ASSESSING THE ANAEROBIC GUT FUNGAL DIVERSITY IN HERBIVORES USING CULTURE-INDEPENDENT SURVEY AND MULTI-YEAR ISOLATION

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Abstract: The overall aim of my dissertation was to investigate the phylogenetic diversity, ecological distribution, and community structure of the anaerobic gut fungi (Neocallimastigomycota) in a wide range of herbivorous animals with special emphasis on hitherto unsampled herbivores. To achieve this aim, two approaches were employed in parallel, anaerobic culturing approaches as well as culture-independent diversity survey. I conducted a multi-year isolation effort utilizing different isolation techniques, which maximized the recovery of multiple AGF isolates. My strategy involved the utilization of multiple carbon sources for AGF enrichment, sequence-guided strategy to target samples enriched in uncultured taxa, and sampling from a wide range of wild, domesticated, and zoo-housed herbivorous animals. This effort has resulted in the characterization of 10 novel AGF genera including five previously uncultured AGF members. This resulted in more than doubling of the number of AGF genera with a cultured representative. This extensive culturing-based study was complemented with a culture-independent survey to evaluate AGF diversity in 27 herbivorous animals. The culture independent survey also served to evaluate the utility of the D1/D2 region of the LSU rRNA as a new phylomarker for the AGF. The analysis produced the first comprehensive reference D1/D2 LSU database encompassing all cultured AGF genera, as well as the majority of candidate genera previously only identified in prior ITS1-based culture-independent surveys. Using the created database, I was able to identify a highly diverse anaerobic fungal community within the sampled animals, with twenty-eight genera and candidate genera, including multiple novel lineages. This work has established a framework for future utilization of D1/D2 LSU amplification and PacBio sequencing for AGF community assessment, and highlight the value of wild herbivores as untapped reservoirs for many yet-uncultured AGF taxa. Collectively, the results from this dissertation show that the genus-level diversity within Neocallimastigomycota is significantly broader than what was previously proposed, and argue that a large number of the yet-uncultured AGF genera are indeed culturable, given the right sampling and isolation conditions.

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PREFACE

The anaerobic gut fungi (AGF, Neocallimastigomycota) belong to a strict anaerobic basal fungal lineage that inhabits the rumen and alimentary tract of a wide range of foregut and hindgut herbivores. Herbivorous animals usually lack the capability to produce cellulolytic and hemicellulolytic enzymes for the degradation of ingested plant materials; instead, they rely on their gut microbial consortium for the digestion process [1]. Within such complex and prokaryote-dominated assemblages, AGF play a crucial role in the anaerobic degradation of the recalcitrant lignocellulosic plant materials through rhizoidal invasion of the plant fibers and production of a wide array of powerful polysaccharide-degrading enzymes, many of which were acquired by horizontal gene transfer from different gut bacteria [2-4]. Their remarkable degradation capacities and vast enzymatic repertoire made them a promising source of enzymes and bioactive molecules for biotechnological applications [5].

Due to the strict anaerobic nature of AGF, lack of a reliable long-term storage protocol, and the frequent occurrence of senescence in AGF strains as a result of prolonged sub-culturing, mycologists often show little interest in working with AGF. Consequently, our understanding of their metabolic capabilities, ecological distributions, evolutionary history, and biotechnological application has been relatively slow [1, 6]. During the last 50 years, multiple efforts have been conducted to explore the phylogenetic diversity and ecological distribution of this understudied group of fungi. Evidences for AGF existence were initially conducted through the use of traditional anaerobic culturing approaches [7, 8]. However, due to the inherent difficulties associated with the isolation and maintenance of anaerobic fungi, efforts to isolate novel AGF lineages have dramatically lagged behind their aerobic counterparts. By the year 2015, only eight genera were described [7-11]. Also, multiple AGF taxa were shown to be extremely fastidious, with complex nutritional and culturing requirements, which significantly hampered isolating them using the current isolation protocols [1, 12].

Furthermore, the majority of AGF isolation and characterization efforts have targeted only a few species of domesticated herbivorous hosts, e.g. cows, horses, goats, sheep, and water buffalos [7, 13-15], with limited studies focusing on undomesticated animals either in captivity or wild. This inadequate host sampling has limited the number of cultured AGF genera.

With the advances in sequencing and the use of DNA barcoding, culture-independent amplicon-based surveys became widely utilized to gauge anaerobic fungal diversity and community structure in herbivores [16-18]. The internal transcribed spacer 1 (ITS1) within the ribosomal RNA locus has been the phylogenetic marker of choice for AGF amplicon-based community analysis [16-18]. These studies have clearly demonstrated that AGF diversity is much broader than previously inferred from culture-based approaches, and have enabled the identification of sixteen different yet-uncultured lineages. Despite the wide use of the ITS1 region in AGF diversity surveys, it is becoming increasingly clear that its use as a phylogenetic marker has fundamental drawbacks [6]. The ITS1 region in the Neocallimastigomycota is polymorphic, exhibiting considerable complex secondary structure [19] and length [20] variability. In addition, there is significant sequence divergence between copies of the ITS1 region within a single strain (up to 12.9%) [11], values that exceed cutoffs utilized for species (even genus in some instances) level delineation from ITS1 data [16, 21-23]. Such limitations render environmental ITS1-based estimates of the AGF diversity unreliable, as it is difficult to decide whether these novel candidate genera are indeed representing new AGF lineages.

Recently, the D1/D2 region of the large ribosomal RNA subunit (D1/D2 LSU) was proposed as an alternative phylomarker for the AGF, and is now widely used for the phylogenetic characterization of AGF pure cultures [10, 11]. Unlike ITS1, D1/D2 LSU displays much lower levels of length heterogeneity [14] and intra-strain sequence divergence [24], and therefore it has the potential to generate a more stable phylogenetic backbone to the AGF [6]. However, the reconciliation between LSU sequence datasets and the datasets previously generated using ITS1 will be challenging. Moreover, the relatively large size of the region (approximately 750 bp) has long hampered the utilization of short read sequencing platforms, e.g. Illumina [6].

The work presented in this dissertation aims to expand our understanding of the AGF diversity while addressing the abovementioned challenges. Chapter I and II demonstrate the isolation and characterization of a novel AGF genus, *Pecoramyces ruminantium*, and a novel AGF species *Anaeromyces contortus*, respectively. This work represents a guiding protocol for using culture-based approaches to uncover multiple novel AGF lineages through sampling a wide range of herbivorous animals. Also, chapter I and II introduce the importance of both morphological characteristics and phylogenetic analysis for confirming the novelty of AGF isolates. These two chapters are now published as two separate manuscripts in the Journal Mycologia.

