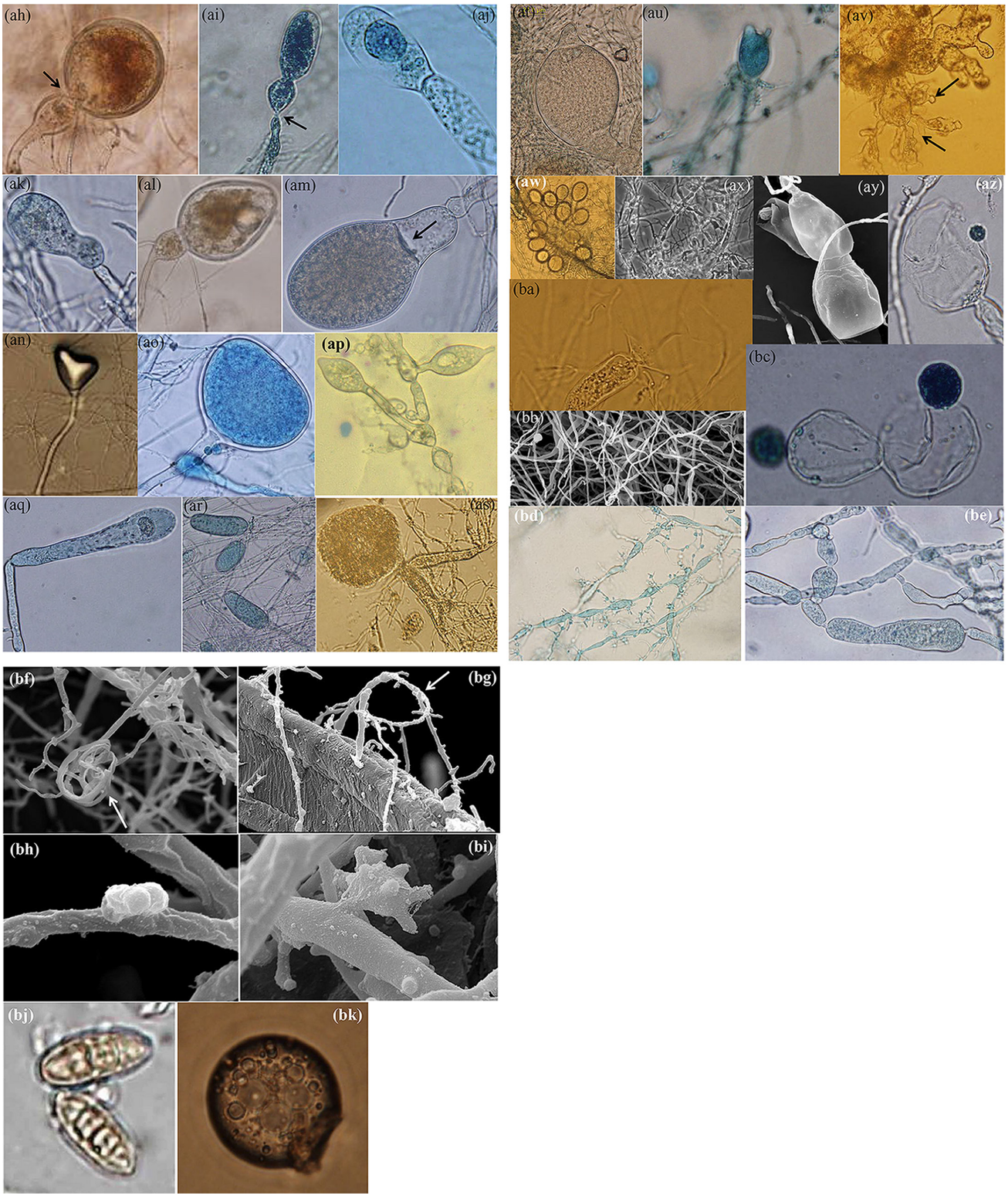


**Fig. 3.** Microscopic features observed in various stages of the *Neocallimastigomycota* life cycle with an emphasis on taxonomically informative traits. Zoospore flagellation (a–d): monoflagellated zoospores with a single (a, *Pecoramyces ruminantium* strain C1A), two (b, *Pecoramyces ruminantium* strain C1A), three (c, *Aklioshbomyces papillarum* strain WT2) or multiple (d, *Neocallimastix cf. frontalis* strain Hef5) flagella. Zoospore encystment (e, f): zoospore cyst and flagellar shedding (e) with the shed flagella forming a bead-like structure at the point of former attachment (arrow, *Pecoramyces ruminantium* strain C1A), (f) shed flagella from a polyflagellated zoospore (*Feramyces austinii* strain F3a), (g–h): development of a single (g) germ tube (*Pecoramyces ruminantium* strain C1A) and two (white arrows) germ

*Fig. 3. Continued*

tubes (*Feramyces austinii* strain F3a). Rhizoidal system development: branching of germ tubes (i–k, *Pecoramyces ruminantium* strain C1A). Monocentric thalli (l–s): endogenous sporangial development (l–o: l, *Joblinomyces apicalis* strain GFH686; m, *Ghazallomyces constrictus* strain Axs-31; n–o, *Pecoramyces ruminantium* strain C1A), pseudo-intercalary sporangial development (arrows indicate the two rhizoidal systems in both figures; p, *Aklioshbomyces papillarum* strain WT2; q, *Feramyces austinii* strain F3a), exogenous sporangial development (r, *Ghazallomyces constrictus* strain Axs-31; s, *Aestipascuomyces dupliciliberans* strain R4) with rhizoidal (Rz) development on one end of the zoospore cyst (Zc), sporangiophore (Sp) development on the other end of the zoospore cyst and a sporangium (s) developing at the end of sporangiophore. In monocentric thalli, nuclei stay within the spore and do not enter the germ tube during germination, resulting in an anucleated rhizoidal system (n and o, *Pecoramyces ruminantium* strain C1A; s, *Aestipascuomyces dupliciliberans* strain R4). Polycentric thalli (t–z): nuclei migrate out of the zoospore cysts into the germ tube, which elongates and branches. Subsequent division of the nuclei give rise to nucleated rhizoids, referred to as rhizomycelia (t, *Paucimyces polynucleatus* strain BB-3; u, *Orpinomyces cf. joyonii* strain D4A). Terminal sporangial development (v–x, *Paucimyces polynucleatus* strain BB-3) at the end of sporangiophores (v), on spherical vesicles (w) or at the end of sporangiophores on spherical vesicles (x). Intercalary sporangial development (y, *Orpinomyces cf. joyonii* strain D4A; z, *Orpinomyces intercalaris* strain SKP4) either as a lateral outgrowth of the hyphae (y), or as a result of hyphal expansion (z). *Sporangiophores* (aa–ag)*:* short sporangiophore (Sp) (aa, arrow, *Tahromyces munnarensis* strain TDFKJa1924). Long sporangiophore (ab, *Aestipascuomyces dupliciliberans* strain R4). Eggcup-shaped sporangiophore (ac, arrow, *Pecoramyces ruminantium* strain S4B). Wide flattened sporangiophore (ad, *Feramyces austinii* strain F3a). Sporangiophore ending with sub-sporangial swelling (ae, arrow, *Pecoramyces ruminantium* strain S4B, arrow). Branched sporangiophore on monocentric multisporangiate thalli with two (af, *Khoyollomyces ramosus* strain ZS-33) and four (ag, *Khoyollomyces ramosus* strain ZS-33) sporangia. Sporangial shapes (ah–as) and features (at–axe): globose sporangium with tightly constricted neck (ah, *Feramyces austinii* strain F3a), ellipsoidal sporangium with constriction at the middle, a tightly constricted neck and a narrow neck port (ai, *Ghazallomyces constrictus* strain Axs-31), ovoid sporangium with a broad neck and wide neck port (aj, *Feramyces austinii* strain F3a), bowling pin-shaped (ak, *Ghazallomyces constrictus* strain Axs-31) sporangium, egg-shaped sporangium (al, *Feramyces austinii* strain F3a) and pyriform sporangium with a basal wall (arrow) forming to separate mature sporangia from sporangiophores (am, *Ghazallomyces constrictus* strain Axs-31). Heart-shaped sporangium (an, *Feramyces austinii* strain F3a), triangular sporangium (ao, *Feramyces austinii* strain F3a), mucronate sporangium with a pointed apex (ap, *Anaeromyces mucronatus* strain BRL7), elongated sporangium (aq, *Ghazallomyces constrictus* strain Axs-31), ellipsoidal sporangia with no constrictions (ar, *Aklioshbomyces papillarum* strain WT2), rhomboidal sporangium (as, *Aestipascuomyces dupliciliberans* strain R4). Sporangia with a single (at) and double (au) papillae (*Aklioshbomyces papillarum* strain WT2). In polycentric species, thalli could lose the ability to produce sporangia and only produce sporangiophores initials (arrows) (av, *Paucimyces polynucleatus* strain BB-3), or could produce sterile sporangia (aw, *Paucimyces polynucleatus* strain BB-3). In such cases, isolates grow by rhizomycelial fragmentation (axe, *Orpinomyces* sp. strain GKP14). Spore released mechanisms (ay–ba): an apical pore formed at the top of the sporangia and the sporangial walls remained intact (ay, *Pecoramyces ruminantium* strain S4B), Sporangial wall rupture (az, *Aestipascuomyces dupliciliberans* strain R4). Complete dissolution of sporangial wall (ba, *Aestipascuomyces dupliciliberans* strain R4). Rhizoidal growth patterns (bb–be) and structures (bf–bi): Filamentous (bb, *Neocallimastix cf. cameroonii* strain G3) versus bulbous (bc, *Caecomyces cf. communis* strain DS1) rhizoidal growth patterns. Wide hyphae with multiple constrictions occurring at regular intervals resulting in a distinctive sausage-shaped morphology (bd, *Anaeromyces contortus* strain O2) or at irregular intervals (be, *Orpinomyces cf. joyonii* strain D4A). Hyphal coils (bf and bg, arrows), and appressoria (bh and bi) in *Anaeromyces contortus* strain O2. Resistant structures (bj, *Khyollomyces ramosus* strain HoCal4.A2.2), and enlarged titan cell-like structures (bk, *Pecoramyces ruminantium* strain C1A).

and even four flagella may be encountered (Fig. 3b, c). A few taxa are steadfastly monoflagellated, and have been referred to as 'uniflagellated' (e.g. genus *Anaeromyces* [45–47]). No genus where zoospores are predominantly bi, tri or tetra flagellate has yet been identified. In the relatively fewer polyflagellated *Neocallimastigomycota* genera described so far, the number of flagella per zoospore ranges between 7–30 (Fig. 3d). The evolution of multiple flagella is a unique trait of these anaerobic fungi within the *Opisthokonta* (Fungi and metazoans) clade.

**Cyst and germ tubes**

Chemical and mechanical cues induce anaerobic fungal zoospores to shed their flagella (Fig. 3e–f), encyst (Fig. 3f) and germinate (Fig. 3g–h). Zoospore encystment involves rounding up of the zoospore body and formation of a thick cell wall between the plasma membrane and the cell surface layer. To our knowledge, no major differences in size and morphology of cysts between taxa have been observed, and hence the cyst shape and size are not taxonomically informative. Cysts germinate by producing germ tubes. Some cysts produce a single germ tube (Fig. 3g), while others produce two germ tubes (Fig. 3h). The number of germ tubes per zoospore cyst is not a taxonomically informative trait as the same isolate can produce one or more germ tubes. Germ tubes eventually grow and branch to develop a rhizoidal system (Fig. 3i–k).

**Thallus development and sporangial formation mechanism**

Thallus developmental pattern is an extremely important trait for genus level delineation in the *Neocallimastigomycota*. Two thallus development patterns have been described: monocentric and polycentric. In monocentric thallus development, the nucleus stays within the spore and does not enter the germ tube during germination, resulting in an anucleated rhizoidal system. As such, one centre of reproduction exists per thallus.

Two distinct mechanisms for sporangial formation are recognized in monocentric thalli: endogenous and exogenous. Usually, monocentric genera exhibit both patterns during growth. In endogenous monocentric thalli, the zoospore cyst enlarges and develops into the sporangium, with anucleated rhizoidal growth originating from one side of the cyst (Fig. 3l–o). However, it

is important to note that since multiple germ tubes often arise from a single cyst in opposite directions as described above, a pseudo-intercalary sporangial development, in which sporangia are positioned in the middle of two main rhizoidal systems (Fig. 3p, q), could occur in some endogenous monocentric species. The production of pseudo-intercalary sporangia could be a taxonomically informative trait for species delineation. On the other hand, in exogenous monocentric thalli, a bipolar germination of the zoospore cyst occurs. Here, anucleated rhizoids develop at one end of the zoospore cyst. At the opposite end, a wider out-growth (sporangiophore) develops at the end of which a sporangium is formed (Fig. 3r). The nucleus migrates out of the zoospore cyst into the sporangium (Fig. 3s).

The second thallus developmental pattern known to occur in the anaerobic gut fungi is polycentric thallus development. Here, the nuclei migrate out of the zoospore cysts into the germ tube, which elongates and branches. Subsequent division of the nuclei in rhizoids give rise to nucleated rhizoids, referred to as rhizomycelia (Fig. 3t, u). The remaining empty zoospore cyst can persist, but has no further function in thallus development.

In polycentric taxa, sporangia are developed terminally or intercalary. Terminal sporangial development occurs in all polycentric taxa with one possible exception (*Orpinomyces intercalaris*). Here, sporangia either develop at the end of sporangiophores (Fig. 3v), or on spherical vesicles (swellings at the hyphal tips). When developing on spherical vesicles, multiple sporangia form, either directly on the vesicles (Fig. 3w) or at the end of sporangiophores (Fig. 3x). Intercalary sporangial development pattern is observed in only a few polycentric taxa. Here, sporangia develop as a lateral outgrowth of the hyphae (Fig. 3y) or from hyphal expansion (Fig. 3z).

### Sporangiophores

In monocentric taxa, sporangiophores are observed only during exogenous, but not endogenous, sporangial formation. Similarly, in polycentric taxa, sporangiophores are only observed in some terminal developmental patterns, as described above, but not in intercalary development patterns. Sporangiophores vary in length from few microns to 600 µm, and various degrees of length variations have been observed between taxa (Fig. 3aa–ab). Some sporangiophores exhibit unique morphology that are informative in delineating genera and species, e.g. eggcup shape (Fig. 3ac, arrow), or wide-flattened (Fig. 3ad). Some sporangiophores end with sub-sporangial swellings (apophysis) (Fig. 3ae, arrow). In some monocentric genera, sporangiophores are branched with two or more sporangia, resulting in monocentric multisporangiate thalli (Fig. 3af–ag). Monocentric multisporangiate thalli can be easily distinguished from polycentric thalli by their anucleated rhizoids.

### Sporangia

As described above, endogenous, pseudo-intercalary endogenous, and exogenous sporangia are observed in genera with monocentric thalli, while terminal and intercalary sporangia are observed in genera with polycentric thalli. The sporangial shape may be highly pleomorphic in some taxa, while others exhibit a highly uniform single sporangial shape. Shapes include globose (Fig. 3ah), ellipsoidal with constriction at the middle (Fig. 3ai), ovoid (Fig. 3aj), bowling pin-shaped (Fig. 3ak), egg-shaped (Fig. 3al), pyriform (Fig. 3am), heart-shaped (Fig. 3an), triangular ((Fig. 3ao), mucronate with a pointed apex (Fig. 3ap), elongated (Fig. 3aq), ellipsoidal without constriction (Fig. 3ar) and rhomboidal (Fig. 3as). Differences in size and shape between exogenous, endogenous and pseudo-intercalary sporangia within a single monocentric isolate is common. Media components and growth conditions could further contribute to such variations. Similarly, differences in size and shape between terminal and intercalary sporangia in a single polycentric isolate have also been frequently observed. In addition to size and shape, some sporangia exhibit additional features that are taxonomically informative. For example, few taxa produce sporangia with one or two papillae, i.e. having pointed protrusion at the distal end of the sporangia (Fig. 3at–au). Upon maturity, basal walls are formed to separate mature sporangia from sporangiophores (Fig. 3am). The sporangial necks (the point between sporangia and sporangiophore or sporangia and rhizoid) may either be tightly constricted (Fig. 3ah–ai), or broad (Fig. 3aj). The neck port (opening) may be narrow (Fig. 3ai) or wide (Fig. 3aj). The size, shape and level of pleomorphy of sporangial necks is potentially taxonomically informative.

It is important to note that polycentric genera often cease to produce sporangia and zoospores. With repeated subculturing, isolates progressively lose the ability to produce sporangia and only produce sporangiophores initials (Fig. 3av). In addition, many polycentric isolates lose their zoosporogenesis ability and produce sterile sporangia (Fig. 3aw), which makes their identification very challenging. In such cases, isolates can reproduce by rhizomycelial fragmentation (Fig. 3ax), which is feasible due to their nucleated nature.

### Spore release

Spore release could occur through an apical pore (Fig. 3ay), via rupturing of the sporangial wall (Fig. 3az), or a combination of both. Spore release may be facilitated by sporangial papillae (Fig. 3at-au). Sporangia could either stay intact (Fig. 3ay), or completely disintegrate (Fig. 3ba) after zoospore discharge. The mechanism(s) by which zoospores are released are taxonomically informative traits for distinguishing taxa at the genus and species levels.

### Rhizoidal growth pattern

Rhizoidal growth pattern is an extremely informative trait for genus-level delineation in the *Neocallimastigomycota*. Taxa either produce filamentous rhizoids (Fig. 3bb) or bulbous rhizoids (Fig. 3bc). Filamentous rhizoids are capable of penetrating the plant fibres by their tapering ends, while bulbous rhizoids expand their spherical holdfasts within plant tissue, physically rupturing the fibres from within.

The majority of genera exhibit a filamentous rhizoidal growth pattern, and produce two types of hyphae (narrow and wide). Narrow hyphae are more frequently observed, especially in monocentric taxa and do not seem to display much variation between taxa. Wide hyphae, usually more readily visible in polycentric taxa, display multiple constrictions, which could either occur at regular intervals resulting in a distinctive and taxonomically informative sausage-shaped morphology (Fig. 3bd), or at irregular intervals (Fig. 3be). On the other hand, only two *Neocallimastigomycota* genera exhibit bulbous rhizoidal growth pattern, with the production of spherical holdfasts (Fig. 3bc). Interestingly, a single isolate (identified as a member of the genus *Cyllamyces* in the original publication, but see the discussion on its identity below) could conceivably represent a transition between both growth patterns, as it produces elongated rhizoids in addition to intermittent bulbous holdfast (see fig. 2e–f in [48]). The prevalence, significance, and dependence on such feature on media composition and substrate remains unclear.

Finally, anaerobic gut fungi can produce additional rhizoidal structures that accelerate plant attachment and colonization, and such structures may be useful in taxonomic delineation. Hyphal coils (Fig. 3bf–bg) wrap around the plant fibres maximizing the contact area between the hyphae and the plant. Appressoria (Fig. 3bh–bi) develop as small protrusions on hyphae that enlarge into multi-lobed vesicles with several penetration pegs, aiding in the penetration and nutrients absorption from plant fibres.