In chapter III, I explored a broader host range to include wild herbivorous animals as hosts to obtain AGF isolates. I was able to isolate a novel AGF genus, *Feramyces austinii*, from the feces of wild Aoudad Barbary sheep (*Ammotragus lervia*) and a wild fallow deer (*Dama dama*). Interestingly, *Feramyces* was the first cultured representative of the previously uncultured Neocallimastigomycota clade AL6, originally identified in culture independent surveys of fecal samples from captive wild animals [16]. Also, chapter III highlights the importance of sampling animals with different lifestyles than the ones usually targeted in traditional AGF studies and shows wild herbivorous animals as untapped reservoirs for many yet-uncultured AGF taxa. This chapter is now published in the Journal Mycologia.

Chapter IV represents my concerted multiyear isolation effort from a wide range of wild, zoo-housed, and domesticated herbivorous mammals. This work highlights the value of intensive sampling and implementation of various isolation strategies in recovering multiple AGF strains. Results from this study have greatly expanded the current Neocallimastigomycota global culture collection by isolating seven novel AGF genera, including the first cultured representatives of the previously uncultured AGF lineages AL1 and AL5. This chapter is now published in the Journal Mycologia.

Chapter V proposes the utilization of D1/D2 LSU as a phylomarker for AGF environmental diversity surveys. In this study, I amplified the region encompassing the ITS1-5.8S-ITS2-D1/D2 LSU from pure isolates (from our culture collection) and environmental fecal samples. To enable sequencing the long amplicon obtained, the single molecular real time (SMRT)-PacBio sequencing approach was used. Ultimately,

this work allowed correlating between D1/D2 LSU data and currently available ITS1 datasets, and generating a reference D1/D2 LSU database for all cultured AGF genera, as well as the majority of candidate genera encountered previously in ITS1-based surveys. In addition, diversity and community structure analyses using the D1/D2 LSU provided interesting clues on the role of host phylogeny and life style in shaping the AGF diversity. This chapter is now published in the Journal Environmental Microbiology.

In chapter VI, I adopted a sequence-guided isolation strategy, in which samples are initially prescreened using culture-independent approaches followed by targeting promising samples exhibiting a high proportion of novel/wanted genera for isolation efforts. Interestingly this effort has yielded the isolation of members of the hitherto uncultured lineage SK4 from a sample (wild Barbary sheep) where SK4 made up 76.6% of the total AGF community. This chapter has been submitted for review in the Journal Microorganisms.

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CHAPTER I

PECORAMYCES RUMINANTIUM, GEN. NOV., SP. NOV., AN ANAEROIBIC GUT FUNGUS FROM THE FECES OF CATTLE AND SHEEP

Abstract

The anaerobic gut fungi (AGF) inhabit the rumen and alimentary tracts of multiple ruminant and nonruminant herbivores, belong to a distinct phylum-level lineage (Neocallimastigomycota), and play an important role in plant biomass degradation in many herbivores. As part of a wider effort to obtain AGF with high lignocellulolytic capacities, we isolated and characterized four different AGF strains from the feces of cattle and sheep. Microscopically, isolates produced monocentric thalli and monoflagellated zoospores. Phylogenetic analysis revealed that all isolates formed a monophyletic cluster with strong bootstrap support as a sister clade to the genus *Orpinomyces* and close to *Neocallimastix*, an unexpected result because these two genera of AGF form poly- flagellated zoospores. Isolates displayed a smooth biofilm-like growth in liquid medium and formed small (0.5–1 mm) pinpoint circular colonies on agar roll tubes. Both endogenous and exogenous sporangia were observed with variable shapes and sizes. Zoospores were mainly spherical, with diameters ranging between 3.8 and 12.5 μm, and mostly a single flagellum. All strains exhibited similar substrate utilization patterns and comparable cellulolytic and xylanolytic activities. Similar ITS1 sequences falling within the same distinctive clade were found on GenBank, with all environmental samples obtained from diverse ruminant and pseudoruminant hosts from three continents, but not from any hindgut-fermenting hosts. Given the high level of sequence divergence between our strains and closest cultured representatives and their distinct microscopic/macroscopic features, we propose a new genus, *Pecoramyces*, from the name of the taxonomic infraorder Pecora ("horned ruminants" or "higher ruminants"; derived from the Latin word for horned livestock), and a new species, *P. ruminantium* (since occurrence seems to be specific to ruminant/pseudoruminant foregut, but not hindgut-fermenting mammals).

Introduction

The anaerobic gut fungi (AGF) inhabit the rumen and alimentary tracts of multiple herbivores. AGF, belonging to a distinct phylum lineage (Neocallimastigomycota), play an important role in plant biomass degradation in many herbivores and represent the only obligately anaerobic fungal group known so far. Currently, the phylum Neocallimastigomycota encompasses eight genera: *Neocallimastix* (Heath et al. 1983), *Orpinomyces* (Barr et al. 1989), *Anaeromyces* (Breton et al. 1990), *Piromyce*s (Gold et al. 1988), *Caecomyces* (Gold et al. 1988), *Cyllamyces* (Ozkose et al. 2001), *Buwchfawromyces* (Callaghan et al. 2015), and *Oontomyces* (Dagar et al. 2015). AGF genera are differentiated based on thallus growth pattern (monocentric versus polycentric growth), rhizoid morphology (filamentous rhizoids versus bulbous holdfasts), and zoospore flagellation (monoflagellated versus polyflagellated). More specific characteristics, e.g., sporangium and sporangiophore morphologies, sporangium development patterns, zoospore release mechanism, zoospore ultrastructure, and size of various structures/life forms, could possibly be used for species-level differentiation (Mountfort and Orpin 1994; Ho and Barr 1995). In addition, phylogenetic analysis based on internal transcribed spacer 1 (ITS1) and large ribosomal subunit (28S rRNA) regions within the rRNA locus is playing an increasingly important role in genus- and species-level delineation in AGF. Such analyses generally con- firm the monophyletic nature of most genera originally proposed based on structural features, with the notable exception of the filamentous, monocentric, and mono- flagellated genus *Piromyces*, which is polyphyletic (Brookman et al. 2000; Fliegerova et al. 2004; Gruninger et al. 2014). Conversely, it is currently unclear whether the bulbous genera Caecomyces and Cyllamyces represent a single (Gruninger et al. 2014; Callaghan et al. 2015) or two (Ozkose et al. 2001) phylogenetically distinct clades.

Multiple culture-independent diversity surveys utilizing ITS1 sequences have been conducted in the past decade to assess the global-level AGF diversity (Liggenstoffer et al. 2010; Nicholson et al. 2010; Kittelmann et al. 2012). These studies have conclusively demonstrated that the scope of Neocallimastigomycota diversity is broader than that deduced based on culture- based approaches. Kittleman et al. analyzed AGF sequences

from a multitude of diversity surveys and identified 14 different clusters of AGF with no cultured representative that are exclusively defined by ITS1 sequence data (Liggenstoffer et al. 2010; Nicholson et al. 2010; Kittelmann et al. 2012). However, the relatively small number of AGF diversity surveys con- ducted so far, as well as the sparse overlap between novel clusters reported in various studies, suggest that this represents a conservative estimate of global AGF diversity.

As part of a wider effort to identify AGF with high lignocellulolytic capacities, we have isolated multiple phylogenetically related AGF strains from sheep (strains S4B, S4E, S4F) and cow (strain C1A) feces. Although strain C1A's genome was sequenced and analyzed before (Youssef et al. 2013), we here characterize in detail the phylogenetic affiliation and morphological characteristics of the ex-type strain C1A and an additional strain S4B. We highlight the key differences between these isolates and currently described AGF taxa, and how such differences justify proposing a new anaerobic fungal genus (*Pecoramyces*) to accommodate these strains.

Materials and methods

1. Isolation source and isolation procedures. All strains described in this study were obtained from fecal samples of herbivores around the city of Stillwater, Oklahoma, USA. Strains S4B, S4E, and S4F were isolated from fecal samples of sheep, whereas isolate C1A was obtained from a fecal sample of an Angus steer. Freshly deposited feces were collected and immediately placed in sterile 15-mL plastic tubes. Tubes were filled to the top with fecal material to displace air and keep the sample as close to anoxic conditions as possible. Samples were transferred to the laboratory within 10 min of collection, where they were immediately suspended in anaerobic liquid medium (rumen-fluid-cellobiose [RFC] medium [Calkins et al. 2016], where cellobiose was replaced with cellulose or a mixture of switchgrass and cellobiose) and serially diluted. Dilutions were incubated at 39º C for 3 d until gaseous bubbles were visible and the plant material was visibly clumped and floated to the surface of the liquid phase in the tubes. From these enrichments, roll tubes (Hungate 1969) were prepared using RFC medium solidified with 2% agar and incubated at 39º C for 2–3 d, after which visible colonies were picked and transferred into liquid RFC medium. This cycle of roll tube preparation and single colony transfer into liquid medium was repeated three times to ensure culture purity.

2. Microscopic observation. Both light and scanning electron microscopy were utilized to observe various life stages of cultures grown in liquid as well as on solid (solidified with 2% agar) RFC medium. For light microscopy, samples were examined after staining with lactophenol cotton blue. To visualize nuclei, samples were incubated for 10 min at room temperature in the dark with the DNA-binding dye 4′,6′-diamidino-2- phenylindole (DAPI) (final concentration of 10 µg/ mL). Excess DAPI was then rinsed off, and the slides were air dried in the dark and then mounted in 4% n-propylgallate in 90% (v/v) glycerol in phosphate- buffered saline (PBS; Davis et al. 2009). All light microscopy examinations were conducted using an Olympus BX51 microscope (Olympus, Center Valley, Pennsylvania) equipped with a Brightline DAPI high- contrast filter set for DAPI fluorescence. Photomicrographs were taken with a DP71 digital camera (Olympus). Measurements of zoospore size and number of flagella were conducted on at least 50 different zoospores. For scanning electron microscopy, samples were fixed using 2% (w/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.0) for 2 h at room

temperature, followed by three successive 15-min washes in a 60 mM sodium cacodylate–180 mM sucrose wash buffer. Samples were then fixed with 1% (w/v) osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 h and then dehydrated in an aqueous ethanol series (50%, 70%, 90%, 95%, and 100% [×3; v/ v] ethanol, 15 min each), and dried by washing in hexamethyldisilazane (×2; 5 min each). Samples were then mounted on stubs, sputter coated with Au-Pb, and examined on a FEI Quanta 600 scanning electron microscope (Hillsboro, Oregon).

3. **Substrate utilizatio**n. Basal medium (RFC medium with no carbon source) was used to test substrate utilization capabilities. Various substrates of different complexities replaced the cellobiose in RFC medium at a final concentration of 0.5% (w/v). These included monomers (arabinose, fructose, fucose, galactose, glucose, glucuronic acid, mannose, ribose, and xylose), dimers (cellobiose, lactose, maltose, sucrose, and trehalose), and polymers (alginate, cellulose, chitin, inulin, pectin, peptone, polygalacturonate, raffinose, starch, tryptone, and xylan). All the above-tested carbon sources were added from a sterile, anoxic 10× stock. In addition, alkali-treated plant materials, including alfalfa, corn stover, energy cane, mixed prairie grass, sorghum, and switchgrass, were also used as the sole carbon and energy sources at a final concentration of 0.5% (w/v). Growth was deemed positive using the specified carbon source when a substrate successfully sustained culture viability through four consecutive subculturing events, as previously suggested (Breton et al. 1990). All results were compared with substrate-unamended control. When using alkali-pretreated plant material as the carbon source, the biomass remaining at the end of incubation was centrifuged and dried overnight at 40º C. Dry weight remaining (DW_f) was used to calculate percentage dry weight loss using the equation: % dry weight loss = $(DW_0 - DW_f)/DW_0 \times 100$, where DW_0 is the starting dry weight of plant biomass.