### Resting stages (resistant body) formation in the *Neocallimastigomycota*

The observations that anaerobic gut fungi can be isolated from the saliva and dry faecal material of foregut and hindgut fermenters [49–51]), that some strains could survive for prolonged time intervals without subculturing [52], and that *Pecoramyces ruminantium* strain C1A could withstand relatively extended times of air exposure [53] have led to postulations that yet-unidentified structure(s) (referred to as resting stages or resistant bodies) could be produced as part of the life cycle of some *Neocallimastigomycota* taxa. Such postulations have been augmented by the microscopic observation of specific structures that do not directly fit into any of the life cycle phases described above. Examples include spherical structures in a strain morphologically similar to *P. communis* (Fig. 6, incorrectly labelled Fig. 5 in the figure legend in reference [54]), multichambered spore-like structures in *Anaeromyces* strains AUC1/AUC2 (Fig. 2 in [52]), *Buwchfawromyces eastonii* strain GE09 (Fig. S1f, g (available in the online version of this article) in [37]) and *Khyollomyces* strain HoCal4.A2.2 [55] (Fig. 3bj), as well as sporangial-like structures developing at the tip of elongated thalli in a *Neocallimastix* sp. strain MC-2 (Figs 7 and 8 in [56]). In addition, the formation of peculiarly enlarged cells in *Pecoramyces ruminantium* has been observed (Fig. 3bk). Remarkably, these cells bear strong morphological resemblance to titan cells: enlarged cells produced by *Cryptococcus neoformans* in response to exposure to the lung environment and shown to enhance the survival and propagation of *C. neoformans* [57]. In addition to putative induction by unfavourable conditions (air exposure or resource depletion), Davies *et al.* [51] suggested that development of such structures could be associated with the transition of fungal-associated biomass from the rumen to the faeces. However, conclusive information on the prevalence, composition, and role of putative resting stages in the *Neocallimastigomycota* life cycle is lacking. As such, the value of their description in current and future classification schemes remains uncertain.

### Caveats associated with microscopic characterization of *Neocallimastigomycota*

As described above, a wide range of microscopic variabilities occur in various life stages within the *Neocallimastigomycota*. However, the sole utilization of microscopic features, especially for species level delineation, could be tenuous due to three main reasons: First, a high level of pleomorphism, especially in sporangia and sporangiophore shapes, is observed within individual *Neocallimastigomycota* strains when grown in different media or even the same media. This is especially true for monocentric genera like *Piromyces* (Fig. S1a–h) and *Neocallimastix* (Fig. S1i–k). Many of such sporangial variations have unfortunately been used as defining criteria for proposing and differentiating species within the genus *Piromyces* (see below). Second, within polycentric taxa, the failure to produce sporangia (Fig. S1l–m) or zoospores, or the production of uncharacteristic sporangial shapes (Fig. S1n) often renders their identification challenging. Finally, difficulty in identifying the number of flagella (an important criterion for generic level differentiation) on some spores might lead to erroneous reporting of mono- and polyflagellation (Fig. S1o–p). Such pleomorphism or paucity in morphological features hence necessitates the simultaneous use of molecular tools for the accurate identification and characterization of anaerobic gut fungi. The concurrent use of both morphological and molecular tools has greatly facilitated the description of most newly described genera, which otherwise would not have been possible.

## Phylogenetic markers for *Neocallimastigomycota* taxonomy

Within the ribosomal operon (Fig. 4a), various loci have been examined for their utility as phylogenetic markers for *Neocallimastigomycota* taxonomy. Studies using the 18S rRNA and 5.8S rRNA subunits suggested their limited value in taxa resolution due to the high level of sequence conservation, or short length, respectively [58]. However, these loci have proven useful for

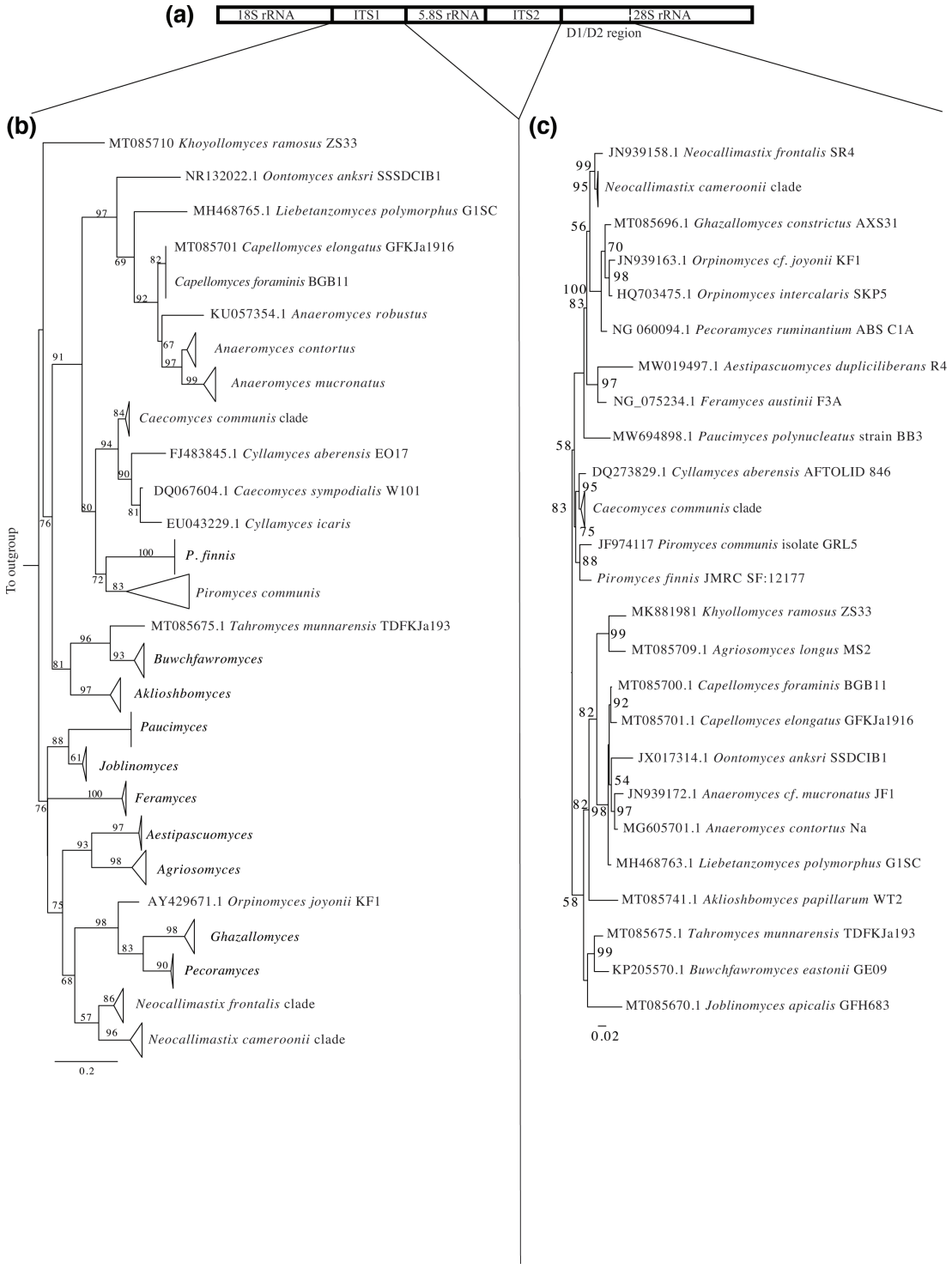


**Fig. 4.** (a) A cartoon of the rRNA locus encompassing the SSU rRNA (18S), the 5.8S rRNA and the LSU rRNA (28S) genes with the two inter-transcribed spacer regions (ITS1 and ITS2). Sizes of various loci are not to scale. (b, c) Maximum-likelihood phylogenetic tree reconstructed using the ITS1 region (b), and D1/D2 region of the LSU rRNA genes (c) of the type species of all described *Neocallimastigomycota* species. For reconstructing both trees, sequences were aligned using the MAFFT aligner with the –auto flag and the default parameters for gap extension and gap opening penalties. The maximum-likelihood tree was reconstructed in FastTree. Bootstrap values are based on 100 replicates and are shown for branches with >50% bootstrap support. (b) ITS1 region sequences of all type species with the exception of *Orpinomyces intercalaris* (for which no available ITS1 sequence was available) were utilized to reconstruct the tree. All clone sequences were used when available. For *Caecomyces churrovis*, *Piromyces finnis* and *Neocallimastix lanati*, the ITS1 regions were extracted from their corresponding genomic sequences. Detailed sub-trees for the genera *Neocallimastix* and *Caecomyces*/*Cyllamyces*, and *Anaeromyces* are shown in Figs. 6–8, respectively. (c) D1/D2 region of the LSU rRNA genes sequences from type species for all described *Neocallimastigomycota* taxa (with the exception of *Anaeromyces robustus*, *Caecomyces sympodialis* and *Cyllamyces icaris* for which D1/D2 sequences are not available) were utilized to construct the tree.

detection [59] and quantification [60] of anaerobic gut fungi in environmental samples. The use of the intergenic spacer region has been restricted to molecular typing methods, e.g. restriction fragment length polymorphism (RFLP), and reported only partial concordance with genus-level assignments using microscopic features [61].

The two most extensively utilized loci for resolving *Neocallimastigomycota* taxonomy are the internal transcribed spacer 1 (ITS1, Fig. 4b), and the 28S large ribosomal subunit (LSU), especially the 5′ region covering the hypervariable D1 and D2 domains in the molecule (D1/D2 LSU, Fig. 4c). ITS1 has been used for ribotyping [62, 63] as well as sequence-based analysis [64], a reflection of its wide utilization in broader mycological research [65]. A threshold of 2 [66] or 3% [31], and a 5 [66] or 6% [66] sequence divergence has often been utilized for species- and genus-level delineation, respectively. However, as previously demonstrated [28, 29, 66], the ITS1 locus in *Neocallimastigomycota* taxonomy suffers from two main flaws: First, considerable sequence divergence is often encountered between copies of the rRNA operon within a single strain [28, 29, 37], rendering accurate species and strain level resolution challenging. Second, ITS1 length varies greatly between different genera [28, 66], limiting its value in assessing inter-genus relationships and supra-genus associations.

The use of the D1/D2 LSU region was first reported in RFLP-based surveys [61, 63], and consequently proposed to resolve relationships between species of a single genus (*Orpinomyces*) [67] and subsequently between various genera [68]. The lack of intra-strain sequence divergence, as well as its uniform length (750–760 bp) across all genera renders it an appealing molecular taxonomic marker [28]. Indeed, all taxa description manuscripts since 2015 reported D1/D2 LSU sequences, and reference sequences for all genera have been generated [28]. Recently, a concerted effort by the scientific community has produced a curated D1/D2 alignment with representatives of all cultured and uncultured clades to facilitate taxonomic assessment and diversity surveys (available at https://anaerobicfungi.org/databases/). Finally, efforts towards utilizing the entire ribosomal operon for taxonomic assessment using long read nanopore sequencing is currently underway (D. Young, personal communication).

Utilization of protein coding genes, e.g. elongation factor 1-α (*EF1α*), and two different RNA polymerase II subunits (*RPB1* and *RPB2*) as phylogenetic markers for *Neocallimastigomycota* taxonomy has been reported, but only as part of larger efforts to resolve interphylum relationships within the kingdom fungi, and always utilizing very few strains [69]. Broader phylogenomic efforts have recently been contributing to resolving the phylum's position within the fungal tree of life, as well as the relationship between various *Neocallimastigomycota* genera. Utilizing a collection of 26 genomic and transcriptomic datasets, Wang *et al*. used a concatenated set of 434 highly conserved and generally single-copy protein-coding genes in fungi for phylogenetic inference using maximum-likelihood and Bayesian approaches [9]. The resulting work has led to a well-resolved phylogenomic tree that was congruent with D1/D2 LSU-generated phylogenetic outline. Future availability of genomes and transcriptomes from *Neocallimastigomycota* taxa would greatly enhance the value of multi locus and genomic-based approaches for resolving *Neocallimastigomycota* taxaonomy.

## KEY FOR THE IDENTIFICATION OF CURRENTLY DESCRIBED *NEOCALLIMASTIGOMYCOTA* GENERA

Twenty anaerobic gut fungal genera have been described so far, based on a combination of growth features, microscopic characteristics of various life cycle stages, and phylogenetic analysis [34–37, 43, 45, 55, 70–75]. A key for the identification and differentiation of members of these genera is presented below (Table 1). The key aims to provide an accurate, fast and convenient procedure for taxa identification. As such, preference is given to readily discernable macroscopic (e.g. colony morphology, liquid growth pattern) and microscopic (thallus development, rhizoidal growth pattern, and zoospore flagellation) features over more challenging characteristics, e.g. zoospore ultrastructure, or genome- and transcriptome-based phylogenomic approaches.

Genera in the *Neocallimastigomycota* exhibit either a monocentric or a polycentric thallus developmental pattern. Such patterns are determined by microscopically examining the distribution of nuclei in preparations stained with nuclear stains, e.g. 4′,6′ diamidino-2-phenylindol (DAPI) or bisbenzimide. Polycentric taxa show nuclei throughout the thalli (Fig. 3t–u), while in monocentric taxa, stained nuclei will be observed only in sporangia (Fig. 3n–p and s), although few are occasionally be observed in the sporangiophores in immature sporangia before septum development.

Four polycentric genera have been described so far: *Orpinomyces*, *Anaeromyces*, *Cyllamyces and Paucimyces*. Further differentiation between these genera is achieved by observing spore flagellation patterns (*Orpinomyces* is unique among polycentric genera in having polyflagellated zoospores *n*=12–24), rhizoidal growth pattern (*Cyllamyces* is unique among polycentric genera in having a distinct bulbous growth pattern), hyphal morphology (*Anaeromyces* is unique in having hyphal constrictions at regular intervals resulting in distinctive sausage-shaped hyphae (Fig. 3bb)), or sporangial developmental pattern (*Paucimyces* is unique in producing spherical vesicles at the hyphal tip from which multiple sporangia develop, Fig. 3v–x). In addition, many of these genera exhibit unique macroscopic features that allow for a quick tentative visual identification. For example, *Orpinomyces* strains generate a unique cottony growth in liquid media that has not been observed in other genera, and *Orpinomyces* colonies on roll tubes also tend to spread out sometimes to >1 cm in diameter (see detailed taxa description and illustrations of the genus *Orpinomyces* below). *Anaeromyces* strains in liquid media form thick pearl-like growth patterns (Fig. S1 in reference [76]).

**Table 1.** Key for Anaerobic gut fungi (AGF) genera characterization

| Key features | | | | Additional key morphological characteristics |
|---|---|---|---|---|
| 1 | Thallus development pattern | Polycentric | 2 | |
| | | Monocentric | 5 | |
| 2 | Zoospore flagellation | Polyflagellated | *Orpinomyces* | Large colony size >1 cm and thick cottony biofilm |
| | | Monoflagellated | 3 | |
| 3 | Rhizoidal system | Bulbous | *Cyllamyces* | |
| | | Filamentous | 4 | |
| 4 | Sausage-shaped hyphae and mucronate sporangia | | *Anaeromyces* | Thick granular pearl-like growth |
| | Spherical vesicles from which multiple sporangia develop | | *Paucimyces* | |
| 5 | Rhizoidal system | Bulbous | *Caecomyces* | Granular colonies and sand-like fungal biofilm |
| | | Filamentous | 6 | |
| 6 | Zoospore flagellation | Polyflagellated | 7 | |
| | | Monoflagellated | 8 | |
| 7 | Dual zoospore release mechanism | | *Aestipascuomyces* | |
| | Colony size of 3–7 mm and thick fungal biofilm in liquid media | | *Feramyces* | |
| | Constricted sporangial neck and narrow neck port | | *Ghazallomyces* | |
| | Colony size up to 2.5 mm and thin fungal biofilm in liquid media | | *Neocallimastix* | |
| 8 | Long flagella (5–6 times longer than the zoospore body) | | *Agriosomyces* | |
| | Sporangia with one or two papillae | | *Aklioshbomyces* | |
| | Swollen sporangiophores and twisted rhizoids | | *Buwchfawromyces* | |
| | Formation of empty cup-shaped sporangia after zoospore release | | *Joblinomyces* | |
| | Branched sporangiophores and multi-sporangiate thalli | | *Khyollomyces* | Loose sand-like fungal biofilm |
| | Different polymorphic sporangial shapes | | *Liebetanzomyces* | |
| | Constriction at the sporangiophore base and intercalary rhizoidal swellings | | *Oontomyces* | |
| | Thin fungal biofilm that attaches firmly to the tube's surface glass | | *Piromyces* | |
| | Sub-sporangial swelling and short sporangiophore | | *Tahromyces* | |
| | Phylogenetic analysis to confirm identity | | *Capellomyces* | |
| | Phylogenetic analysis to confirm identity | | *Pecoramyces* | |

The majority of genera (16/20) are monocentric, and their rapid macroscopic or microscopic differentiation is more challenging. In monocentric genera, bulbous rhizoidal growth pattern has been observed in one genus (*Caecomyces*), allowing for a relatively straightforward identification. In addition, this genus is characterized by a granular colony morphology, and a 'sandy' growth in liquid media (Fig. 5n) that resembles bacterial, rather than fungal growth, and differentiates *Caecomyces* from other monocentric filamentous genera (see detailed taxa description and illustrations of the genera *Caecomyces* and *Cyllamyces* below).