4. **Enzyme assays.** All enzymatic activities were conducted on both cell pellet and cell-free supernatant of strains C1A and S4B cultures grown on alkali- pretreated corn stover, and also on cell pellet and cell- free supernatant of strain S4B grown on alkali- pretreated alfalfa, energy cane, mixed prairie grass, sorghum, and switchgrass. Cultures were incubated at 39º C for 5 d, after which the cell-free supernatant was separated from the cell pellet by centrifugation. The cell pellet fraction was lysed by crushing in a sterile

mortar upon submersion in liquid nitrogen, and the lysate was used for total protein extraction using Tris-glycine extraction buffer (Tris base, 3 g/L; glycine, 14.4 g/L; pH 8.3). Cell-free supernatant was used directly for enzyme assays without extraction. Total protein concentration in both fractions was measured using the Qubit protein assay kit (Life Technologies, Carlsbad, California). Activities for six core enzymes required for cellulose (endoglucanase, exoglucanase, cellobiohydrolase, and β-glucosidase) and xylan (xylanase and β-xylosidase) deconstruction were measured in both fractions. Endoglucanase, exoglucanase, and xylanase activities were determined using a DNS (3,5-dinitrosalicyclic acid)-based assay (Breuil and Saddler 1985) employing carboxymethyl cellulose sodium salt (CMC; 1.25% w/v), avicel microcrystalline cellulose (1.25% w/v), and beechwood xylan (1.25% w/v), respectively, as substrates. These assays were conducted for 2 h in a sodium acetate buffer (100 mM). Cellobiohydrolase and β-xylosidase activities were assayed using 10 mM p-nitrophenyl-β-D- cellobioside (PNPC) and p-nitrophenyl-β-D- xylopyranoside (PNPX), respectively, as a substrate (Kubicek 1982; Dashtban et al. 2010). The assay was conducted for 15 min in 50 mM sodium acetate buffer, with 1 M sodium carbonate as a stop reagent. β- Glucosidase activity was assayed using the β- Glucosidase Activity Assay Kit (Sigma-Aldrich, St. Louis, Missouri). All enzyme activities were reported as units/mg protein, where a unit is defined as the number of μmoles of product released per minute.

5. **Phylogenetic analysis and ecological distribution**. DNA was extracted from cellobiose-grown cultures using a modified CTAB (cetyl trimethylammonium bromide) procedure as described previously (Youssef et al. 2013) and used as a template for amplifying partial nuc rDNA ITS1 region (hereafter ITS1) using the modified MN100 and MNGM2 primers as described before (Liggenstoffer et al. 2010), as well as the D1/D2 region of the nuc 28S rRNA (hereafter 28S rRNA) using NL1 and NL4 primers as described previously (Dagar et al. 2011). Polymerase chain reaction (PCR) reactions were examined by gel electrophoresis (Osterman 1984), and products were purified using PureLink PCR Purification Kit (ThermoFisher Scientific, Waltham, Massachusetts) according to manufacturer's instructions and Sanger- sequenced using the services of Eurofins MWG Operon, LLC (Louisville, Kentucky).

Sequences were aligned with ITS1 and 28S rRNA sequences reported from AGF isolates

using ClustalW, and the alignment was manually curated in MEGA6 (Tamura et al. 2013). Trees were constructed in MEGA6 using a maximum likelihood approach with *Chytriomyces* sp. WB235A isolate AFTOL-ID 1536 as the outgroup (accession numbers DQ536498.1 for ITS1 and DQ536493.1 for the 28S rRNA). Bootstrap values were calculated based on 100 replicates.

To evaluate the ecological distribution of this novel line- age, we queried the ITS1 sequences of both strain C1A and strain S4B against the GenBank nr (nonredundant) data- base using BLASTn and modified the output to display 5000 instead of the default 100 aligned sequences. Since the lowest ITS1 sequence pairwise similarity between all four isolates was 94%, we used this cutoff to filter the BLASTn output and considered those sequences with similarity ≥94% to either strains to be potential uncultured representatives of this novel genus. Sequences were truncated to the ITS1 amplicon size, as opposed to the entire ITS1, and inserted into pure culture isolates tree where their phylogenetic positions were evaluated. The ecological distribution (animal and geographical location) of sequences that remained reproducibly affiliated with the clade with a boot- strap support >50% was evaluated.

6. Data and culture accession. ITS1 partial sequences are deposited in GenBank under accession numbers KX961615, KX961613, and KX961614 for strains S4B, S4E, and S4F, respectively. 28S rRNA partial sequences are deposited in GenBank under accession numbers KX961618, KX961616, and KX961617 for strains S4B, S4E, and S4F, respectively. Strain C1A genome was previously sequenced (Youssef et al. 2013), and the nuc rDNA ITS1-5.8S-ITS2 region (partial ITS1, partial ITS2) are under GenBank accession number JN943056.1, whereas the nuc 28S rRNA gene partial sequence is under GenBank accession number JN939127.1. Alignments were deposited in TreeBASE under study accession URL: http://purl.org/phylo/treebase/phylows/study/TB2:S20067. The cultures are stored in our collection on agar medium according to the procedure described by (Calkins et al. 2016).

Results

1. Isolation. Four isolates (C1A, S4B, S4E, and S4F) were obtained from fecal material of cow and sheep. Two of these isolates (C1A and S4B) were chosen for in-depth characterization, with strain C1A chosen as the ex-type strain and described below. When applicable, differences between the two strains are noted.

2. Microscopic characteristics.

Zoospores. Microscopic examination revealed that zoospores of both strains were spherical, with an average diameter (± SD) of 7.5 ± 1.5 μm (strain C1A; n = 55, range: 3.8–10.5 μm) to 7.7 ± 1.5 μm (strain S4B; n = 110, range: 5.5–12.5 μm). The majority of the examined zoospores were monoflagellated, with an average flagellum length (± SD) ranging from 17 ± 3.2 μm (for strain C1A; n = 60, range: 11.5–27 μm) to 19.5 ± 4.6 μm (for strain S4B; n = 120, range: 14.5–36.0 μm) (FIG. 1-1A– B). Approximately 6–9% of the examined zoospores were biflagellated (FIG. 1-1C).

Zoospore encystment and germination. Zoospore encystment was associated with flagellar shedding. The discarded flagella exhibited a bead-like structure at the former point of attachment (FIG. 1-1D). Zoospore cysts germinated to produce a germ tube (FIG. 1-1E) that formed a branched rhizoidal system that emerged from one (FIG. 1-1J) or two (FIG. 1-1K) points on the developing sporangium. No nuclei were observed within the rhizoids (FIG. 1-1F–I), and no intercalary rhizoidal swellings or rhizoidal constrictions were observed.

Sporangial structures. Both endogenous and exogenous germination were observed, the former due to expansion of the zoospore cysts (where the zoospore cyst enlarges into a zoosporangium with rhizoidal growth originating from one side and no sporangiophore forming on the other side). These varied widely in size, with the smaller endogenous sporangia mainly subglobose (7–20 μm diam.) (FIG. 1-1J) and large endogenous sporangia ovoid (65– 140 μm L × 45 μm W) (FIG. 1-1L) in shape.

Exogenous sporangial formation was also observed, following bipolar germination of the zoospore cyst, with rhizoids developing on one side and a wider out- growth later differentiating into a sporangiophore on the opposite side. Young exogenous sporangia were mainly spherical (10–60 μm diam.) (FIG. 1-1M) or ovoid (10–50 μm L × 7–30 μm W) (FIG. 1-1N).