Within the remaining 15 monocentric filamentous genera, four produce polyflagellated zoospores (*Aestipascuomyces*, *Feramyces*, *Ghazallomyces* and *Neocallimastix*). These genera could be differentiated by their unique macroscopic or microscopic traits. *Aestipascuomyces* strains are unique in their dual spore release mechanism through an apical pore and sporangial dissolution. *Feramyces* strains produce medium-sized (3–7 mm), circular, beige colonies with well-defined dark centre and filamentous edges

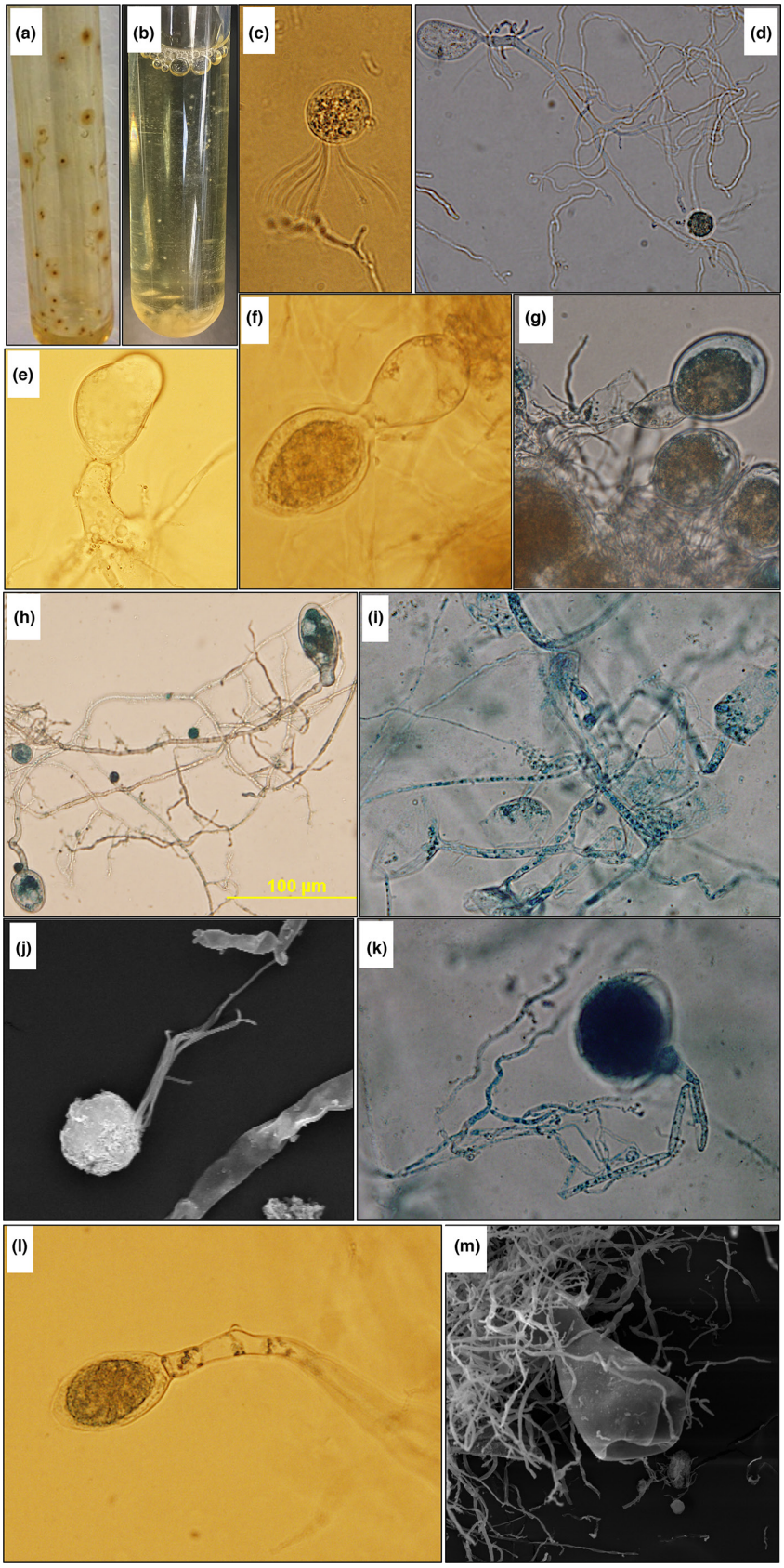


*Fig. 5. Continued*

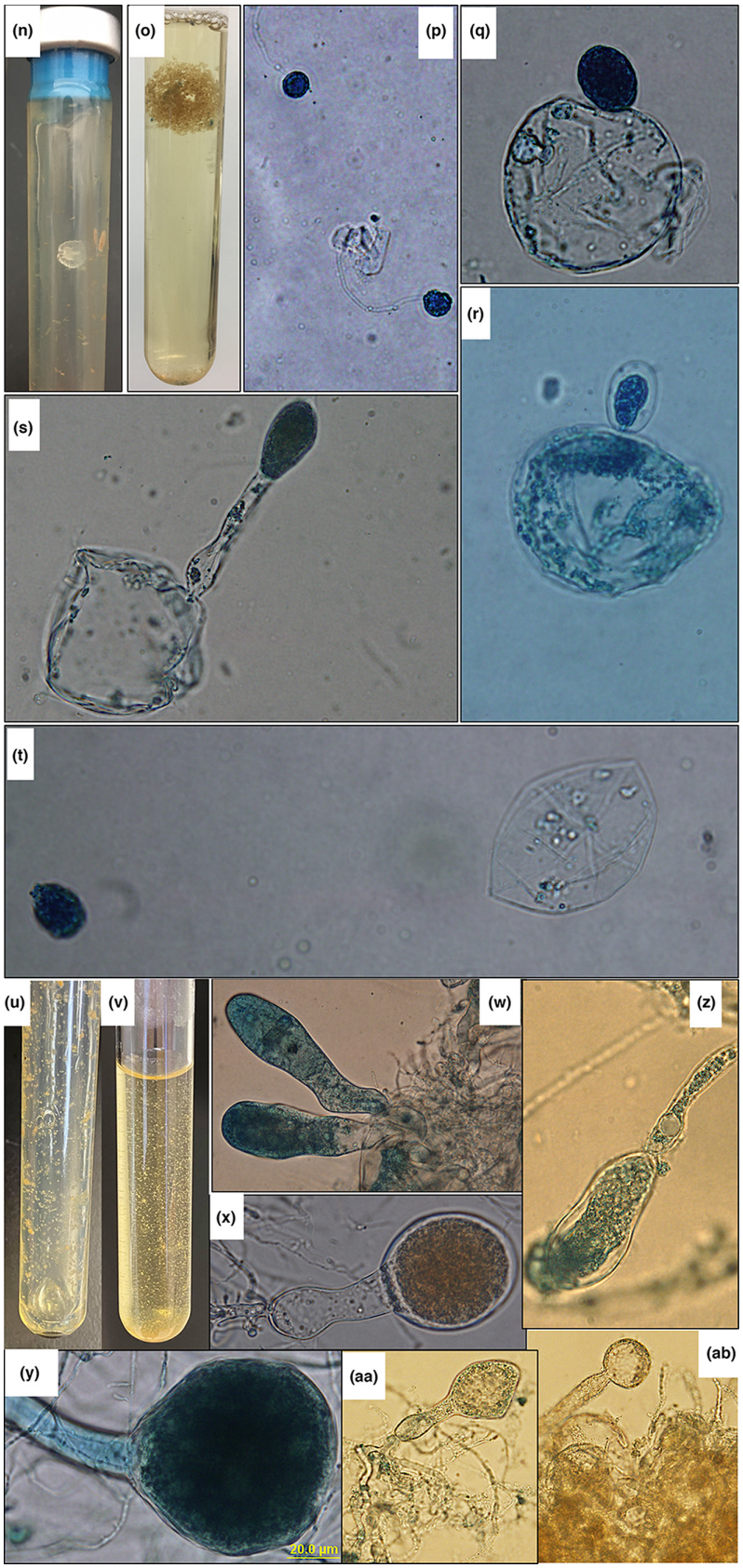


*Fig. 5. Continued*

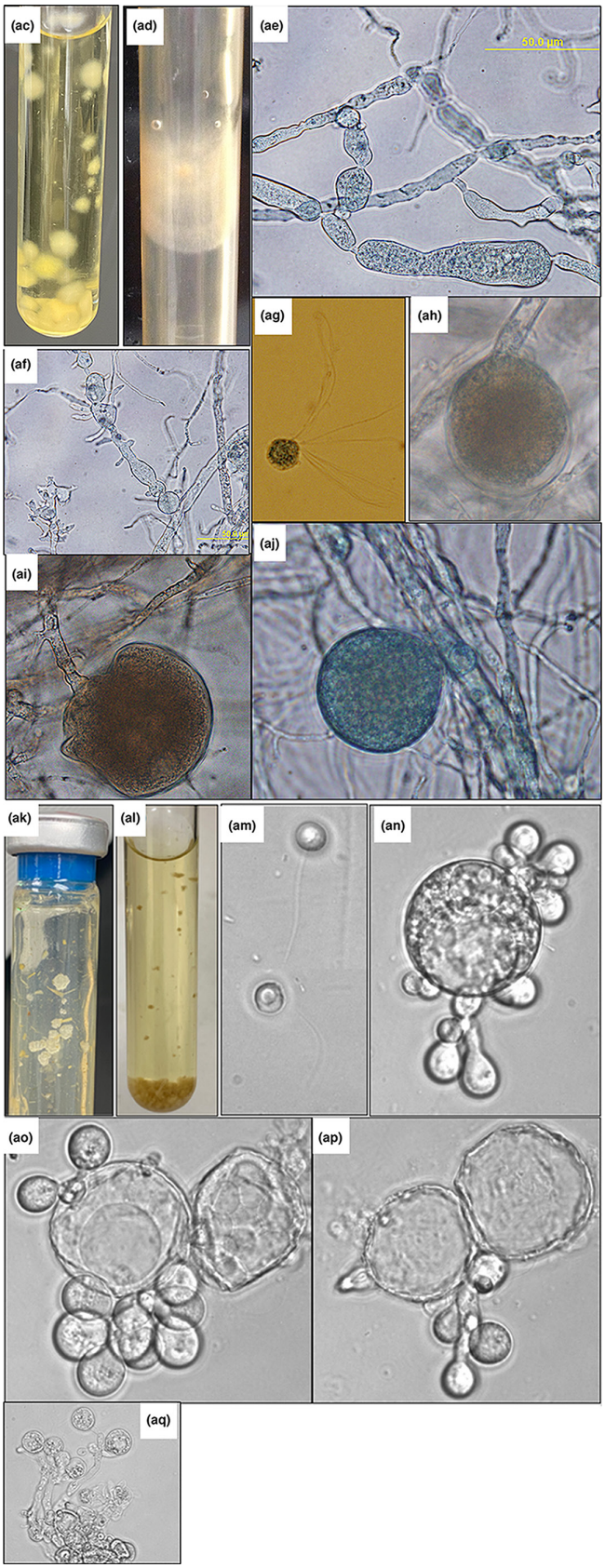


*Fig. 5. Continued*

**Fig. 5.** Additional illustrations of multiple *Neocallimastigomycota* species using *cf*. strains from the authors' culture collections. *Neocallimastix frontalis* strain Hef5 (a–m): beige circular colonies (up to 2.5 mm) with a dark centre of sporangial structure on agar roll tubes (a), and thin biofilm growth in liquid media (b); globose zoospores with 9–17 flagella (c); endogenous ellipsoidal (d) and mitten-shaped (e) sporangia; ovoid exogenous sporangia at the end of a short sporangiophore with an egg-cup like morphology (f); globose exogenous sporangia at the end of a short sporangiophore with an egg-cup like morphology (g); ellipsoidal exogenous sporangia at the end of a long sporangiophore (h); zoospores release through dissolution and rupturing of the entire sporangial wall (i). *Neocallimastix cf. cameroonii* strain G3 (j–m): globose zoospores with 9–17 flagella (j); globose (k) and ovoid (l) mature sporangia; zoospore release through a wide apical pore with the sporangial wall stays intact (m). *Caecomyces cf. communis* strain DS1 (n–t): *Caecomyces* produces small white granular colonies (n), and sandy morphology growth in liquid media (o). *Caecomyces cf. communis* strain DS1: globose monoflagellated zoospores (p). Bulbous rhizoidal system with spherical holdfasts and both endogenous and exogenous; globose endogenous sporangia (q), ovoid endogenous sporangia (r), ovoid exogenous sporangia at the end of long tubular sporangiophore (s). Sporangia detached from the rhizoidal system (t). *Piromyces cf. communis* strain Jen1 (u–y), and *Piromyces cf. finnis* strain DonB11 (z–ab): *Piromyces* produces small circular colonies with a dark centre of sporangial structure (u), and exhibits thin biofilm-like growth in liquid medium that firmly attaches to the glass walls of the tube (v). Sporangia develop both endogenously and exogenously with variable shapes including elongated (w), spherical (x and ab), ovoid (y), ellipsoidal (z), and diamond-shaped (aa). Some sporangia have broad neck with broad neck port (o). Short egg-cup shaped sporangiophores are frequently observed (x and aa). *Orpinomyces cf. joyonii* strain D4A (ac-aj): *Orpinomyces* forms large (usually >1 cm diameter) white colonies on agar roll tubes (ac), and a characteristic thick cottony fungal growth (ad) in liquid media. Narrow and wide hyphae; with the wide hyphae displaying multiple constrictions at irregular intervals (ae). Old cultures producing sporangiophores initials and no sporangia (af). (ag–aj) *Orpinomyces cf. joyonii* strain D4A: globose polyflagellated zoospores with 10–25 flagella (ag). Globose terminal sporangia developed at the tip of sporangiophore (ah–ai). Sporangia developed as a lateral outgrowth of hyphae (aj). *Cyllamyces cf. aberensis* strains TSB2 (ak–al), CFH681 (am–ap) and BFH688 (aq): *Cyllamyces* produces granular colonies on agar tubes (ak), and thin loose biofilm-like growth in liquid media (al). *Cyllamyces* displays monoflagellated zoospores (am), bulbous rhizoidal system with multiple sporangia, and polycentric thallus with nuclei in the rhizoidal holdfast and the sporangia (an–ap). In the presence of rice straw, *Cyllamyces* sometimes produces elongated rhizoids and bulbous holdfast (aq).

on solid media, and a thick biofilm growth pattern in liquid mediain reference (see fig. 1 in [71]). In contrast, *Neocallimastix* strains produce smaller beige circular colonies (up to 2.5 mm) with a dark centre of sporangial structure, and a thin biofilm growth pattern in liquid media (see detailed taxa description and illustrations of the genus *Neocallimastix* below). Finally, *Ghazallomyces* strains produce a characteristic sporangial neck constrictionin reference (see Fig. 5d–g in [55]]).

The 11 remaining genera all exhibit the same thallus development (monocentric), rhizoidal growth (filamentous) and zoospore flagellation (monoflagellated) patterns. Most of these genera could be identified based on unique microscopic features, e.g. extremely long flagella in *Agriosomyces* zoospores(Fig 2a in [55]), papilla on the sporangia of *Aklioshbomyces* (Fig. 3at–au), presence of swollen sporangiophorein (Fig. 1e–h in [37]) and twisted rhizoidsin (Fig. 1b in [37]) in *Buwchfawromyces*, formation of empty cup-shaped sporangia after zoospore release in *Joblinomyces* (Fig. 6c–f in [55]), branched sporangiophores and multisporangiate thallus in *Khoyollomyces* (Fig. 3af-ag), highly pleomorphic sporangial shapes in *Liebetanzomyces* (Figs 2c–l and 3a–f in [73]), constriction in the base of sporangiophores (Fig. 1d, e in [36]) and intercalary rhizoidal swelling (Fig. 1f–g in [36]) in *Oontomyces*, formation of thin fungal biofilm that attaches firmly to the tube's glass surface in *Piromyces*, and formation of short sporangiophores and sub-sporangial swellings in *Tahromyces* (Fig. 9j–k in [55]). Nevertheless, the subtlety of many of these characteristics, and the fact that, in most cases, only one species has been described for each of the genera described above, renders molecular sequence identification necessary for confirmation.

## *NEOCALLIMASTIGOMYCOTA* GENERA AND SPECIES

Progress in isolation and characterization of anaerobic gut fungal taxa has been a continuous, yet uneven, process during the last ≈45 years. Their strict anaerobic nature and extreme oxygen sensitivity necessitate the mastery of the anaerobic Hungate techniques [77, 78] for their isolation and maintenance. Additionally, although long-term storage procedures for various taxa have been described [79–81], their efficacy and reliability vary greatly between strains. Finally, senescence is frequently observed, with repeated subculturing often leading to either the production of sporangia that do not differentiate to zoospores, or the outright failure to produce sporangia [33]. Such difficulties have resulted in the loss of multiple historic strains, and are reflected by the inability of many culture collections to maintain and preserve *Neocallimastigomycota* isolates. Orpin was the first to report on obtaining a culture with a single fungal strain (*P. communis*) in 1976, but the culture was not axenic but rather a mixed bacterial-fungal culture [27]. Successful isolation of anaerobic gut fungi as a pure culture was first reported for *Sphaeromonas* and *Piromonas* from the horse caecum [54], and *Neocallimastix* from the cow rumen [17, 82] in 1981. Heath *et al*. [34] described the ultrastructure of the polyflagellate zoospore of a pure culture of *Neocallimastix frontalis* [44]; and the manuscript represents the first formal description of an anaerobic gut fungal genus. Gold *et al*. [43] used flagellar ultrastructure and life cycle description to characterize two bulbous isolates, and proposed the name *Caecomyces* for the flagellated structures previously named *Sphaeromonas*. The same manuscript also proposed the name *Piromyces* as a substitute for *Piromonas*, although no characterization was reported for *Piromyces* in this paper. The genus *Piromyces*, together with the novel genus *Orpinomyces* were described the following year [70], and a fifth genus (*Anaeoromyces*) was subsequently described in 1990 [45]. A notable

lull in the description of new genera followed, with only one genus (*Cyllamyces*) [74] described in the next quarter century. Recently, a rapid acceleration of the pace of taxa description has occurred, with the description of 14 new genera from India, China, the UK, Germany, and the USA since 2015 [35–37, 55, 71, 72, 75] (Fig. 1). Such expansion has been spurred by renewed interest in exploiting the biotechnological potential of anaerobic gut fungi, for example for biofuel [83], value-added products [84] and secondary metabolites [85].

Here, we synthesize data from taxa description manuscripts, comparative studies (e.g. [46, 86, 87]), molecular sequence data, and examination of strains from our culture collection to provide an updated list of *Neocallimastigomycota* genera and species. Genera are presented by the chronological order of their discovery. Synonyms are proposed based on available molecular and phenotypic data. In cases where the information to assess synonymy is inadequate, both names are retained with a proposition to use a single name for describing future strains. Uncertain affiliations based on inadequate descriptions are noted. Currently uncultured alphanumeric genus-level clades identified only in culture-independent surveys (e.g. AL3, MN4, SK3) are not included.

## *NEOCALLIMASTIX* BRAUNE 1913 [20], VAVRA AND JOYON 1966 [88], HEATH *ET AL.* 1983 [34]

*History*. The name '*Neocallimastix*' predates discovering the fungal nature of 'rumen flagellates' by Orpin [7]. Polyflagellated structures similar to *Neocallimastix* zoospores were observed in 1913 [20], for which the name *Callimastix frontalis* was proposed. Vavra and Joyon proposed that the *Callimastix* flagellate *C. cyclopsis* represents the dispersal phase of zoosporic fungi and renamed it as *Neocallimastix* with *frontalis* as the type species [88]. Isolates belonging to the genus *Neocallimastix* were first reported in 1981 [17] and the formal description of the first isolate (*N. frontalis*, strain PN1) was provided by Heath *et al* in 1983 [34].

*Type: Neocallimastix frontalis* strain PN1, Figs 20–25 in [34]

*GenBank sequences accession numbers:* No sequence data are available from the type strain. Multiple morphologically identical *cf.* strains were subsequently isolated; and the earliest deposited sequence data for *N. frontalis* is *N.* cf. *frontalis* isolate RE1 isolated from sheep rumen [89], clones obtained in [66] MK036660.1-MK036676.1 (ITS1, 5.8S, ITS2), and KR920744 (LSU) obtained in [90].

*ID in Databases:* MycoBank ID: MB25486; Index Fungorum: IF25486; NCBI taxon ID: 4756.

*Diagnosis*. Description of the genus *Neocallimastix* is provided in [34], and expanded in [86] and [33]. On solid agar media, *Neocallimastix* strains produce beige circular colonies (up to 2.5 mm) with a dark centre of sporangial structure (Fig. 5a). In liquid media, *Neocallimastix* strains usually exhibit thin biofilm growth (Fig. 5b). The genus *Neocallimastix* is characterized by its polyflagellated zoospores (7–30 flagella, Fig. 5c), monocentric thalli, and filamentous rhizoids, with both endogenous (Fig. 5d–e) and exogenous (Fig. 5f–h) sporangial development patterns.

*Occurrence*. Members of the genus *Neocallimastix* have been isolated from faecal and/or rumen samples of cows (*Bos taurus*) [91], water buffalo (*Bubalus bubalis*) [33], goat (*Capra hircus*) [39], sheep (*Ovis aries*) [34, 38, 41, 82, 92–94], deer [28], sika deer (*Cervus nippon*) [95], gaur (*Bos gaurus*) [96], elk (*Cervus canadensis*) [28], yak (*Bos grunniens*) [64], chamois (*Rupicapra rupicapra*) [97] and alpine Ibex (*Capra ibex*) [98]. Culture-independent surveys examining *Neocallimastigomycota* diversity in a large number of animal species suggest a ubiquitous and highly abundant pattern of occurrence of the genus *Neocallimastix*, being consistently encountered in the majority of examined animal species, and often representing a large fraction of the overall community [28]. A higher prevalence in foregut over hindgut fermenters [31], mirroring the isolation sources reported for most taxa, as well in captive over wild animals [28], has also been proposed.

*List of Neocallimastix species*. The following names have been proposed for various *Neocallimastix* isolates: *N. frontalis* [34, 88], *N. patriciarum* [93], *N. hurleyensis* [92], *N. variabilis* [91], *N. cameroonii* [38], *N. californiae* [39] and *N. lanati* [41]. The potential synonymy between the first four species (*N. frontalis*, *N. hurleyensis*, *N. patriciarum* and *N. variabilis* [91]) has previously been debated. *N. frontalis* strain PN1 was isolated from the ovine rumen, as previously described. *N. hurleyensis* was also isolated from the ovine rumen in 1985 [99], morphologically described in 1987 [100], but formally named in 1991 [92] as a *Neocallimastix* species distinct from *N. frontalis* based on observed differences in flagellar numbers and ultrastructure [92], and differences in zoospore release mechanisms, with *N. hurleyensis* releasing zoospores through a single apical pore, as opposed to the dissolution of the entire zoosporangial wall in *N. frontalis* [100]. Additional differences include the lack of reference to exogenous sporangial development in *N. hurleyensis* description manuscript [92]. However, Ho and Barr [33] suggested that such differences could be attributed to differences in growth conditions, inter-laboratory variations, or failure to observe a specific feature, and proposed *N. hurleyensis* as a possible synonym of *N. frontalis*. Similarly, Ho and Barr [33] argued for the lack of differences between *N. variabilis* and *N. frontalis*. *N. patriciarum*, originally isolated by Orpin in 1975 and named *N. frontalis* [7], was subsequently assigned to *N. patriciarum* [93] following the Latin description of *N. frontalis* by Heath *et al.* [34], and the distinction between *N. patriciarum* (isolate CX of Orpin [7]) and *N. frontalis* (isolate PN1 of Heath [34]) was mainly based on number of zoospore flagella, the presence or absence of an equatorial constriction of the zoospore, flagellar ultrastructure differences, as well as major fermentation end products and carbon source utilization patterns differences [93]. Finally, Wubah and Fuller [86] compared type

strain (PN1) of *N. frontalis* (isolate PN1 of Heath [34]) and *N. patriciarum* (isolate CX of Orpin [93]), and concluded that there are insufficient microscopic differences to justify separating the two strains into two species.

We sought to further assess this potential synonymy using phylogenetic analysis. We recognize that multiple gaps exist in availability of sequence data from original or *cf*. strains for some taxa. We utilized ITS1 (Fig. 6) and D1/D2 LSU sequence data (when available) from *cf*. strains of *N. frontalis* (strain Re1, ITS1; strain SR4, LSU), *N. hurleyensis* (strain R1, ITS1), and *N. patriciarum* (strain CX, LSU) to further examine potential synonymy. To our knowledge, no sequence data from original or *cf*. strains are available for *N. variabilis*. Nineteen clones are available for the *N. cf. frontalis* strain Re1. These clones are 0–5.65% divergent (average 0.82%). The single ITS1 sequence available for the original *N. hurleyensis* strain R1 [52] diverges by 0.96–6.3% to the 19 *N. cf. frontalis* isolate Re1 clones (average 1.69%) (Fig. 6), confirming earlier morphological data that proposed synonymy of *N. hurleyensis* with *N. frontalis*.

The only available sequence for isolate CX, Orpin's original culture from which the *N. patriciarum* type material was derived, covers the D1/D2 region of LSU and shows only 0.43% divergence to *N. cf. frontalis* strain SR4 (currently the oldest *N. cf. frontalis* isolate with an available D1/D2 LSU sequence in the database), again confirming earlier morphological data that proposed synonymy of *N. patriciarum* to *N. frontalis*. As such, phylogenetic analysis confirms potential synonymy previously proposed by microscopic examination for all four species. We propose using the variety (var.) rank for retaining informative names in strains where synonymy is proposed i.e. referring to *N. variabilis* as *N. frontalis* var. *variabilis*, and to *N. hurleyensis* as *N. frontalis* var. *hurleyensis*.

Synonymy is also proposed for the three subsequently proposed species (*N. cameroonii*, *N. californiae* and *N. lanati*). Available molecular sequence data for these three species include: LSU data from the type *N. cameroonii* isolate ABS CaDo3a, the whole region encompassing ITS1-5.8S rRNA-ITS2-D1/D2 28S rRNA for an additional *N. cf. cameroonii* strain (strain G3), ITS1 data as well as genomic sequences encompassing several rRNA loci from *N. californiae* strain G1, and genomic sequences encompassing several rRNA loci from *N. lanati*. Comparison of the ITS1 and the D1/D2 LSU regions of these *Neocallimastix* species showed a high degree of similarity with only 0.58–1.72% divergence in the ITS1 region (Figs. 4a and 6), and 0.15–0.56% divergence in the D1/D2 LSU region (Fig. 4b). The available microscopic data for *N. californiae* [39] does not provide conclusive evidence for clear phenotypic differences when compared to *N. cameroonii*, and currently no microscopic data are available for the effectively, but not yet validly, published *N. lanati*.

Interestingly, prior studies appear to have isolated, but misclassified, strains that are highly related to *N. cameroonii*. Li and Heath [96] isolated a *Neocallimastix* species for which they sequenced the ITS1 region, and designated this species as *N. cf. patriciarum*, but this designation was not morphologically justified in the manuscript. Other strains with available ITS1 sequences that were also assigned as *N. cf. patriciarum* strains are isolates NMW1 obtained from Malaysian water buffalo [62], and isolate BTN1 (GQ355329.1) obtained from Indian cattle. The % divergence between the ITS1 regions of these three *N. cf. patriciarum* ranged between 0.52–5.6% (Fig. 6), suggesting they belong to the same species. The ITS1 sequences for all three isolates were similar to *N. cameroonii* (1.2–4.3% divergence) (see below) (Fig. 6), suggesting that they are most probably synonyms to *N. cameroonii*. The variety (var.) designation for retaining prior suggested species names (i.e. referring to *N. californiae* and *N. lanati* as *N. cameronii* var. *californiae* and *N. cameronii* var. *lanati*, respectively) is proposed.

As such, we propose retaining the following species for the genus *Neocallimastix*: *N. frontalis* (synonyms *N. hurleyensis*, *N. variabilis*, *N. patriciarum* (Orpin) and *N. cameronii* (syn. *N. californiae*, *N. cf. patriciarum* and *N. lanati*).

### ***Neocallimastix frontalis*** **Vavra and Joyon [88], Heath *et al.* [34]**

=*Callimastix frontalis* Braune [20]

=*Neocallimastix variabilis* [91]

=*Neocallimastix hurleyensis* [92]

*Type strain*: strain PN1, Figs 20–25 in [34].

*GenBank sequence accession numbers: N. cf. frontalis* isolate RE1: MK036660.1–MK036676.1 (ITS1, 5.8S, ITS2) clones obtained in [66], and KR920744 (LSU) obtained in [90]. No sequence data are available from type strains. Multiple morphologically identical *cf.* strains were subsequently isolated; and the earliest deposited sequence data is for *N. cf. frontalis* isolate RE1 isolated from sheep rumen [89].

*ID in databases:* MycoBank: MB107058; Index Fungorum: IF: 107058 l; NCBI taxon ID: 4757.

*Diagnosis.* Detailed descriptions are provided in [34, 86, 91]. Briefly, in addition to characteristics provided in the description of the genus *Neocallimastix* above, the species is characterized by endogenous and exogenous sporangial development; occasionally branched sporangiophore; ellipsoidal, pyriform or ovoid sporangia; lack of constriction or slight constriction in sporangial necks; and release of zoospores through a sporangial rupture and dissolution.

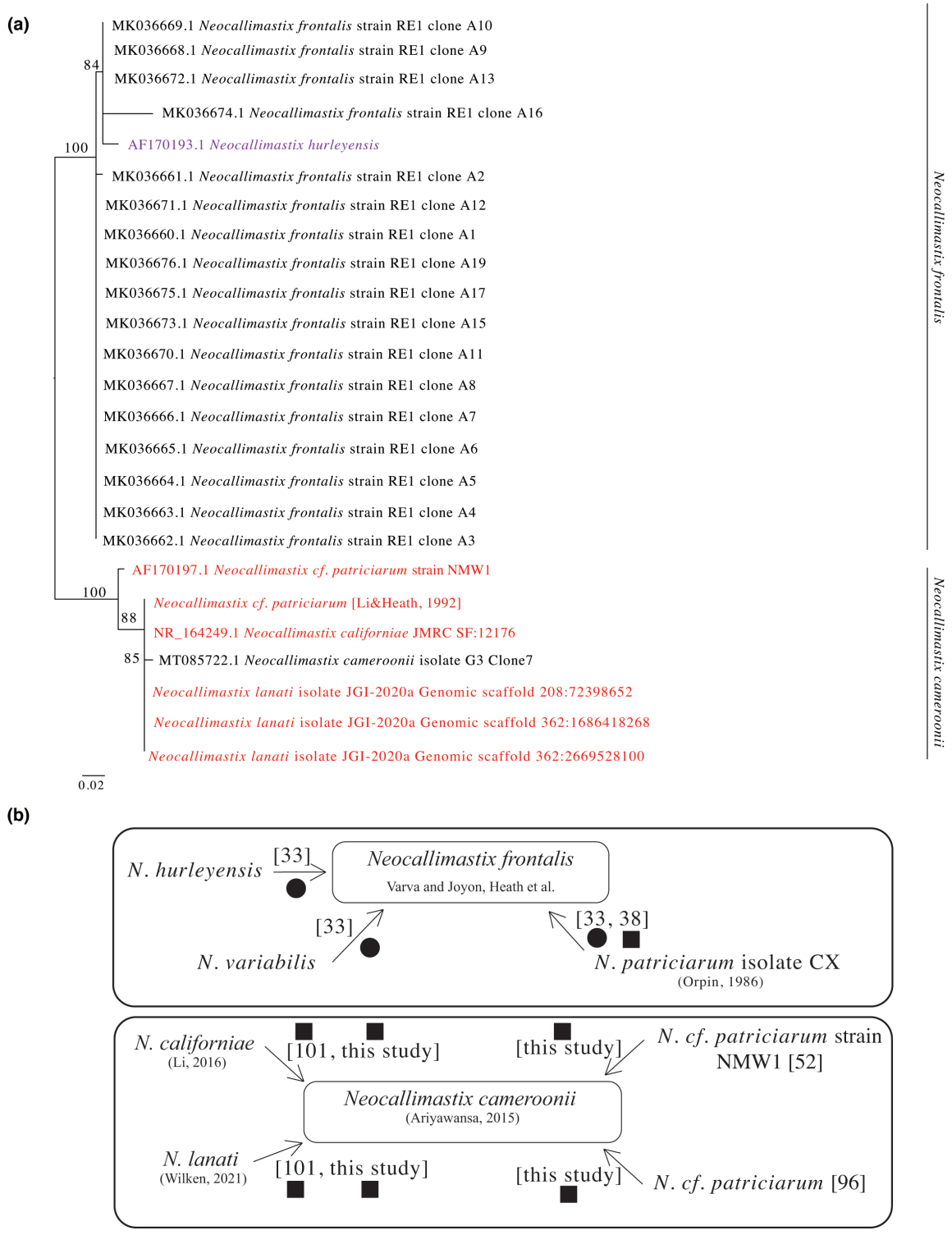


**Fig. 6.** (a). Maximum-likelihood phylogenetic tree reconstructed using the ITS1 region of *Neocallimastix* species highlighting the synonymy of *N. hurleyensis* (shown in purple text) to *N. frontalis*, and the synonymy of *N. californiae*, *N. lanate* and *N. cf. patriciarum* (shown in red text) to *N. cameronii*. Sequences were aligned using the MAFFT aligner with the –auto flag and the default parameters for gap extension and gap opening penalties. The maximum-likelihood tree was reconstructed in FastTree. Bootstrap values are based on 100 replicates and are shown for branches with >50% bootstrap support. (b) Cartoon highlighting the studies that proposed synonymy of the above species to either *N. frontalis* or *N. cameroonii* using phylogenetic (■), and/or morphological (●) evidences.

*Additional illustrations*: *N. frontalis* strain Hef5, Oklahoma State University Culture Collection. (Fig. 5a–i).

### ***Neocallimastix cameroonii* Ariyawansa *et al.* [38]**

=*Neocallimastix californiae.* Li *et al.* [39]

=*Neocallimastix lanati.* Wilken *et al.* [41]

*Type strain*: ABS CaDo3a [38]

*GenBank sequence accession numbers:* NG_060329.1 (D1/D2 LSU for type strain ABS CaDo3a), MT085722.1 (ITS1-5.8S-ITS2-D1/D2 LSU for *N. cf. cameroonii* strain G3).

*ID in databases:* MycoBank: MB51212; Index Fungorum: IF51212; NCBI taxon ID: 17 640 372.

*Diagnosis.* Original description provided in [38]. In addition to its phylogenetic distinction from *N. frontalis* (Fig. 6b–c) [38, 101]. The species is characterized by bifurcated rhizoidal system at the base of sporangia (Fig. 176f in [38]), ovoid and spherical sporangia (Fig. 176d–f in [38]), and the release of zoospores through a wide apical pore with the sporangial wall staying intact (Fig. 5m).

*Additional illustrations*: *N. cf. cameroonii* strain G3 Oklahoma State University Culture Collection (Fig. 5j–m).

## ***CAECOMYCES* (GOLD *ET AL.* 1988) [43]**

*History.* Similar to the genus *Neocallimastix*, identification of members of the genus *Caecomyces* predates the discovery of the fungal nature of 'rumen flagellates'. zoospores of the genus *Caecomyces* were first observed by Liebetanz [19] and Braune [20], for which the name *Sphaeromonas* was proposed. It was not until 1976, when the affiliation of *Sphaeromonas communis* with the fungi was confirmed [16], and its isolation in pure culture [54] soon followed. The genus was formally described by Gold *et al.*, based on the examination of two isolates obtained from the faeces of a horse [43]. A name change from *Sphaeromonas* to *Sphaeromyces* to emphasize its fungal nature was unfeasible, since the name *Sphaeromyces* was already in use. Therefore, the name *Caecomyces* was proposed for the genus based on the habitat from which the isolates were obtained (the horse caecum).

*Type: Caecomyces communis* strain PN3. Figs 1, 2 and 5–10 in [54].

*GenBank sequence accession numbers*: JF974109 (*Caecomyces cf. communis* GRL-11, ITS1), JF974124 (*Caecomyces cf. communis* GRL-12, LSU). No sequence data are available from type strains. Multiple morphologically identical *cf.* strains were subsequently isolated; and the earliest deposited sequence data is for *Caecomyces cf. communis* GRL-11 and GRL-12.

*ID in databases.* MycoBank: MB25287; Index Fungorum: I IF25287; NCBI taxon ID: 4823.

*Diagnosis.* Description of the genus *Caecomyces* is provided in [43], and expanded in [87]. On solid media, *Caecomyces* produces small white granular colonies (Fig. 5n). It exhibits sandy morphology growth in liquid medium (Fig. 5o); a reflection of its bulbous, non-filamentous, rhizoidal growth, and its monocentric determinate growth pattern. *Caecomyces* produces monoflagellated zoospores (Fig. 5p), and exhibits a bulbous rhizoidal system with spherical holdfasts, and monocentric thalli that are either uni- or multisporangiate.

*Occurrence.* Isolates of *Caecomyces* have been reported from horse (*Equus ferus*) faeces [43, 54], cow rumen [13, 43, 102] and faeces [87], Sheep salvia [94], rumen [103] and faeces [40, 94], fallow deer (*Dama dama*) faeces [28], Yak rumen and faeces [64], rumen and faeces of water buffalo [104], goat rumen [66], and alpine ibex faeces [98]. Culture-independent diversity surveys suggest the ubiquity of members of the genus *Caecomyces* in a wide range of foregut and hindgut animals [28], although rarely representing the most dominant member of the community [31], although a recent study has identified *Caecomyces* as the most abundant genus in faecal samples from donkeys [105]). Further, it was observed that *Caecomyce*s could form the majority (30–90%) of colonies produced on-roll tubes from samples originating from animals that were fed a specific diet (e.g. rice straw with molasses or palm press fibre [33]). Such observation suggests an *in vivo* niche selection and the ability of rapid propagation under specific nutritional regimes.

*List of Caecomyces species.* The following *Caecomyces* species have been proposed: *C. communis* [54], *C. equi* [43], *C. sympodialis* [102] and *C. churrovis* [40]. *Caecomyces communis* [54] and *Caecomyces equi* [43] were initially distinguished by Gold *et al.* [43] based on the presence of single bulbous rhizoid in *C. equi* as opposed to multiple bulbous rhizoids in *C. communis*. However, subsequent studies [87] cast doubt on the distinctiveness of the two species and argued that the number of bulbous rhizoids depends on the age of the culture, with younger cultures more commonly display single bulbous holdfast, while multiple holdfasts develop as the cultures age. Unfortunately, the unavailability of the type strain and lack of sequence data for *C. equi* hampers further investigation into whether *C. equi* is a separate species from *C. communis*. As such, we propose retaining the name *C. equi,* while using the more common species name '*C. communis*' to describe future strains showing similar phylogenetic affiliation and phenotypic traits.