Mature exogenous sporangia had a size range of 40–155 μm L × 20–50 μm W and varied in shape, with spherical (FIG. 1-1O), ovoid (FIG. 1-1P), pyriform (FIG. 1-1Q), and ellipsoid (FIG. 1-1R) forms observed. The latter two shapes were frequently encountered in strain S4B but rarely observed in strain C1A cultures. Maturation of exogenous ellipsoid sporangia was frequently associated with a single (FIG. 1-1R) or double (FIG. 1-1S) constriction at their middle section. Such constrictions were more frequently encountered in strain S4B but were rarely observed in strain C1A. Liberation of zoospores occurred through a wide apical pore, with the sporangial wall staying intact after zoospore discharge (FIG. 1-1T–U).

Sporangiophores. Exogenous sporangia were typically observed at the end of unbranched sporangiophores ranging in length from 5 to 150 μm. Short sporangiophores frequently (FIG. 1-1V), but not always (FIG. 1-1W), displayed an eggcup-like (14–70 μm L × 14 –27 μm W) morphology. Within longer sporangiophores, three distinct morphologies were observed: sporangiophores ending in an eggcup-shaped structure (observed only in S4B cultures) (FIG. 1-1P), sporangiophores ending in subsporangial swelling (apophysis), encountered frequently in strain S4B and occasionally observed in strain C1A cultures (FIG. 1-1X), and naked sporangiophores without constrictions or swellings (FIG. 1-1Y).

3. Macroscopic growth characteristics. Strains C1A and S4B exhibited a smooth biofilm-like growth in cellobiose-containing liquid medium (FIG. 1-2 A–B) and formed small pinpoint circular colonies (0.5–1 mm diam.) on agar roll tubes (FIG. 1-2 C).

4. Substrate utilization. In cases where the same substrate was tested on both strains, isolates C1A and S4B displayed similar substrate utilization pattern (TABLE 1-1). The polymers cellulose, inulin, polygalacturonate, raffinose, starch, and xylan all supported growth as the sole carbon and energy sources, whereas alginate, chitin, pectin, peptone, and tryptone failed to support growth. Out of the dimers tested, cellobiose, maltose, sucrose, trehalose, but not lactose, supported growth. The majority of monomers tested supported growth of both strains. These included fucose, fructose, glucose, mannose, and xylose. However, arabinose, galactose, glucuronic acid, and ribose were not utilized by either strain. In addition to the above substrates, strains C1A and S4B were capable of utilizing complex alkali-pretreated plant material, including alfalfa, corn stover, energy

cane, mixed prairie grass, sorghum, and switchgrass as the sole carbon source. The process was accompanied with 30–50% dry weight loss after 5 d of incubation (FIG. 1-3A).

5. Enzymatic activities. When grown on alkali- pretreated corn stover, both isolates displayed considerable cellulolytic and xylanolytic enzymatic activities in cell-free as well as the cell-associated fractions (FIG. 1-3B). The nature of the plant material used as the carbon source had a noticeable effect on the total enzymatic activities detected in the cell-free and cell-associated fractions (TABLE 1-2).

6. Phylogenetic analysis and ecological distribution. Phylogenetic analysis using the 28S rRNA gene reproducibly grouped strains C1A and S4B along with the two other isolates (S4E and S4F) into a single, monophyletic, and bootstrap-supported sister clade to the genus *Orpinomyces*, regardless of the tree-building algorithm and the taxa included in the analysis (FIG. 1-4). Strains S4B, S4E, and S4F had identical 28S rRNA sequences, whereas strain C1A displayed 0.54% 28S rRNA sequence divergence from the other three strains. Using the amplified ITS1 sequence, the above four isolates grouped together with a strong bootstrap support, albeit with less clear delineation of their relationship to other groups (*Orpinomyces, Neocallimastix, Cyllamyces, Ceacomyces,* and *Piromyces*) in the tree (FIG. 1-5). Strains S4E and S4F displayed 98.6% ITS1 sequence similarity and were 97.8%, and 96.8%, respectively, similar to strain S4B ITS1 amplified sequence. On the other hand, the ITS1 sequence of strain C1A was 94% similar to the amplified ITS1 sequences of isolates S4B and S4E, and 94.9% similar to that of isolate S4F.

In an attempt to identify the ecological distribution of members of this novel AGF clade, we queried publicly available culture-independent ITS1 sequences in the nr database using the amplified ITS1 sequence of isolates C1A and S4B. A total of 1090 sequences were identified as potentially affiliated with this novel clade with strong bootstrap support regardless of the tree-building algorithm and the taxa included in the analysis (FIG. 1-5). These sequences originated from five different studies, including three published (Tuckwell et al. 2005; Liggenstoffer et al. 2010; Herrera et al. 2011) and two unpublished studies (GenBank popset accession numbers 443910245 and 411146349). Thus, similar sequences were observed from three continents (North America, Australia, and Asia), and from fecal or rumen samples of 11 different animals (cow, yak, bactrian camel, llama,

pygmy hippo, sable antelope, sheep, sika deer, southern gerenuk, America bison, and bontebok). This attests to the widespread distribution of this novel clade. Interestingly, these sequences were only identified in ruminant (cow, yak, sable antelope, sheep, sika deer, southern gerenuk, America bison, and bontebok) and pseudoruminant (llama, pygmy hippo, and camel) foregut, but not in hindgut-fermenting hosts.

TAXONOMY

Pecoramyces Radwa Hanafy, Noha Youssef, G.W. Griff. & Mostafa Elshahed., gen. nov. *Index Fungorum registration:* IF552530

Typification: Pecoramyces ruminantium Radwa Hanafy, Noha Youssef, G.W. Griff. & Mostafa Elshahed (holotype).

Etymology: Pecora = derived from the Latin word for horned livestock (also the name of the infraorder Pecora within the suborder Ruminantia, which comprises even-toed hoofed mammals with ruminant digestion); myces = the Greek name for fungus. Obligately anaerobic fungus with determinate, monocentric thallus with single terminal sporangium (variable in shape, including spherical, ovoid, ellipsoid, and pyriform and sometimes bearing constrictions) and forming mainly monoflagellate zoospores. Zoospores may germinate either endogenously or exogenously. The clade is defined by the sequences JN943056/KX961613 (ITS1, 5.8S, ITS2 complete) and also JN939127/KX961616 (28S rRNA, partial sequence; D1/D2 regions). The most genetically similar genus is *Orpinomyces*, which is defined as forming a polycentric thallus (Barr et al. 1989), bearing multiple sporangia that release polyflagellated zoospores. This contrasts with the monocentric growth of *Pecoramyces* and the monoflagellated zoospores, which it produces. *Pecoramyces ruminantium* Radwa Hanafy, Noha Youssef, G.W. Griff. & Mostafa Elshahed., sp. nov. FIG. 1-1A–Y

Index Fungorum registration: IF552531

Typification: U.S.A. OKLAHOMA: Stillwater, 36.12′N, 97.06′W, ~300 m above sea level, isolated from the freshly deposited feces of an Angus steer (*Bos taurus*), collected in Sep 2009 by Audra Liggenstoffer. Holotype material from culture of isolate C1A (3-d-old cultures, killed and preserved in 5% glutaraldehyde) is lodged in the Department of Microbiology and Molecular Genetics, Oklahoma State University, with isotype material

at the Aberystwyth University biorepository (code ABS).

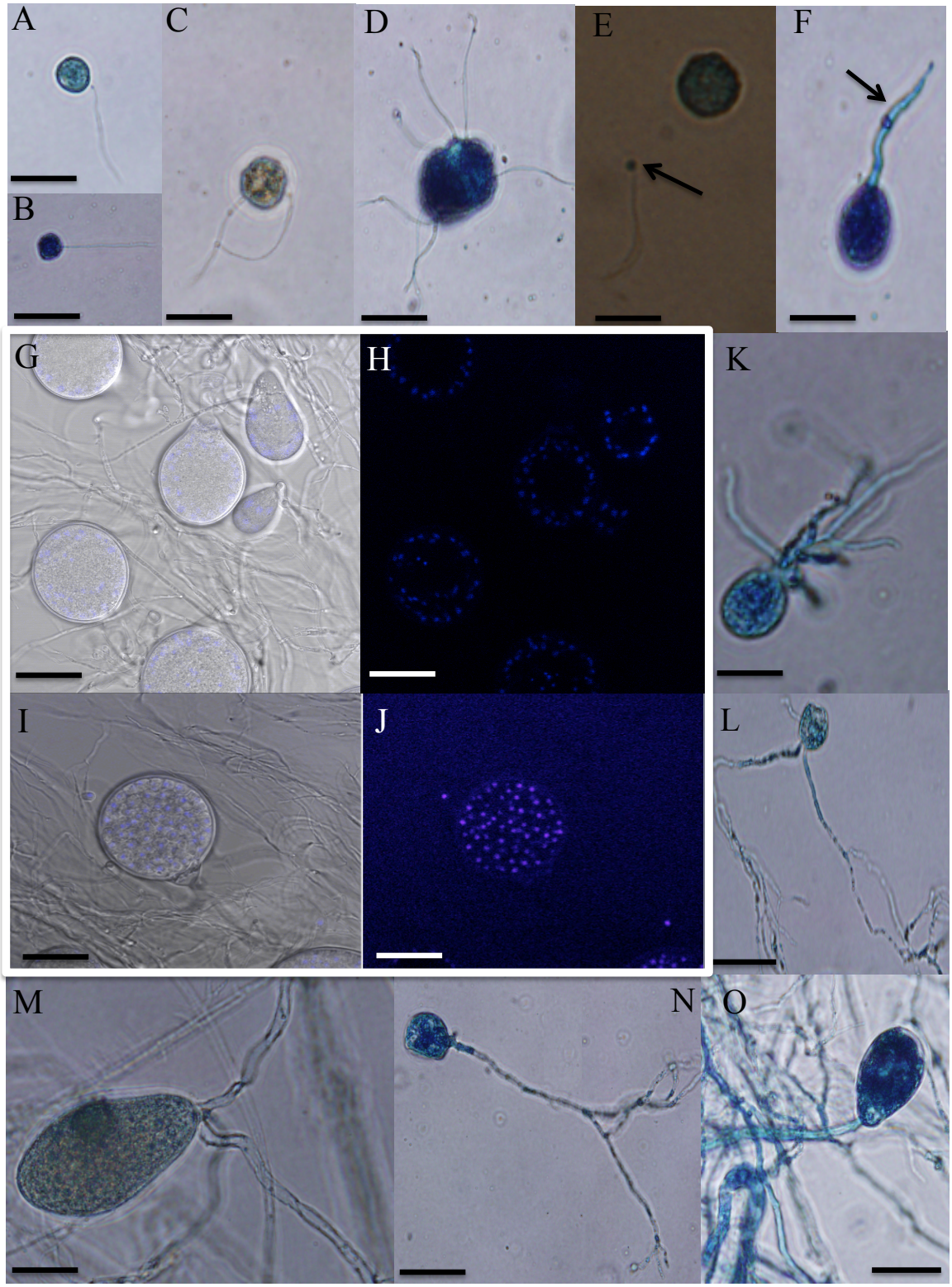
Ex-type culture C1A type culture (isolate C1A) is stored cryogenically in liquid nitrogen at Aberystwyth University. GenBank accession no. JN943056 (ITS1, 5.8S, ITS2 complete) and JN939127 (28S rRNA, partial sequence; D1/D2 regions).

Etymology: The species epithet reflects the fact that this fungus appears to be specific to ruminant/pseudoruminant hosts and absent in hindgut-fermenting mammals.

An obligately anaerobic fungus with determinate monocentric thallus and single terminal sporangium. Extensive anucleate rhizoidal system, lacking rhizoidal swellings or constrictions. Mature sporangia ovoid, spherical, ellipsoid, or pyriform (40–155 μm L × 20–50 μm W), nonpapillate. Sporangiophores unbranched, variable in length (5–150 μm), often forming an apophysis-like or eggcup-like swelling below the sporangium. Zoospores formed abundantly, spherical (3.8–12.5 μm diam.) with single flagellum (15–36 μm long). Occasional (6–9%) biflagellate zoospores are formed. Colonies grown on cellobiose exhibit a smooth biofilm-like growth and form small pinpoint circular colonies (<1 mm diam.) on agar roll tubes. The clade is defined by the sequences JN943056/KX961613 (ITS1, 5.8S, ITS2 complete), and also JN939127/KX961616 (28S rRNA, partial sequence; D1/D2 regions).

Additional specimens examined: U.S.A. OKLAHOMA: Stillwater, 36.12′N, 97.06′W at ~300 m above sea level, isolated from the freshly deposited feces of sheep (*Ovis aries*), in Sep 2015 by Radwa Hanafy. These cultures are named S4B, S4E, and S4F.