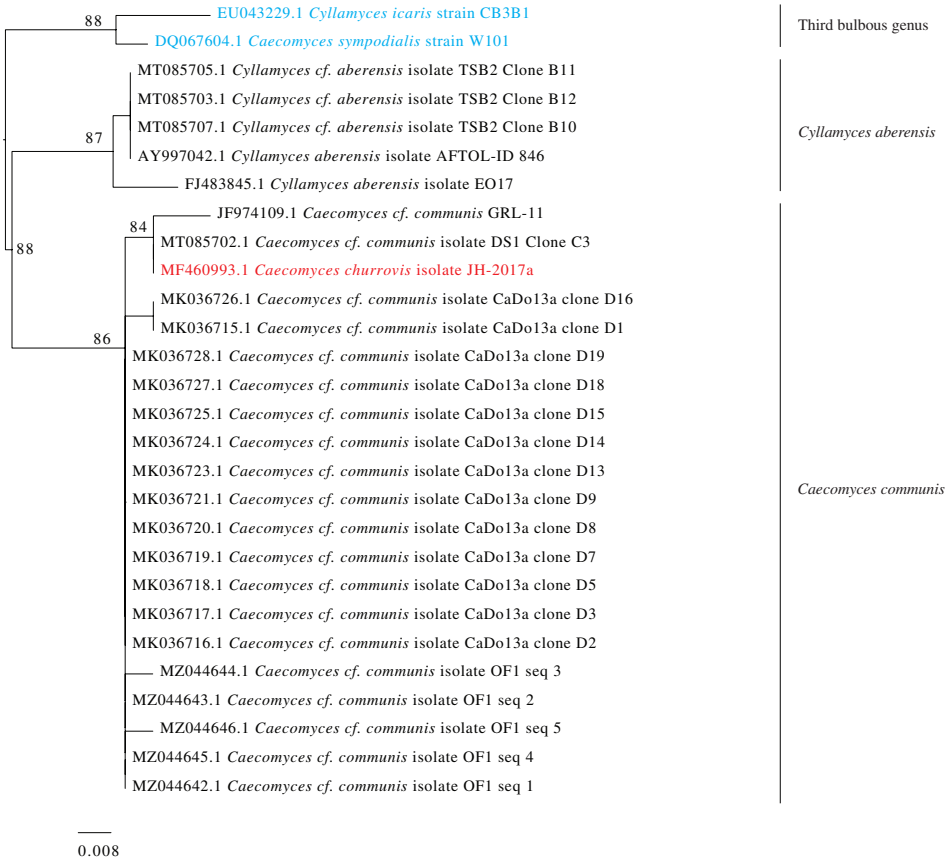


**Fig. 7.** Maximum-likelihood phylogenetic tree reconstructed using the ITS1 region of *Caecomyces* and *Cyllamyces* species highlighting the clear separation between the two clades comprising *Caecomyces communis* and *Cyllamyces aberensis*, and the synonymy of *Caecomyces churrovis* (shown in red text) to *Caecomyces communis*. The tree also highlights the peculiar position of *Cyllamyces icaris* and *Caecomyces sympodialis* (shown in cyan text) separate from either of the two clades, and suggesting they could belong to a third clade. Sequences were aligned using the MAFFT aligner with the –auto flag and the default parameters for gap extension and gap opening penalties. The maximum-likelihood tree was reconstructed in FastTree. Bootstrap values are based on 100 replicates and are shown for branches with >50% bootstrap support.

Descriptions of *C. sympodialis* in [102] and *C. churrovis* in [40] demonstrate their bulbous rhizoidal nature. Phylogenetically, *C. sympodialis* is distinct from *C. communis* (3.62% divergence at the ITS1 level), and *C. churrovis* (4.25% divergence at the ITS1 level) (Fig. 7). However, only one *C. sympodialis* ITS1 sequence is available (and no D1/D2 LSU sequences), rendering such assessment of phylogenetic distinction uncertain, given within strain ITS1 divergence commonly encountered in the *Neocallimastigomycota*. Microscopic characterization also suggests a distinct pattern of thallus development (multi-sporangiate with sympodial distribution of multiple sporangia on unbranched sporangiophores, see below) that is probably not a function of growth condition or culture age. As such, the position of *C. sympodialis* as a distinct species appears probable, in spite of the limited sequence data available.

Description of *C. churrovis* provided in [40] does not adequately ascertain phenotypic distinction from *C. communis*. Further, *C. communis* and *C. churrovis* cluster together in both the ITS1 (Figs 4a and 7), and the D1/D2 LSU trees (Fig. 4b) with only 0.89 and 0.77% average sequence divergence, respectively. *C. churrovis* should hence be considered a synonym for *C. communis*. The variety (var.) designation (i.e. referring to *C. churrovis* as *C. communis* var. *churrovis*) is proposed.

As such we propose retaining the following species for the genus *Caecomyces*: *C. communis* (synonym *C. churrovis*), *C. equi* and *C. sympodialis*.

### ***Caecomyces communis comb. nov.* Gold *et al.* 1988 [43]**

= *Sphaemonas communis* Orpin 1981 [54]

=*C. churrovis* [40]

*Type strain*: strain PN3 Figs 1, 2 and 5–10 in [16].

*GenBank sequences accession numbers:* JF974109 (*Caecomyces cf. communis* GRL-11, ITS1), JF974124 (*Caecomyces cf. communis* GRL-12, LSU).

ID in databases MycoBank: MB1335565; Index Fungorum: IF135565; NCBI taxon ID: 4824.

*Diagnosis.* Original description is provided in [16] using *in vivo* observations and enrichments, with a brief description provided for the first isolate (designated H3) in [54]. Wubah and Fuller [87] provide a more detailed description using a *cf.* strain isolated from cattle faeces in Athens, Georgia. *C. communis*, as described in all three studies display monoflagellated zoospores (Fig. 5p), monocentric thalli that are either uni- or multisporangiate, and a bulbous rhizoidal system with spherical holdfasts (Fig. 5q-s). Both endogenous and exogenous sporangia are observed. Endogenous sporangia are mainly globose (Fig. 5q) and ovoid (Fig. 5r). Exogenous sporangia are typically observed at the end of long tubular sporangiophores and are ovoid in shape (Fig. 5s). Zoospore release mechanism was not observed, but sporangia were frequently seen detached from the sporangiophore or the rhizoid (Fig. 5t).

*Additional illustrations. Caecomyces cf. communis* strain DS1 from Oklahoma State University Culture Collection (Fig. 5n–t).

### *Caecomyces equi* Gold *et al.* 1988 [43]

*Type strain*: strain PN3 from horse caecum.

*Nucleotide accession number*: NA.

*ID in databases*: MycoBank: MB135566, Index Fungorum: IF135566; NCBI taxon ID: NA.

*Diagnosis.* The description is similar to that of *C. communis*, with the possible differences of the presence of single bulbous rhizoid (*C. equi*) or many bulbous rhizoids (*C. communis*), and the production of predominantly unisporangiate thalli (*C. equi*) as opposed to unisporangiate or multisporangiate thalli by *C. communis* (but see [87] as stated above).

*Caecomyces sympodialis* Chen *et al.* 2007 [102]

*Type strain*: W101 from rumen of yellow cow (*Bos indicus*).

*GenBank sequence accession number:* DQ067604.1 (ITS1).

*ID in databases:* MycoBank: MB504777; Index Fungorum: IF504777; NCBI taxon ID: NA.

*Diagnosis.* Detailed description is provided in [102]. In addition to the general characteristics of the genus *Caecomyces*, the species is unique in producing thalli with multiple sporangia (more than three) that are sympodially distributed on long and tubular unbranched sporangiophores (Figs i–k in [102]).

## *PIROMYCES* GOLD *ET AL.* 1988 [43]

*History.* Monoflagellated spores that are morphologically distinct from *Sphaeromonas communis* (larger and more elongate) were reported by Liebetanez [19] and Braune [20], and named *Piromonas communis*. Orpin characterized optimal growth conditions for *P. communis* in rumen samples, partly described its sporangial and spore morphologies, and obtained a mixed bacteria–fungal culture with *P. communis* as the sole fungal strain [27]. The name *Piromyces* was suggested by Gold *et al*. in 1988 to emphasize the fungal, rather than protozoan, nature of members of this genus; and a detailed description of *P. communis* using isolates obtained from a Holstein steer was published in in 1989 [70].

*Type*: *Piromyces communis* Figs 1, 5–10 in [27].

*GenBank sequence accession numbers.* The full ITS region of the original *P. communis* strain P isolated by Orpin in 1977 from Clun Forest sheep rumen and preserved as a specimen voucher at the Aberystwyth University biorepository (code ABS20-Pcomm01) was recently (2020) sequenced and deposited in GenBank (accession number MW341535.1). The earliest deposited D1/D2 LSU sequence data from a strain with the highest ITS1 sequence similarity to *P. communis* strain P is for *Piromyces cf. communis* BRL-3 (GenBank accession number JF974096).

*ID in database:* MycoBank ID: MB25332; Index Fungorum ID: IF25332; NCBI Taxon ID: 4821.

*Diagnosis.* Description of the genus *Piromyces* is provided in [70], and expanded in [33]. On solid agar media, *Piromyces* produces small circular colonies with a dark centre of sporangial structure (Fig. 5u). It exhibits thin biofilm-like growth in liquid medium that firmly attaches to the glass walls of the tube (Fig. 5v). Members of the genus *Piromyces* are characterized by monocentric thalli (with both endogenous and exogenous sporangial development), filamentous rhizoidal system, and monoflagellated zoospores (occasionally bi- to tetra-flagellated). Sporangia display a variety of shapes, including globose, ovoid, pyriform, ellipsoidal, and elongated (Fig. 5w–ab).

*Occurrence.* Members of the genus *Piromyces* have been isolated from the faeces, rumen and caecum of a wide range of animals, including, but not limited to, cows [28, 70], sheep [27, 28], deer [28, 106], horses [28, 39, 107], donkeys (*Equus africanus*) [28, 108, 109], pony [109], asian elephant (*Elephas maximus*) [107], water buffalo [110] and goats [111]. Similarly, culture-independent diversity surveys often report a ubiquitous distribution for members of the genus *Piromyces*, being consistently encountered in the majority of examined animal species, and often representing a large fraction of the overall community [28, 31]. Samples where members of the genus *Piromyces* constitute the entire AGF community has also been reported, e.g. faecal samples of black rhinoceros and pronghorn in [31].

*List of Piromyces species.* Traditionally, the genus *Piromyces* accommodated strains displaying moncentric thalli, monoflagellated zoospores and filamentous rhizoids. Following the description of *P. communis* [70], several *Piromyces* species (*P. mae* [107], *P. dumbonicus* [107], *P. rhizinflatus* [108], *P. spiralis* [111], *P. minutus* [106] and *P. citronii* [109]) displaying such features were described between 1990 and 2000. While the type strain of *P. communis* originally isolated by Orpin in 1977 [27] was preserved as a specimen voucher at the Aberystwyth University biorepository (code ABS20-Pcomm01) from which ITS1 molecular sequence data has been generated; no molecular sequence data are available to ascertain the phylogenetic affiliation of *P. mae* [107], *P. dumbonicus* [107], *P. rhizinflatus* [108], *P. spiralis* [111], *P. minutus* [106] and *P. citronii* [109]. Further, subsequent research has clearly demonstrated that the phenotype of filamentous rhizoids, monocentric thalli and monoflagellated zoospores is polyphyletic in the *Neocallimastigomycota* [62]. Indeed, such phenotype was reported for 10 additional genera since 1995 [35–37, 55, 73]. Further, broad microscopic similarities between earlier described *Piromyces* species and some of these newer genera have been noted [36, 55]. As such, the affiliation of many of the earlier described species with the genus *Piromyces*, circumscribed based on phylogenetic similarity to *P. communis*, could not be confirmed; and the fact that all these isolates are currently extinct further hampers *Piromyces* taxonomy. For two of the *Piromyces* species described since 2002 (*P. polycephalus* [110] and *P. irregularis* [38]), molecular sequence data is available. However, low ITS1 sequence similarity (78.6, and 77.1%, respectively) to *Piromyces communis* type strain P (MW341535.1) negates their affiliation to the genus *Piromyces*. On the other hand, molecular sequence data confirm the affiliation of the latest described *Piromyces* species (*P. finnis* [39]) with the genus *Piromyces*.

### ***Piromyces communis* Gold *et al.* [43]**

=*Piromonas communis* [19, 27]

*Type strain*: strain P, Figs 1, 5-10 in [27].

*GenBank sequence accession numbers:* MW341535.1 (ITS1, strain P isolated by Orpin and preserved as a voucher specimen at Aberystwhth University) and JF974096 (D1/D2 region of 28S rRNA, *Piromyces cf. communis* BRL-3).

*ID in databases:* MycoBank: MB 135567; Index Fungorum: IF135567; NCBI taxon ID: 4822.

*Diagnosis.* Detailed descriptions are provided in [70]. Briefly, in addition to characteristics provided in the description of the genus *Piromyces* above, the species is characterized by mainly globose zoospores that are monoflagellated. Biflagellated zoospores are also frequently encountered. Endogenous sporangia are mostly globose and occasionally pyriform. Exogenous sporangia are mostly elongated, although globose, ovoid and irregular-shaped sporangia are also observed. Sporangial neck is broad and either not constricted or slightly constricted. Sporangiophores vary in length, and could also display an eggcup shape. Zoospores are released via an apical pore, followed by the dissolution of the entire sporangia wall.

*Additional illustrations. Piromyces cf. communis* strain Jen1 from Oklahoma State University Culture Collection (Fig. 5u–y).

### ***Piromyces mae* Li *et al.* (1990) [107]**

*Type strain*: strain PN11.

*GenBank sequence accession numbers:* NA.

*ID in databases:* MycoBank: MB 126402; Index Fungorum: IF1 126402; NCBI taxon ID: NA.

*Diagnosis.* Detailed description is provided in [107]. Briefly, in addition to characteristics provided in the description of the genus *Piromyces* above, the species is characterized by developing one, rarely two, papillae at their apex, through which spore release occurs; as well as the formation of subsporangial swelling just below the sporangia.

### ***Piromyces dumbonicus* Li *et al.* (1990) [107]**

Type strain: strain PN12.

*GenBank sequence accession numbers*: NA.

*ID in databases*: MycoBank: MB 126401; Index Fungorum: IF1 126401; NCBI taxon ID: NA.

*Diagnosis.* Detailed description is provided in [107]. The distinction between *P. dumbonicus* and *P. communis* was mostly based on ultrastructural assessment, with no clear morphological or microscopic differences reported.

#### ***Piromyces rhizinflatus* Breton *et al.* 1991 [108]**

*Type strain:* strain PS, Figs 1 and 2 in [108].

*GenBank sequence accession numbers*: NA.

*ID in databases*: MycoBank: MB 276850; Index Fungorum: IF 276850; NCBI taxon ID: NA.

*Diagnosis*. Detailed description is provided in [108]. The species was isolated from dried faeces of Saharan donkey that was stored up to 150 days, implying a possible level of air-tolerance [108]. The species was differentiated from other *Piromyces* species by the possession of a pronounced neck constriction (to differentiate it from *P. communis*) and the lack of papilla (to differentiate it from *P. mae*).

#### ***Piromyces minutus* Ho *et al.* 1993 [106]**

Type strain: D2.

*GenBank sequence accession numbers*: NA.

ID in databases: MycoBank: MB: 360153; Index Fungorum: IF:360153; NCBI taxon ID: NA.

*Diagnosis*. Detailed description is provided in [106]. The species could be differentiated from other *Piromyces* species by its sparingly branched rhizoidal structure and its markedly smaller sporangia [106]

#### ***Piromyces spiralis* Ho *et al.* 1993 [111]**

*Type strain:* strain G34.

*GenBank sequence accession numbers:* NA.

*ID in databases:* MycoBank: MB: 360467; Index Fungorum: IF: 360467; NCBI taxon ID: NA.

*Diagnosis*. Detailed description is provided in [111]. The species is characterized by extensive coiling of the main rhizoid, the rapid dissolution of the sporangial wall; often leaving zoospores clustered. As well, *P. spiralis* sporangia are mostly spherical, with a marked paucity of other sporangial shapes in examined cultures.

#### ***Piromyces citronii* Gaillard-Martinie *et al.* 1995 [109]**

*Type strain*: strain A1.

*GenBank sequence accession numbers*: NA.

*ID in databases*: MycoBank: MB: 434478; Index Fungorum: IF: 434478; NCBI taxon ID: NA.

*Diagnosis*. Detailed description is provided in [109]. The main difference between *P. citronii* and most other *Piromyces* species is the production of a monocentric bi-, tri- or multi-sporangiate thallus (i.e. two, three, or more exogenous sporangia developing on the same sporangiophore).

#### ***Piromyces finnis* Li *et al.* 2016 [39]**

*Type strain: Piromyces finnis* strain KS-2015.

*GenBank sequence accession number:* KY399730 (ITS1).

*ID in databases:* MycoBank: MB: 551677; Index Fungorum: IF: 551677; NCBI taxon ID: 1754191.

*Diagnosis.* Description is provided in [39]. The species is phylogenetically distinct from *P. communis*. In addition to general characteristics of the genus *Piromyces*, the species is characterized by pleomorphic (mainly oval and club-shaped) sporangia, and the development of a septum at the base of the sporangia.

*Additional illustrations. Piromyces cf. finnis* strain DonB11 from Oklahoma State University Culture Collection (Fig. 5z–ab).

### *ORPINOMYCES* (BRETON *ET AL.* 1989, BARR *ET AL.* 1989)

*History.* Polycentric thallus development pattern has long been recognized in the Chytrid genera *Nowakowskiella* and *Catenaria* [112, 113]. Within the *Neocallimastigomycota*, polycentric thallus development was first reported in enrichments set up using cow's rumen fluid and alfalfa or Bermuda grass as substrate [114, 115]. The first polycentric isolate was reported by Breton *et al.* in

1989 [116] from the rumen of sheep. Although its polycentric nature was recognized by the authors, the isolate was nevertheless described as a new species within the genus *Neocallimastix*. Barr *et al.* [70] isolated a closely related strain from the cow rumen in 1989, and reasoned that the observed polycentric thallus merits the proposition of a new genus to accommodate this strain. The genus name *Orpinomyces* (to honour Dr. Colin Orpin's contributions) and the species name *bovis* were proposed.

*Type*: *Neocallimastix joyonii (Orpinomyces joyonii)* NJ1 [116]. Figs 1 and 2 in [116].

*GenBank sequence accession numbers:* AY429671 (ITS1, 5.8S, ITS2), JN939163 (D1/D2 LSU) (*Orpinomyces cf. joyonii* strain KF1). No sequence data are available from the type strains. Multiple morphologically identical *cf.* strains were subsequently isolated; and the earliest deposited sequence data is for an *O. cf. joyonii* strain KF1 is hence utilized as reference sequence.

*ID in databases*: MycoBank ID: MB25326; Index Fungorum: IF25326; NCBI taxon ID: 37163.

*Diagnosis.* Description of the genus *Orpinomyces* is provided in [70] and expanded in [33]. *Orpinomyces* forms large (usually >1 cm diameter) white colonies on agar roll tubes (Fig. 5ac). In liquid media, *Orpinomyces* displays a characteristic thick cottony fungal growth (Fig. 5ad). These unique growth patterns allow tentative identification based on visual examination of colonies and liquid growth. *Orpinomyces* produces polyflagellated zoospores, exhibits polycentric thallus development and a filamentous rhizoidal growth pattern. Wide hyphae usually displaying multiple constrictions at irregular intervals (Fig. 5ae). Old cultures of *Orpinomyces* tend to lose their ability to produce sporangia and only produce sporangiophores initials (Fig. 5af).

*Occurrence.* Species of *Orpinomyces* have been isolated from cow rumen [33, 70, 117], buffalo rumen [33], sheep rumen [116], as well as faecal samples of American bison (*Bison bison*) and alpaca (*Lama pacos*) [28], cows [28, 118], sheep [52], water buffalo [67] and yak [64]. Culture-independent diversity surveys suggest that members of the genus *Orpinomyces* almost invariably constitute a minor fraction of the community when encountered, and are more prevalent in foregut herbivores [28, 31].

*List of Orpinomyces species*. Two *Orpinomyces* species are currently recognized: *Orpinomyces joyonii* [70, 116, 119] and *Orpinomyces intercalaris* [117] (Fig. 4b). Breton *et al.* [116] reported on the isolation and characterization of a novel strain with polycentric thallus and polyflagellated zoospores, for which the name *Neocallimastix joyonii* was proposed. Shortly thereafter, Barr *et al.* [70] isolated another strain from the rumen of a Holstein steer for which the name *Orpinomyces bovis* was first proposed. A detailed comparison between *O. bovis* and *N. joyonii* was conducted by Li *et al.* [119], and the authors concluded that both isolates represented the same genus and species based on their morphological similarities. The combination *Orpinomyces joyonii* was hence proposed: *Orpinomyces* for the genus name to recognize these strains as the first representative of a new genus, and *joyonii* for the species name to recognize the priority of Breton *et al.* over Barr *et al.*). The second species *Orpinomyces intercalaris* was subsequently isolated and characterized in 1994 [117].

### ***Orpinomyces joyonii* Breton *et al.* [108], Li *et al.* [116, 119]**

=*Orpinomyces bovis* Barr *et al.* [70]

=*Neocallimastix joyonii* Breton *et al.* [119]

*Type: Neocallimastix joyonii* strain NJ1 *(=Orpinomyces joyonii)=Orpinomyces bovis.* [70, 116]. Figs 1 and 2 in [116].

*GenBank sequence accession numbers. O. cf joyonii* strain KF1: AY429671 (ITS1-5.8S-ITS2), JN939163 (D1/D2 LSU). Sequence data from strains originally isolated by [70, 116] are not available. The earliest deposited sequence data for an *O. cf joyonii* strain (strain KF1 from rumen fluid of cows in Prague, Czech Republic) is hence utilized as reference sequence.

*ID in databases:* MycoBank ID: MB127934, Index Fungorum: IF127934; NCBI taxon ID: 48 250.

*Diagnosis.* Detailed description is provided in [70, 116] as well as [118] for *O. cf. joyonii* strain KF1. In addition to the general characteristics of the genus described above, *O. joyonii* produces globose polyflagellated zoospores with 10–25 flagella (Fig. 5ag). Sporangia are mainly globose (Fig. 5ah–aj) and developed either terminally at the tip of sporangiophores (Fig. 5ah–ai) or as a lateral outgrowth of hyphae (Fig. 5aj).

*Additional illustration. O. joyonii* strain D4A, from Oklahoma State University Culture collection (Fig. 5ac–aj).

### ***Orpinomyces intercalaris* Ho *et al.* 1994 [117]**

*Type strain*: strain 19-2 a [117]. Figs. 1–10 in [117].

*GenBank sequence accession numbers*: *O. cf. intercalaris* strain SKP1: HQ703471 (D1/D2 LSU). ITS1: NA. Original molecular data from strain 19-2 a [117] are not available. The earliest deposited sequence data for an *O.* cf. *intercalaris* (strain SKP1 from water buffalo rumen, Haryana, India) is hence utilized as the reference sequence.

*ID in databases.* MycoBank ID: MB 357919, Index Fungorum: IF 357919; NCBI taxon ID: 1049955.

*Diagnosis.* Detailed description is provided in [117] and expanded in [33]. In addition to the general characteristics of the genus, *O. intercalaris* is characterized by the production of globose and intercalary sporangia (developed as expansion of the hyphae or as a lateral outgrowth of hyphae), while terminal sporangia are rarely observed. Also, *O. intercalaris* is characterized by the presence of zoospore cyst as an empty persistent structure attached to the rhizomycelium. These criteria differentiate it from *O. joyonii*, in addition to their phylogenetic distinction. Upon maturation, two septa are formed to separate the intercalary sporangium from the hyphae. Zoospores are released through rupturing the sporangial wall followed by complete collapse of the sporangial wall. Hyphae of *O. intercalaris* are constricted at regular intervals resulting in a sausage-shaped appearance, as opposed to the constrictions observed at irregular intervals in *O. joyonii* hyphae.

## *ANAEROMYCES* BRETON *ET AL.* 1990 [45]

=*Ruminomyces* Ho *et al.* 1990 [47]

*History.* The first representative of the genus *Anaeromyces* (*A. mucronatus*) was isolated from the cow rumen in 1990 [45]. Shortly thereafter, a highly similar strain was reported (also from the cow rumen), for which the name *Ruminomyces elegans* [47] was proposed. Precedence was given to the genus name *Anaeromyces* over *Ruminomyces* [33].

*Type*: strain BF2 from the rumen of a cow [45]. Fig. 1 in [47].

*GenBank sequence accession numbers. A. cf. mucronatus* strain (strain JF1 MW899528 (ITS1-5.8S-ITS2-D1/D2 LSU). No sequence data are available from type strains. Multiple morphologically identical *cf.* strains were subsequently isolated; and the earliest deposited sequence data is for *A. cf. mucronatus* strain (strain JF1 isolated from deer faecal samples).

*ID in databases*: MycoBank ID: MB27188; Index Fungorum: IF27188; NCBI taxon ID: 105135.

*Diagnosis.* Description of the genus *Anaeromyces* is provided in [45, 47], and expanded in [33]. On solid media, *Anaeromyces* forms circular white colonies (4–6 mm in diam., Fig. S1a in [76]). In liquid, it forms thick, pearl-like growth (Fig. S1b in [76]). *Anaeromyces* produces monoflagellated zoospores (Fig. 3e in [76]), exhibits polycentric thallus development (Fig. 2a, b in [76]), and filamentous rhizoidal growth pattern (Fig. 2c in [76]). Zoospores are typically small, globose and always monoflagellated (uniflagellated) with a flagellum length 16–20 µm), with a rough, uneven surface. Terminal sporangia are typically mucronate with an acuminate apex (Fig. 3c in [76]). *Anaeromyces* produces wide and narrow hyphae, with frequent constrictions in the wide hyphae resulting in a unique sausage-like appearance (Fig. 1d, e in [76]). Sporangiogenesis and spore formation often cease after repeated subculturing, with new biomass development and formation of new nuclei occurring through hyphal propagation.

*Occurrence. Anaeromyces* strains have been isolated from the cow rumen [45, 47], sheep rumen [120] and faeces [39], deer, European bison (*Bison bonasus*) and American bison [118], cows, goats [76], American bison and alpaca [28], as well as cattle and water buffalo [121]. Culture-independent analysis reported the presence of *Anaeromyces*-affiliated sequences in 26/30, and 8/21 of the samples examined in [31] and [28], respectively. These studies also documented the occurrence of *Anaeromyces* beyond the typical foregut ruminants from which they were isolated by detecting them in faecal samples from some hindgut fermenters (e.g. horses and donkeys). In all studies, *Anaeromyces*-affiliated sequences typically represent a minor component of the overall community in examined samples.

*List of Anaeromyces species.* Four species of *Anaeromyces* have so far been described: *A. mucronatus* [45], *A. elegans* [47], *A. robustus* [39] and *A. contortus* [76]. Similar to the situation of *Caecomyces communis* and *C. equi*, the lack of sequence data in the original papers that described *A. mucronatus* [45] and *A. elegans* [47], the relatively minor differences between representatives of both species, which could be a function of the growth conditions or mere overlooking specific characteristics in the original description, and the absence of original cultures, render confident ascertainment of the relationship between *A. mucorantus* and *A. elegans* impossible. Therefore, we propose retaining the names *A. mucronatus* and *A. elegans*, but only using *A. mucronatus* (and refraining from usage of the name *A. elegans*) to describe any new related isolates, a practice that has already been followed in subsequent isolation efforts [28, 118]. *A. contortus* is phylogenetically and morphotypically distinct from *A. mucronatus/elegans*.

*A. robustus* represents a unique case that might justify future assignment to another genus (*Capellomyces*) or a new genus pending additional availability of data. The *A. robustus* description [39] reports multiple unique features when compared to all other *Anaeromyces* species. The zoosporangia have a distinct morphology previously unreported within the *Neocallimastigomycota*, where they are club-shaped and occasionally fuse to form a whale's tail-like appearance. This is in contrast to the mucronate sporangia with acuminate apex typically observed in other *Anaeromyces* species. The zoospores are described as having several posteriorly directed flagella [39], implying a polyflagellated strain, in contrast to the monoflagellated zoospores observed in other *Anaeromyces* spp. Further, the species appear to lack the unique sausage-like hyphal morphology observed in other *Anaeromyces* species [39], and a convincing demonstration of the polycentric nature of *A. robustus* via DAPI staining is lacking in [39]. Interestingly, ITS-1 sequence data of *A. robustus* (KU057354.1) places it outside of the *Anaeromyces* clade, and closer to members of the genus *Capellomyces* (Fig. 8). D1/D2 LSU sequences data for this strain is currently unavailable. Pending availability of additional information, the status of this species remains questionable. The unique morphology of sporangia, coupled to the apparent lack

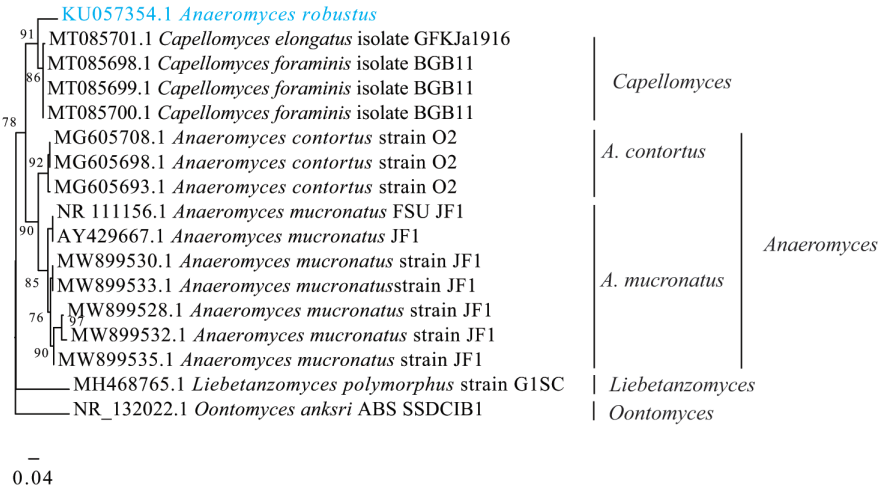


**Fig. 8.** Maximum-likelihood phylogenetic tree reconstructed using the ITS1 region of representatives of *Anaeromyces–Liebetanzomyces–Capellomyces–Oontomyces* clade. Sequences were aligned using the MAFFT aligner with the –auto flag and the default parameters for gap extension and gap opening penalties. The maximum-likelihood tree was reconstructed in FastTree. Bootstrap values are based on 100 replicates and are shown for branches with >50 % bootstrap support.

of fundamental *Anaeromyces* traits could either suggest affiliation with the genus *Capellomyces*, known to exhibit a wide range of sporangial morphology, or as a distinct new genus within the *Anaeromyces–Liebetanzomyces–Capellomyces–Oontomyces* clade.

### ***Anaeromyces mucronatus* Breton *et al.* 1990 [45]**

*Type strain*: strain BF2 from the rumen of a cow [45], Fig. 1 in [47].

*GenBank sequence accession numbers.* MW899528 (ITS1-5.8S-ITS2-D1/D2 LSU). Sequence data from strain BF2 originally isolated by is not available. The earliest deposited sequence data for an *A. cf. mucorantis* strain (strain JF1 isolated from deer faecal samples from Prague, Czech Republic) is hence utilized as a reference sequence.

*ID in databases*: MycoBank ID: MB 361273; Index Fungorum Registration Identifier IF 361273; NCBI taxon ID: 994854.

*Diagnosis.* Detailed description is given in [45] and expanded in [33, 47], In addition to general genus-level characteristics, the species is characterized by terminal elliptical sporangia with pointed apex, spherical exclusively monoflagellated (uniflagellated) zoospores, and sausage-shaped hyphae.

### ***Anaeromyces elegans* Ho *et al.* 1990 [47]**

=*Ruminomyces elegans*

*Type strain*: Lectotype, Figs 1 and 3 in [47].

*GenBank sequence accession numbers.* NA.

*ID in databases*: MycoBank ID: MB 360152; Index Fungorum: IF360152; NCBI taxon ID: NA.

*Diagnosis.* The species show high similarity to *A. mucronatus*. The main difference is the presence of an appressorium structure, i.e. lobed-like hyphal structures thought to facilitate the penetration of plant cell walls. However, as described above, such presence could have been overlooked in the original description of *A. mucronatus*. Zoospores are monoflagellated and spherical (Fig. 3c, d in [47]). Mature sporangia are mainly ellipsoidal and fusiform with acuminate apices (Figs 2,b and 3a in [47]). Sporangia are developed terminally at the tip of sporangiophores. Zoospores are released through a transverse slit of the sporangial wall which stays intact after the zoospore release (Fig. 3e in [47]). Hyphae exhibit the unique sausage-like appearance with tight constrictions at regular intervals (Fig. 3a in [47]).

### ***Anaeromyces robustus* Li *et al.* 2016 [39]**

*Type strain*: S4 (University of California Santa Barbara Culture Collection).

*GenBank sequence accession numbers*: NR_148182.1 (ITS). No D1/D2 LSU sequence available.

*ID in databases*: MycoBank ID: MB 551676; Index Fungorum: IF 551676; NCBI taxon ID:1 754 192.

*Diagnosis. A. robustus* is differentiated from other *Anaeromyces s*pecies by having distinct club-shaped sporangia that occasionally fuse to form a whale's tail-like appearance. The zoospores possess several posteriorly directed flagella [39], implying a polyflagellated strain. The hyphae lack the unique sausage-like morphology observed in other *Anaeromyces* species [39],

### *Anaeromyces contortus* Hanafy *et al.* 2018 [76]

*Type strain*: O2, Fig. 1e in [76]

*GenBank sequence accession numbers.* MG605693, MG605698, MG605708 (ITS1), and MF121931 (D1/D2 LSU) sequence available.

*ID in databases*: MycoBank ID: MB 821369; Index Fungorum Registration Identifier: IF 821369; NCBI taxon ID: 2170304.

*Diagnosis.* Detailed description is given in Hanafy *et al.* [76]. In addition to phylogenetic distinction from the *A. mucronatus* (Figs. 4b–c and 8), the species differs from *A. mucronatus* and *A. elegans* in two main features: First: the production of the globose intercalary sporangia in addition to the mucronate terminal sporangia observed in *A. mucronatus* and *A. elegans* sporangia, and second: The formation of highly coiled and entangled hyphal structures that are similar to traps produced by many nematophagous fungi [76].

## *CYLLAMYCES* OZKOSE *ET AL.* 2001

*History.* The first representative of the genus *Cyllamyces* (*C. aberensis*) was described in 2001 [74] from cow faeces, and a second species (*C. icaris*) was reported in 2014 from the faeces of a water buffalo [48].

*Type*: strain EO14 (AFTOL 846), Figs. 1–11 in [74].

*GenBank sequence accession numbers:* AY997042 (ITS1, 5.8S, ITS2) and DQ273829 (D1/D2 LSU).

*ID in databases*: MycoBank ID: MB28540; Index Fungorum: IF28540; NCBI taxon ID: 324620.

*Diagnosis.* The genus *Cyllamyces* accommodates strains that produce bulbous holdfasts (similar to the genus *Caecomyces*), but display polycentric thallus developmental pattern (as opposed to the monocentric pattern in *Caecomyces*). Differences in morphological and microscopic characteristics between members of the two genera are majorly reflecting the difference between their determinate (monocentric) versus indeterminate (polycentric) thallus developmental patterns. *Cyllamyces* colonies on solid media (Fig. 5ac) are granular and beige-coloured colonies, similar to *Caecomyces*, but are typically larger in size, a characteristic of all polycentric genera. In liquid media, *Cyllamyces* isolates usually aggregate into relatively large and loose granular brown clumps (Fig. 5ad, compared to the fine granular, often sandy-like, smaller clumps in *Caecomyces* (Fig. 6o). However, the bulbous nature of the rhizoidal growth results in much smaller clumps when compared to filamentous polycentric genera (*Orpinomyces*, *Anaeromyces* and *Paucimyces*).

*Cyllamyces* strains produce bulbous holdfast, with one or multiple sporangiophores arising from the holdfast. Each sporangiophore produces multiple spherical sporangia. The strains' polycentric nature is reflected by their capacity to produce as many as 12 sporangia per thallus (and up to five per a single sporangiophore) as well as the observation of nuclei throughout the entire thallus (i.e. in the holdfast, sporangiophores, and sporangia (Figs. 8–9 in [74]). In comparison, *Caecomyces* species are monocentric, with no observed nuclear migration through the thallus. In addition, *Caecomyces* produces single holdfasts per thallus each bearing a single sporangium, although the production of more than one holdfast and multiple sporangia has also been observed, as described above [87]. Similar to *Caecomyces*, *Cyllamyces* produces monoflagellated zoospores (Fig. 6ae).

*Phylogenetic relationship between the bulbous genera Caecomyces and Cyllamyces.* Phylogenetic analysis using ITS1 and D1/D2 LSU regions indicate that the bulbous genera *Caecomyces* and *Cyllamyces* constitute a single monophyletic clade within the *Neocallimastigomycota* (Fig. 4b–c). A recent study that timed *Neocallimastigomycota* diversification events using highly conserved and generally single-copy genes from transcriptomic datasets strongly indicates that bulbous rhizoidal growth pattern evolved relatively recently (≈10 Mya) [9]. However, while the monophyly of bulbous Neocallimastigomycota is undisputed, the relationship between the genera *Caecomyces* and *Cyllamyces* is less clear. Phylogenetic analyses using ITS1 and D1/D2 LSU loci often provide conflicting tree topologies. For example [37] and [101] place *Cyllamyces* as an internal node within the broader *Caecomyces* in ITS1 [37], and D1/D2 LSU [37, 101] phylogenetic trees, respectively. Such pattern has led to suggestions of either splitting the genus *Caecomyces* into two genera [66], or reassigning *Cyllamyces* isolates as members of the genus *Caecomyces*. On the other hand, the analysis by Wang *et al.* using ITS1, entire ITS and D1/D2 LSU regions produced variable topologies that were dependent on the marker and tree-building algorithm utilized [64]. As well, topologies presented in [28, 31, 55] places both genera as two distinct separate clades. These variable outcomes are clearly a reflection of the paucity of strains included in the analysis; and interpretation of the observed results are often complicated by the unclear and possibly erroneous designations of strains in public databases. As well, the availability of only a single ITS1 sequence for some strains provides an incomplete picture of the breadth of diversity of the strain.