Figure 1-1. Microscopic features of *Pecoramyces ruminantium* ex-type strain C1A (unless otherwise indicated). Both light microscopy (A–Q, S–T, and V–W) and scanning electron microscopy (R, U, X, and Y) pictures are shown. Light microscopy pictures were examined after staining with lactophenol cotton blue (A–E, J–Q, S–T, and V–W), as well as following nuclei staining with DAPI and visualizing using a fluorescence microscope equipped with a Brightline DAPI high-contrast filter set (G, I). Overlay images are shown in F and H. A–B. Monoflagellated zoospore of strains C1A (A) and S4B (B). C. Biflagellated zoospore. D. Zoospore cyst. Arrow points to the shed flagellum with bead-like structure. E. Germinating zoospore cyst producing a germ tube (arrow). F–I. Monocentric thallus; nuclei were observed in sporangium, not in rhizoids or sporangiophore. J. Young subglobose endogenous sporangium with one main rhizoidal system. K. Rhizoidal system with two main rhizoids. L. A mature ovoid endogenous sporangium. M. A young spherical exogenous sporangium. N. A young ovoid exogenous sporangium. O. A mature spherical exogenous sporangium on a short sporangiophore (strain S4B). P. A mature ovoid exogenous sporangium on a long sporangiophore ending in an eggcup-like structure (strain S4B). Q. A mature pyriform exogenous sporangium. R. A mature ellipsoid exogenous sporangium of strain S4B with a single constriction at the middle (arrow). S. A mature ellipsoid exogenous sporangium of strain S4B with two constrictions (arrows). T–U. An empty sporangium with one remaining zoospore after zoospore release through a wide apical pore (arrows). V. A spherical exogenous sporangium of strain S4B on a short sporangiophore appearing like an eggcup. W. An ovoid exogenous sporangium on a short sporangiophore. X. A long sporangiophore of strain S4B ending in a subsporangial swelling, apophysis (arrow). Y. A long naked sporangiophore without constrictions or swellings. All scale bars = 20 μm.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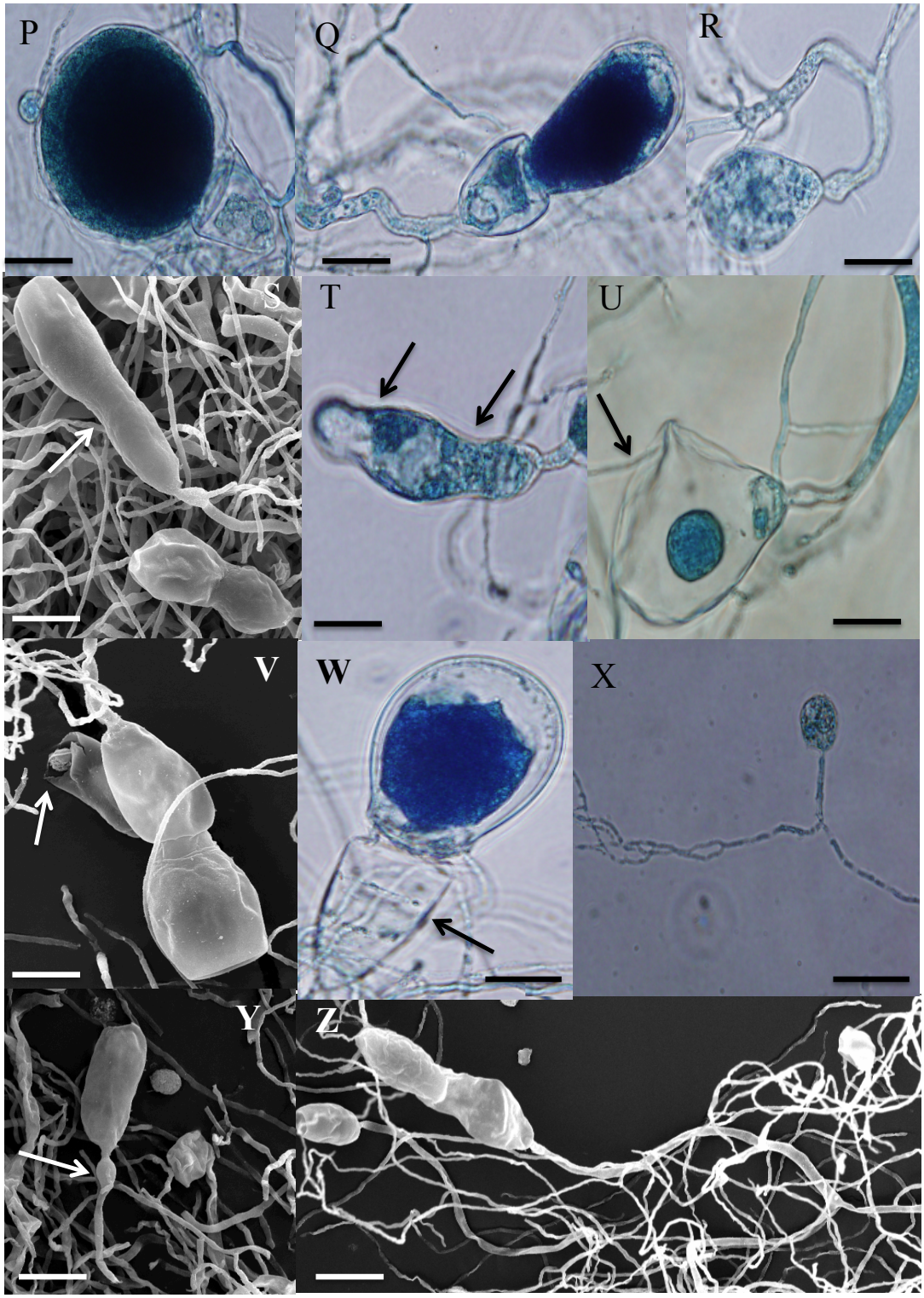
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Figure 1-2. Macroscopic and microscopic features, and phylogenetic relatedness of *Pecoramyces ruminatum* strain S4B and *Orpinomyces* sp. D4A from our culture collection. **Panel A:** Macroscopic features of (left) *Pecoramyces ruminatum* strain S4B and (right) *Orpinomyces* sp. D4A in liquid culture and on agar roll tubes. **Panel B:** Microscopic features of *Orpinomyces* sp. D4A showing (left) thick rhizoidal growth, (middle) spherical sporangia, and (right) DAPI-stained nuclei detected in the rhizoids of attesting to the production of polycentric thallus. For comparison please refer to Figure 1 for *Pecoramyces ruminatum* strain S4B microscopic features. **Panel C:** Phylogenetic relatedness of the genus *Pecoramyces* (represented by strains S4B and C1A) as a sister group to the genus *Orpinomyces* (represented by several isolates including four *Orpinomyces* strains from our culture collection (D4A, D4C, D3A, and D3B) based on the sequences of the rRNA LSU gene. The tree was obtained using a maximum likelihood approach with *Chytriomyces* sp. WB235A isolate AFTOL-ID 1536 as an outgroup (not shown). Bootstrap values (from 100 replicates) are shown for nodes with more than 50 bootstrap support. Analysis was conducted in Mega 6.