Here, we conducted phylogenetic analysis using ITS1 using 10 strains of *Caecomyces* and *Cyllamyces* reliably characterized as monocentric (*Caecomyces* strains Cado13a, OF1, DS1, GRL-11, JH-2017a, W101) or polycentric (*Cyllamyces* strains AFTOL-ID 846, EO17, CB3B1, and TSB2) using maximum likelihood (Fig. 7). The resulting topology shows clear separation between the two clades comprising *Caecomyces communis* and *Cyllamyces aberensis* (Fig. 7). The positions of *Cyllamyces icaris* and *Caecomyces sympodialis* are the exception. Only one ITS1 sequence is available for each of these species, and these two sequences cluster together (with only 1.83 % divergence). The two sequences diverge from *Caecomyces communis* clade by 5.36, and 3.95%, respectively, and from *Cyllamyces aberensis* clade by 4.6, and 2.68%, respectively. This suggests that *Cyllamyces icaris* and *Caecomyces sympodialis* could putatively represent a third distinct bulbous genus (Fig. 7). However, the presence of only a single ITS1 sequence from each of these species, and the absence of any LSU data preclude any further phylogenetic delineation. Indeed, microscopic descriptions in both manuscripts show a remarkable morphological resemblance, e.g. development of multiple sporangiophores from the bulbous holdfast, with multiple sporangia (usually more than three) growing on the end of these sporangiophores (Fig. 5 in [102] and (Fig. 4 in [48]), and the thallus developmental patterns (monocentric in *Caecomyces sympodialis* and polycentric in *Cyllamyces icaris*) are not convincingly demonstrated in either manuscripts.

Therefore, we reason that phylogenetic analysis (using the admittedly relatively limited sequence data available), as well as morphological and microscopic distinctions described above that reflect differences in thallus development patterns (a trait consistently regarded as a genus-level differentiation phenotype) support retaining *Cyllamyces* as a distinct separate genus within the Neocallimastigomycota. Future assessments would require isolation and thorough characterization, loci sequencing, and comparative genomic and transcriptomic analysis of multiple strains from both clades in a single comparative study. A third bulbous genus comprised of *Cyllamyces icaris* and *Caecomyces sympodialis* is also possible given the available limited phylogenetic data, and the morphological and microscopic similarities between the two isolates [48, 102].

*Occurrence. Cyllamyces* strains have been isolated from cow faeces [74], buffalo faeces [48] and rumen, and tunis sheep (R.A. Hanafy, unpublished results). Sequences affiliated with the genus *Cyllamyces* has previously been assigned to the alphanumeric candidate genera MN1 [122] and SP8 [30]. Culture-independent surveys identified members of the genus *Cyllamyces* (based on monophyly and high sequence identity to *C. aberensis* ITS1 [31], or LSU [28]) sequences in a wider range of animals, but still detected a clear preference to members of the Bovidae, especially domesticated cows.

### ***Cyllamyces aberensis* Ozkose *et al*. 2001 [74]**

*Type strain*: EO14 (=AFTOL ID 846), Figs. 1–11 in [74].

*GenBank sequence accession numbers:* AY997042 (ITS1, 5.8S, ITS2) and DQ273829 (D1/D2 LSU).

*ID in databases.* MycoBank ID: MB28540; Index Fungorum Registration Identifier: IF28540; NCBI taxon ID: 324620.

*Diagnosis.* Description is provided in detail in Ozkose *et al.* 2001 [74], *C. aberensis* produces spherical monoflagellated zoospores, polycentric thalli, and a single bulbous holdfast with multiple sporangia (Figs. 1–4 in [74]). Multiple unbranched sporangiophores are developed from different part of the bulbous holdfast (Fig. 7 in [74]). Branched sporangiophores were frequently encountered (Fig. 5 in [74]). Sporangia are spherical or ovoid born terminally at the end of the sporangiophore (Figs 5 and 7 in [74]). Nuclei are observed in sporangia, sporangiophores, and the bulbous holdfasts (Figs 8 and 9 in [74]).

*Additional Illustration*: *Cyllamyces cf. aberensis* strain TSB2 from Oklahoma State University Culture collection (Fig. 5ak-al), and CFH681 (Fig. 5am-ap) and BFH688 (Fig. 5aq) from Agharkar Research Institute, Pune, India culture collection.

### ***Cyllamyces icaris* Sridhar *et al.* [48]**

*Type strain*: CB3B1.

*GenBank sequence accession numbers*: EU043229 (ITS1, 5.8S, ITS2).

*ID in databases*: MycoBank ID: 629693; Index Fungorum Registration Identifier: IF 629693; NCBI taxon ID: NA.

*Diagnosis.* The description of *C. icaris* is provided in [48]. The authors differentiate *C. icaris* from *C. aberensis* based on its production of multiple holdfasts, larger sporangia, smaller number of sporangia per sporangiophore, and rudimentary filamentous growth structure in addition to bulbous growth pattern [48]. Phylogenetic analysis based on a single ITS1 sequence proves distinction from *C. aberensis* (average sequence divergence of 4.6%). However, while the described strain certainly appears distinct from *C. aberensis*, its affiliation as member of the genus *Cyllamyces*, rather than *Caecomyces*, or its affiliation with a third bulbous genus altogether, could not be conclusively ascertained from the provided information as detailed above. No evidence for polycentric thallus development in strain CB3B1 (i.e. DAPI stain showing nuclear migration to holdfasts and sporangiophores) is provided; and data on colony characteristics and liquid growth are also absent. The described patterns of multiple holdfast and multisporangiate growth have been observed in *Caecomyces* species, as stated above [33, 87].

## *BUWCHFAWROMYCES* CALLAGHAN *ET AL.* 2015

*History.* The genus has one species, *B. eastonii*, isolated from faeces of an asian water buffalo [37]. The strain was originally classified as a member of the genus *Anaeromyce*s. However, clear differences in morphological and microscopic features, as well as phylogenetic analysis using ITS1 and D1/D2 LSU loci clearly justified its accommodation in a new genus, for which the name *Buwchfawromyces* was proposed [37].

*Diagnosis.* Detailed description is provided in [37]. *Buwchfawromyces* produces monoflagellated zoospores, exhibits monocentric thalli development, and a filamentous rhizoidal growth pattern. Zoospores are relatively large (9–11 µm) with a long (30–40 µm) single flagellum. Fusion of zoospore results in zoospore-like structures with multiple flagella (supplemental Fig. 1a in [37]). Zoospore release mechanism is unclear. Single, spherical to ovoid terminal sporangia are produced. Large, swollen sporangiophores are reported (Fig. 1e, f in [37]), with a septum observed at the base of the sporangia. Sporangial neck is constricted with a narrow neck port (Fig. 1h in [37]). Capable of prolonged survival at 39 °C. Thick-walled septated structures, possibly a resting stage, observed in older but not younger cultures (Fig. S1f, g in [37]).

*Occurrence. Buwchfawromyces* have been isolated from faeces of an Asian water buffalo (*Bubalus bubalis*, Type species GE09), as well as from cow, sheep, and horse faeces [37]. Culture-independent surveys identified *Buwchfawromyces*-affiliated sequences in cow and red deer from New Zealand to which the alphanumeric designation SK2 was assigned prior to the isolation of GE09. Due to a possible mismatch to the utilized primers, no *Buwchfawromyces*-affiliated sequences were identified in the study by [31]. *Buwchfawromyces*-affiliated sequences were identified in six out of 21 examined faecal samples of wild and domesticated herbivores, mostly as a small fraction of the community [28].

### *Buwchfawromyces eastonii* Callaghan *et al.* 2015 [37]

*Type strain*: GE09 Aberystwyth University biorepositories (Wales), Isotype at Royal Botanic Gardens, Kew, London (K); and Friedrich-Schiller-Universität Jena, Germany (JE).

*GenBank sequence accession numbers.* EU414755 and EU414756 (ITS1, 5.8S, ITS2), and KP205570 (D1/D2 LSU)

*ID in databases*: MycoBank ID: MB550797 (genus) and MB550798 (species); Index Fungorum: IF550797 (genus) and IF550798 (species); NCBI Taxon ID: 1 623 672 (genus) and 512 029 (species).

## *OONTOMYCES* DAGAR *ET AL.* 2015

*History.* The genus has one species, *O. anksri*, isolated from the forestomach of an indian camel (*Camelus dromedarius*) [36].

*Diagnosis.* Detailed description is provided in [36]. *Oontomyces* produces monoflagellated zoospores, exhibits monocentric thalli development, and filamentous rhizoidal growth pattern. Zoospores are spherical with a single long flagellum (Fig. 1a, b in [36]). *Oontomyces* produces long non-swollen sporangiophores that are separated from the hyphae by a distinct constriction (Fig. 1d, e in [36]). Sporangia are terminal ovoid to elongate (Fig. 1e in [36]). Intercalary rhizoidal swellings also observed (Fig. 1g in [36]).

*Occurrence.* Isolated from the forestomach of a camel. Sequences affiliated with *Oontomyces* have been detected in one culture-independent study targeting anaerobic gut fungi from the bactrian camel (*Camelus bactrianus*) forestomach in China: Xinjiang (unpublished; GenBank accessions JX944831, JX944837, JX944839, JX944843, JX944846, JX944847, JX944848, JX944853, JX944859, JX944860, JX944862, JX944864, JX944871, JX944872, JX944873, JX944879, JX944883, JX944884, JX944887, JX944888, JX944889, JX944891, JX944895, JX944899, JX944900, JX944902, JX944904, JX944911, JX944912, JX944913, JX944920, JX944921, JX944925, JX944934, JX944937, JX944940, JX944942, JX944944, JX944946, JX944947, JX944962, JX944964, JX944967, JX944968, JX944969, JX944976, JX944977). Broader culture-independent analysis from wide range of herbivores failed to detect *Oontomyces*-affiliated sequences from all samples examined, suggesting a very narrow host (camel) or geographic (Asia) distribution.

### *Oontomyces anksri* Dagar *et al.* 2015 [36]

*Type strain*: SSD-CIB1, Fig. 1 in [36].

*GenBank sequence accession numbers*: JX017310 (ITS1, 5.8S, ITS2 complete) and JX017314 (D1/D2 LSU).

*ID in databases*: MycoBank ID: MB550795 (genus) and MB550796 (species); Index Fungorum: IF550795 (genus) and IF550796 (species); NCBI taxon ID: 1 650 676 (genus) and 1 212 493 (species).

## *PECORAMYCES* HANAFY *ET AL.* 2017

*History.* A *Pecoramyces* species was isolated from the faeces of an Angus steer, and its genome and transcriptome were sequenced and analysed in 2013 [12]. The strain was erroneously classified as a member of the genus *Orpinomyces*. However, subsequent analysis identified clear morphological and microscopic differences when compared to *Orpinomyces*; and

phylogenetic analysis using ITS1 and D1/D2 LSU loci further affirmed its phylogenetic distinction. Such results hence justified its accommodation in a new genus, for which the name *Pecoramyces* was proposed [35]. So far, only one species (*P. ruminantium*) has been described [35].

*Diagnosis.* Detailed description is provided in [35]. On solid media, *Pecoramyces* forms small (0.5–1 mm diameter) pinpoint-like colonies. In liquid media, it forms a thick smooth biofilm-like growth pattern. *Pecoramyces* produces monoflagellated zoospores, exhibits monocentric thalli development, and filamentous rhizoidal growth pattern. Zoospores are relatively large (7.5±1.5 µm) and are released through a wide apical pore, with the sporangial wall staying intact after zoospore discharge. Both endogenous and exogenous sporangia are encountered. Endogenous sporangia are subglobose and ovoid, while exogenous sporangia are spherical or ovoid. Sporangia display both long and short sporangiophores, with some exhibiting eggcup morphology or subsporangial swellings. The formation of peculiarly enlarged cells in *Pecoramyces* with strong morphological resemblance to titan cells (enlarged cells produced by *Cryptococcus neoformans* in response to exposure to the lung environment and shown to enhance the survival and propagation of *C. neoformans* [57]) has been observed (Fig. 3bi), and could possibly be a resting stage in its life cycle.

*Occurrence.* Isolates belonging to the genus *Pecoramyces* have been isolated from faecal samples of cow and sheep [12, 35], goat rumen [123, 124], oryx [28, 101], and nilgiri tahr (*Nilgiritragus hylocrius*) faeces. Culture-independent diversity surveys suggest a widespread distribution pattern and a preference to foregut fermenters [35], although occurrence in hindgut samples has also been observed [28] as an occasional minor fraction of the community.

### *Pecoramyces ruminantium* Hanafy *et al.* 2017 [35]

*Type strain*: C1A, Fig. 1a–v in [35].

*GenBank sequence accession numbers*: NG_060094.1 (D1/D2 LSU), NR_152323.1 (ITS1, 5.8S, ITS2).

*ID in databases*: MycoBank ID: MB552530 (genus) and MB552531 (species); Index Fungorum: IF552530 (genus) and IF55231 (species); NCBI taxon ID: 1987567 (genus) and 1987568 (species).

## *FERAMYCES* HANFAY *ET AL.* 2018

*History.* The genus has one species, *F. austinii*, isolated from the rumen and faecal samples of deer and wild Barbary sheep (*Ammotragus lervia*) [71].

*Diagnosis.* Detailed description is provided in [71]. On solid media, *Feramyces* produces medium-sized (3–7 mm), circular, beige colonies with well-defined dark centre and filamentous edges on solid media. In liquid media, *Feramyces* displays a thick biofilm growth pattern.

*Feramyces* produces polyflagellated zoospores, exhibits monocentric thalli development, and filamentous rhizoidal growth pattern. Zoospores are relatively large (6.5–13 µm), with 7–16 flagella. Zoospore release occurs through sporangial apical pore, with sporangia staying intact. Long, coiled sporangiophores with subsporangial swelling are observed. Both endogenous and exogenous sporangial development are produced, with endogenous sporangia displaying globose or pyriform morphology, and exogenous sporangia displaying highly pleomorphic morphologies. Pseudointercalary sporangia are sometimes observed. Hyphal growth is extensive with constrictions observed at regular intervals in wide hyphae.

*Occurrence*. Isolated from the rumen and faecal samples of deer and wild barbary sheep [71] in the USA and the Czech Republic (K. Fliegerová, personal communication). Prior to its isolation, sequences affiliated with the genus *Feramyces* were identified in faecal samples from giraffe (*Giraffa camelopardalis*), okapi (*Okapi johnstoni*) and greater kudu (*Tragelaphus strepsiceros*) with the alphanumeric designation AL6 [31]. Subsequent work identified its occurrence in multiple wild and domesticated samples, with preference to sheep and deer hosts [28].

### *Feramyces austinii* Hanfay *et al.* 2018 [71]

*Type strain*: F3a (Oklahoma State University Culture Collection, Fig. 2h in [71]).

*GenBank sequence accession numbers*: MG584193 (ITS1-5.8S-ITS2-D1/D2 LSU).

*ID in databases*: MycoBank ID MB823650 (genus) and MB823651 (species); Index Fungorum Registration Identifier: IF823650 (genus) and IF823651 (species); NCBI taxon ID: 2 683 847 (genus) and 2170546 (species).

## *LIEBETANZOMYCES* JOSHI *ET AL.* 2018

*History.* The genus currently has one species, *L. polymorphus*, isolated from goat rumen in 2018 [73].

*Diagnosis.* Detailed description is provided in [73]. On solid media, colonies are small, beige to brown (1–2 mm) with a dense central core of sporangial structures. Young newly formed sporangia are developed at the edges of colonies (Fig. 1b–c Fig. 1b, c in [73]). In liquid media, it produces a thin loose beige biofilm (Fig. 1d in [73]). Isolation from enrichments is usually achieved after prolonged incubation (3 weeks), indicating slow growth. *Liebetanzomyces* produces monoflagellated zoospores, exhibits monocentric thalli development, and filamentous rhizoidal growth pattern. Zoospores are average sized (5–6 µm), monoflagellated. Zoospore release mechanism is yet unclear. Endogenous, exogenous, and occasionally pseudointercalary sporangia are observed with highly pleomorphic morphology including globose, ellipsoid, clavate, ovoid and irregular shaped (Fig. 2 in [73]). Sporangia with papillae is observed (Fig.3d in [73]). Upon maturity, a septum is developed at the base of sporangia (Fig. 3b–e in [73], note the star). Both short and long sporangiophores are observed for exogenous sporangia, with occasional constriction at the base of the sporangiophores (Fig. 3e in [73]). Rhizoids are filamentous and highly branched.

*Occurrence. Liebetanzomyces* strains were isolated from goat rumen [73], as well as cow faeces, and barbary sheep (Hanafy, unpublished). Culture-independent analysis indicated a relatively rare distribution pattern in nature, being identified only in a few samples usually in larger studies (cow, sheep and llama in [31], cow in [30], and alpaca, aoudad sheep, blackbuck antelope and oryx in [28]), and invariably constituting an minor fraction of the community when encountered. Members of this clade have been given the alphanumeric designation SP4 prior to their isolation [30].

#### *Liebetanzomyces polymorphus* Joshi *et al.* 2018 [73]

*Type strain.* Holotype: strain G1SC (collection of micro-organisms at Agharkar Research Institute, Pune, India). An isotype was deposited at Aberystwyth University biorepository, Wales.

*GenBank sequence accession numbers*: MH468765 (ITS1, 5.8S, ITS2) and MH468763 (D1/D2 LSU).

*ID in databases*: MycoBank ID MB554794 (genus) and MB554795 (species); Index Fungorum: IF554794 (genus) and IF554795 (species); NCBI taxon ID 547816 (genus), and 2219670 (species).

### *AGRIOSOMYCES* HANAFY *ET AL.* 2021

*History.* The genus currently has one species, *A. longus*, isolated from faeces of a wild sheep (mouflon sheep, *Ovis gmelini*) as well as a boer goat (*Capra hircus*) [55].

*Diagnosis.* Detailed description is provided in [55]. On solid media, *Agriosomyces* forms small, light brown, circular colonies. In liquid media, it forms a thin biofilm-like growth. *Agriosomyces* produces monoflagellated zoospores, exhibits monocentric thalli development, and a filamentous rhizoidal growth pattern. Zoospores are small (4±1.1 µm), monoflagellated, with extremely long flagella (5–6 times the zoospore size). Zoospores are released through dissolution and rupturing of the sporangial wall. Sporangiophores exhibit sub-sporangial swelling and the sporangial neck is constricted with a narrow neck port. Endogenous and exogenous sporangia are observed. Sporangia are globose and very homogenous, with very low level of pleomorphism observed.

*Occurrence.* Isolated from faecal samples of wild (mouflon) sheep and Boer goat [55]. A recent culture-independent survey suggests a limited ecological distribution, being encountered in few (6/21; goat, deer, sheep and alpaca) animals, and representing >1 % of the community in only two samples (the Mouflon and Boer goat samples from which they were isolated).

*Agriosomyces longus* Hanafy *et al.* 2020 [55]

*Type strain*: MS2 (Oklahoma State University Culture Collection), Fig. 2g in [55].

*GenBank sequence accession numbers.* MK882010–MK882013 (ITS1), MK881996 (D1–D2 LSU); and MT085708–MT085709 (ITS1-5.8S-ITS2-D1/D2 LSU).

*ID in databases*: MycoBank ID: MB830737 (genus) and MB830738 (species); Index Fungorum: IF830737 (genus) and IF830738 (species); NCBI taxon ID: 2710854 (genus) and 2710868 (species).

### *AKLIOSHBOMYCES* HANAFY *ET AL.* 2021

*History.* The genus currently has one species, *A. papillarum*, isolated from faeces of a white tailed deer (*Odocoileus virginianus*) [55].

*Diagnosis.* Detailed description is provided in [55]. On solid media, *Aklioshbomyces* forms small beige, circular colonies with a brown central core. In liquid media, it produces heavy growth of thick biofilms that firmly attaches to the tube's glass surface. *Aklioshbomyces* produces monoflagellated zoospores, exhibits monocentric thalli development, and filamentous rhizoidal growth pattern. Zoospores are medium sized (4.5–7.4 µm) and mostly monoflagellated. Exogenous and endogenous sporangia, as well as pseudointercalary sporangia are observed. No morphological differences were detected between endogenous and exogenous sporangia, with the most common morphologies being ovoid, globose, obpyriform and ellipsoidal. Most Sporangia were papillated

with one or two papillae, thought to facilitate zoospore release. Sporangiophores are unbranched with widely varying lengths from a few microns to 230 µm.

*Occurrence.* The *Aklioshbomyces* type strain was isolated from white tailed deer faeces. Interestingly, culture-independent analysis suggests an extremely limited distribution: in a survey of 21 animals, it was only identified in high proportion in the same white-tailed deer faecal sample from which it was isolated. In the four additional samples it was identified, it represented a minor (<1%) fraction of the anaerobic fungal community [55]. This preference to a single deer species is extremely rare within the *Neocallimastigomycota*, in contrast to the more common observed preferences to an animal family (e.g. *Cyllamyces* preference for Bovidae or *Khoyollomyces* for Equidae) or a gut type (e.g. *Pecoramyces* for foregut fermenters).

### ***Aklioshbomyces papillarum* Hanafy *et al.* 2020 [55]**

*Type strain*: WT-2 (Oklahoma State University Culture Collection, Fig. 3m in [55].

*GenBank sequence accession numbers*: MT085737–MT085741 (ITS1-5.8S-ITS2-D1/D2 LSU).

*ID in databases*: MycoBank ID: MB830735 (genus) and MB830736 (species); Index Fungorum IF830735 (genus), and IF830736 (species); NCBI taxon ID: 2710855 (genus) and 2710861 (species).

## ***CAPELLOMYCES* HANAFY *ET AL.* 2021**

*History.* Isolates representing two different species (*C. foraminis* and *C. elongatus*) were isolated from faecal samples of a wild boar goat (USA), and domesticated goat (India), respectively, and reported in the same manuscript [55]. While displaying a high level of ITS1 and D1/D2 LSU sequence identity, multiple growth and microscopic differences were identified, justifying their accommodation into two distinct species.

*Type*: BGB-11 (Oklahoma State University Culture Collection, Fig. 4n in [55]).

*GenBank sequence accession numbers*: AY997042 (ITS1, 5.8S, ITS2) and DQ273829 (D1/D2 LSU).

*ID in databases*: MycoBank ID: MB830739; Index Fungorum: IF830739; NCBI taxon ID: 2710856.

*Diagnosis.* Detailed description is provided in [55]. Members of the genus *Capellomyces* form small white to beige colonies on solid media, and thin biofilm growth in liquid media. Strains exhibit monocentric thalli development, filamentous rhizoids, and monoflagellated zoopores. Endogenous and exogenous sporangial development patterns have been observed.

*Occurrence.* The genus *Capellomyces* was isolated from faecal samples of Boer goat and domestic goat, suggesting a preference to goats' alimentary tract. An isolate assigned to the genus *Anaeromyces* and obtained from goat rumen (*Anaeromyces* sp. GA-04 GenBank accession FJ912851.1) exhibits high sequence similarity to *Capellomyces* (1.2 % average ITS1 sequence divergence between various clones of *Capellomyces* and *Anaeromyces* sp. GA-04). Peculiarly, sequences affiliated with the genus *Capellomyces* were not identified in a recent D1/D2 LSU-based culture-independent diversity survey of 21 animals, which might be attributed to a mismatch to the Neocallimastigomycota-specific reverse primer employed [28].

### ***Capellomyces foraminis* Hanafy *et al.* 2020 [55]**

*Type strain*: BGB-11 (Oklahoma State University Culture Collection, Fig. 4n in [55].

*GenBank sequence accession numbers.* MK882007–MK882009 (ITS1) and MK881975 (D1/D2 LSU).

*ID in databases*: MycoBank ID: MB830740; Index Fungorum Registration Identified: IF830740; NCBI taxon ID: 2710863.

*Diagnosis.* Detailed description in provided in Hanafy *et al.* 2020 [55]. In addition to features provided in the genus description, the following features were observed: Zoospores are average-sized (5.5 µm), and liberated through a wide apical pore at the top of the sporangia followed by sporangial wall collapse. Endogenous sporangia are ellipsoidal and ovoid. Sporangiophores are unbranched, ranging in length between 20 and 150 µm, with some ending in subsporangial swellings. Exogenous sporangia are ovoid, ellipsoidal or globose.

### ***Capellomyces elongatus* Hanafy *et al.* 2020 [55]**

*Type strain*: GFKJa1916 (Agharkar Research Institute, Pune, India culture collection, Fig. 7k in [55]).

*GenBank sequence accession numbers*: MK775315 (ITS1); MK775304 (D1/D2 LSU), MT085701.1 (ITS1-5.8S-ITS2-D1/D2 LSU).

*ID in databases:* MycoBank ID: MB830869; Index Fungorum Registration Identified: IF830869; NCBI taxon ID: 2710862.

*Diagnosis.* Detailed description in Hanafy *et al.* [55]. In addition to features provided in the genus description, the following features were observed: Zoospores are average-sized (4–5 µm). Endogenous sporangia are more pleomorphic when compared to *C. foraminis*

displaying cylindrical, elongate, globose, subglobose, ellipsoid and obovoid morphologies. Sporangiophores are extremely long (up to 300 µm in some cases), and multisporangiate thalli are commonly observed (i.e. two exogenous sporangia developing on the same sporangiophore). Exogenous sporangia are ovoid and globose

## *GHAZALLOMYCES* HANAFY *ET AL.* 2021

*History.* The genus has one species *G. constrictus*, isolated from the faeces of an Axis deer (*Axis axis*) [55].

*Diagnosis.* Detailed description is provided in [55]. On solid media, *Ghazallomyces* produces small circular colonies with a white to light brown central core. In liquid media, *Ghazallomyces* produces a thick white biofilm growth. *Ghazallomyces* produces polyflagellated zoospores, exhibits monocentric thallus development, and filamentous rhizoidal growth pattern. Zoospores are average-sized, with 7–14 flagella, and are released through an apical pore followed by collapse of the sporangial wall. Endogenous and exogenous sporangia are produced, both of which are highly pleomorphic, and displaying tightly constricted necks with narrow ports. Fine septum formation at the base of the sporangia is observed. The empty zoospore cyst remains as a persistent swollen structure at the base of sporangiophore during exogenous thallus development.

*Occurrence. Ghazallomyces* was isolated from the faeces of an axis deer. Interestingly, culture-independent survey of 21 samples only identified it in the sample from which it was isolated, suggesting an extremely rare ecological distribution pattern.

### *Ghazallomyces constrictus* Hanafy *et al.* 2020 [55]

*Type strain*: Axs-31 (Oklahoma State University Culture Collection), Fig. 5h in [55].

*GenBank sequence accession numbers*: MK882043-MK882046 (ITS1) and MK881971 (D1/D2 LSU).

*ID in databases*: MycoBank ID MB830733 (genus) and MB830734 (species); Index Fungorum Registration Identifier IF830733 (genus), and IF830734 (species); NCBI taxon ID: 2 710 857.

## *JOBLINOMYCES* HANAFY *ET AL.* 2021

*History.* The genus has one species, *J. apicalis*, isolated from the faeces of domesticated goat and sheep [55].

*Diagnosis.* Detailed description is provided in [55]. On solid media, *Joblinomyces* produces small colonies with a dark central core surrounded by long and thin radiating rhizoids. In liquid media, *Joblinomyces* produces a thin biofilm. *Joblinomyces* produces monoflagellated zoospores, exhibits monocentric thallus development, and a filamentous rhizoidal growth pattern. Zoospores are medium-sized (5–6 µm), monoflagellated (1–2 flagella), and are released through the gradual dissolution of a wide apical portion of the sporangial wall, resulting in the formation of an empty cup-shaped sporangium. Long unbranched sporangiophores (20–80 µm) are observed. Endogenous and exogenous sporangia are globose, subglobose, ovoid and obovoid.

*Occurrence.* Isolated from domestic goats and sheep faeces [55]. Members of this clade have been given the alphanumeric designation AL5 prior to their isolation [31]. *Joblinomyces* sequences were encountered in 14/30 samples examined in [31] mainly in foregut fermenters where they typically represented a minor fraction of the overall anaerobic gut fungal community within a specific sample, with the exception of a domesticated goat sample where the genus represented 19.6 % of the total anaerobic gut fungal community [31]. In a subsequent culture-independent study, *Joblinomyces* sequences were encountered in 3/21 samples (mouflon sheep, aoudad sheep, and fallow deer), only representing a significant member of the anaerobic gut fungal community in the Fallow deer sample (77.12%). This suggests a fairly limited distribution pattern, and a relative preference for foregut over hindgut fermenters.

### *Joblinomyces apicalis* Hanafy *et al.* 2020 [55]

*Type strain*: GFH683, Fig. 6c in [55].

*GenBank sequence accession numbers* : MK910278–MK910282 (ITS1) and MK910268–MK910272 (D1/D2 LSU).

*ID in databases*: MycoBank ID: MB830867 (genus) and MB830868 (species), Index Fungorum Registration Identifier IF830867 (genus), and IF830868 (species), NCBI Taxon ID: 2710858 (genus) and 2710865 (species).

## *KHOYOLLOMYCES* HANAFY *ET AL.* 2021

*History.* The genus has one species, *K. ramosus*, isolated from the faeces of zebra and domesticated horses [55].

*Diagnosis.* Detailed description is provided in [55]. On solid media, *Khoyollomyces* forms small yellow to yellowish brown irregularly shaped colonies. In liquid media, it generates a loose growth pattern, and exhibits a sand-like appearance resembling patterns generally observed with bulbous genera. *Khoyollomyces* produces monoflagellated zoospores, exhibits monocentric thallus development, and a highly branched filamentous rhizoidal system. Zoospores are average-sized (6 µm) and are liberated through a wide apical pore at

the top of the sporangia. Long sporangiophores ranging in length from 20–400 µm are typically observed. The genus is characterized by multisporangiate thalli, with the majority of sporangiophores being branched and bearing two to four sporangia. Endogenous, exogenous, and pseudo-intercalacry sporangia are all observed. Endogenous sporangia are small and subglobose, while exogenous sproragnia are larger and more pleomorphic (heart, ovoid, pyriform).

*Occurrence.* Isolated from the faeces of zebra and horses [55, 101] (Callaghan and Griffith, unpublished). Members of this clade have been given the alphanumeric designation AL1 prior to their isolation [31]. Culture-independent analysis identified their prevalence as a significant component of the fungal community in multiple hindgut herbivores, especially those associated with the family Equidae e.g. horses and zebras [28, 31]

#### ***Khoyollomyces ramosus* Hanafy *et al.* 2020 [55]**

*Type strain*: ZS-33, Fig. 8j in [55]

*GenBank sequence accession numbers*: MK882019 (ITS1) and MK881981 (D1–D2 28SrDNA)

*ID in databases*: MycoBank ID: MB830741 (genus) and MB830742 (species), Index Fungorum Registration Identifier IF830741 (genus) and IF830742 (species). NCBI taxon ID: 2710859 (genus) and 2710867 (species).

### *TAHROMYCES* HANAFY *ET AL.* 2021

*History.* The genus has one species *T. munnarensis* isolated from the faeces of Nilgiri tahr goats [55].

*Diagnosis.* Detailed description is provided in [55]. On solid media, *Tahromyces* produces small colonies with a compact centre and filamentous edges. In liquid media, growth occurs as a thin biofilm attached to the glass surface. *Tahromyces* produces monoflagellated zoospores, exhibits monocentric thallus development, and a filamentous rhizoidal growth pattern. Zoospores are relatively small (3–4 µm) and are released via irregular dissolution of the sporangial wall. Endogenous sporangia are globose, ovoid and obovoid, with some showing subsporangial swelling. Exogenous sporangia are also globose, ovoid and obovoid. Sporangiophores are short, some of which ending with subsporangial swellings with or without a constricted neck (between 12 and 20 µm). Septum formation at the base of some mature sporangia is observed.

*Occurrence.* Isolated from the faeces of Nilgiri tahr, a mountain goat indigenous to India. Members have not been identified so far in culture-independent analysis, possibly reflecting its restricted host (Nigliri tahr), or geographical (India) distribution.

#### ***Tahromyces munnarensis* Hanafy *et al.* 2020 [55]**

*Type strain*: TDFKJa193, Fig. 9h in [55].

*GenBank sequence accession numbers*: MT085675 (ITS1-5.8S-ITS2-D1/D2 LSU); MK775321 (ITS1); MK775310 (D1–D2 28S rRNA).

*ID in databases*: MycoBank ID MB830865 (genus) and MB830866 (species); Index Fungorum Registration Identifier IF830865 (genus), and IF830866 (species); NCBI taxon ID: 2710860 (genus), and 2710866 (species).

### *AESTIPASCUOMYCES* STABEL *ET AL.* 2020

*History.* Members of the genus *Aestipascuomyces* were simultaneously and independently isolated by two research groups in 2020 from faecal samples of alpaca and sheep [75]. Only one species, *A. dupliciliberans*, has been described.

*Diagnosis.* Detailed description is provided in [75]. On solid media, *Aestipascuomyces* forms medium-sized (2–5 mm) white filamentous colonies with a white centre of sporangia. In liquid media, *Aestipascuomyces* forms a distinctly heavy biofilm growth. *Aestipascuomyces* produces polyflagellated zoospores, exhibits monocentric thallus development, and a filamentous rhizoidal growth pattern. Zoospores are relatively large (5–14 µm), with 7–20 flagella, and are released through an apical pore as well as by sporangial wall rupturing. Utilization of this dual spore release mechanism in a single strain has not been observed in other *Neocallimastigomycota* genera. Unbranched sporangiophores ranging in length between 10 and 300 µm, many of which displaying subsporangial swelling. Pleomorphic endogenous and exogenous sporangia are observed. Sporangial necks are often tightly constricted.

*Occurrence.* Isolated from the faeces of alpaca and sheep [75]. Members of this clade have been identified in faecal samples of sheep and deer and given the alphanumeric designation SK4 prior to their isolation [125]. A recent survey of 21 faecal samples identified its occurrence as a major component in the sheep sample from which it was isolated, and as an extremely minor component of five additional samples. A possible positive correlation between *Aestipacsuomyces* abundance and grazing in summer pastures has been suggested [75].

#### ***Aestipascuomyces dupliciliberans* Stabel *et al.* 2020 [75]**

*Type strain*: R4 (Oklahoma State University Culture Collection, Fig. 2b in [75].

*GenBank sequence accession numbers*: MW019494–MW0194497 (ITS1-5.8S-ITS2-D1/D2 LSU).

*ID in databases*: MycoBank ID: MB837524 (genus) and MB837526 (species); Index Fungorum Registration Identifier IF837524 (genus), and IF837526 (species), NCBI taxon ID: 2789199 (genus), and 2789200 (species).

## *PAUCIMYCES* HANAFY *ET AL.* 2021

*History.* The genus has one species, *P. polynucleatus*, isolated from the faeces of a wild blackbuck antelope (Indian Antelope, *Antilope cervicapra*) [72].

*Diagnosis.* Detailed description is provided in [72]. On solid media, *Paucimyces* produces medium sized white compact circular colonies with no dark centre. In liquid media, thin loose biofilm-like growth is observed, with occasional clumping that appears as small, loose, fragile spherical structures at the bottom of the culture tube. *Paucimyces* produces monoflagellated zoospores, exhibits polycentric thallus development, and a filamentous rhizoidal growth pattern. The relatively large (6–10 µm) zoospores are released through a wide apical pore at the top of the sporangia, with the sporangial wall staying intact after the discharge. The polycentric thalli exhibit a highly branched nucleated filamentous rhizomycelium. During thallus development, hyphal tips swell to form spherical vesicles, from which multiple sporangiophores arise (Fig. 2h–i in [72]). Each sporangiophore bears one sporangium at its end. In many cases, sporangia develop on the spherical vesicles without sporangiophores. Sporangia are mainly ovoid. Basal walls are formed to separate the mature sporangia from the sporangiophores (Fig. 2i–k in [72]). Old cultures lose the capacity for the production of sporangia and only produce sporangiophores initials.

*Occurrence.* Isolated from the faeces of a wild blackbuck (Indian antelope) [72]. Members of this clade have been identified in faecal samples in a recent culture-independent study [28], and were given the alphanumeric designation RH5 prior to their isolation. Interestingly, *Paucimyces*-affiliated sequences were identified almost entirely in sheep samples in this study, suggesting a possible genus preference for the ovine alimentary tract.

### *Paucimyces polynucleatus* Hanafy *et al.* 2021 [72]

*Type strain*: strain BB-3 (Oklahoma State University Culture Collection), Fig. 2c in [72].

*GenBank sequence accession numbers*: MW694896–MW64898 (ITS1-5.8S-ITS2-D1/D2 LSU).

*ID in databases*: MycoBank ID: MB838953 (genus) and MB838954 (species); Index Fungorum Registration Identifier: IF838953 (genus), and IF838954 (species), NCBI taxon ID: NA.

Funding information
This work has been supported by NSF grant 2029478 to N.H.Y. and M.S.E.

Acknowledgements
We thank Joan E. Edwards (Palital Feed Additives) for critically reviewing the manuscript.

Conflicts of interest
The authors declare that there are no conflicts of interest.

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