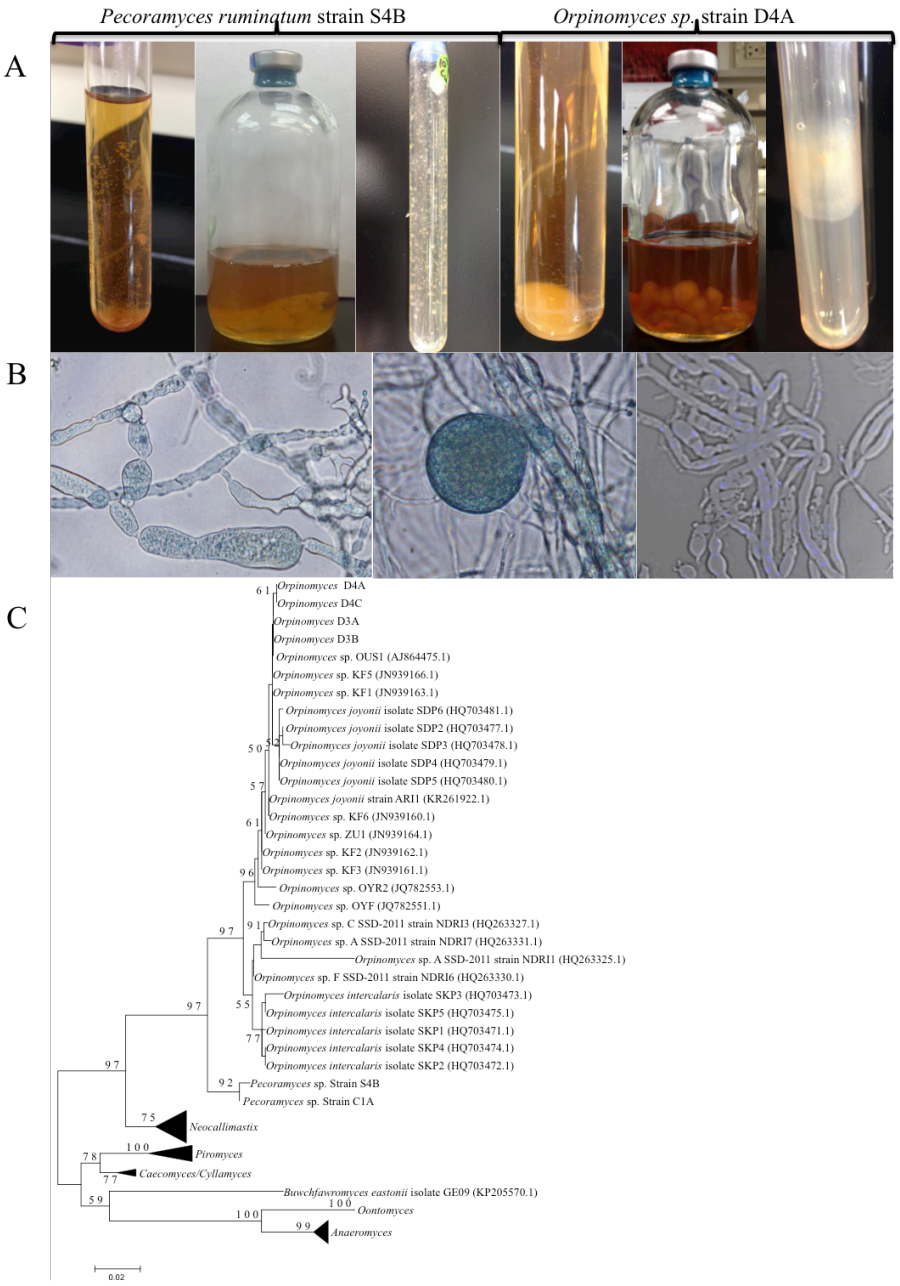

Pecoramyces ruminatum strain S4B
Orpinomyces sp. strain D4A
A
B
C
Orpinomyces D4A
Orpinomyces D4C
Orpinomyces D3A
Orpinomyces D3B
Orpinomyces sp. OUS1 (AJ864475.1)
Orpinomyces sp. KF5 (JN939166.1)
Orpinomyces sp. KF1 (JN939163.1)
Orpinomyces joyonii isolate SDP6 (HQ703481.1)
Orpinomyces joyonii isolate SDP2 (HQ703477.1)
Orpinomyces joyonii isolate SDP3 (HQ703478.1)
Orpinomyces joyonii isolate SDP4 (HQ703479.1)
Orpinomyces joyonii isolate SDP5 (HQ703480.1)
Orpinomyces joyonii strain ARI1 (KR261922.1)
Orpinomyces sp. KF6 (JN939160.1)
Orpinomyces sp. ZU1 (JN939164.1)
Orpinomyces sp. KF2 (JN939162.1)
Orpinomyces sp. KF3 (JN939161.1)
Orpinomyces sp. OYR2 (JQ782553.1)
Orpinomyces sp. OYF (JQ782551.1)
Orpinomyces sp. C SSD-2011 strain NDRI3 (HQ263327.1)
Orpinomyces sp. A SSD-2011 strain NDRI7 (HQ263331.1)
Orpinomyces sp. A SSD-2011 strain NDRI1 (HQ263325.1)
Orpinomyces sp. F SSD-2011 strain NDRI6 (HQ263330.1)
Orpinomyces intercalaris isolate SKP3 (HQ703473.1)
Orpinomyces intercalaris isolate SKP5 (HQ703475.1)
Orpinomyces intercalaris isolate SKP1 (HQ703471.1)
Orpinomyces intercalaris isolate SKP4 (HQ703474.1)
Orpinomyces intercalaris isolate SKP2 (HQ703472.1)
Pecoramyces sp. Strain S4B
Pecoramyces sp. Strain C1A
Neocallimastix
Piromyces
Caecomyces/Cyllamyces
Buwchfawromyces eastonii isolate GE09 (KP205570.1)
Oontomyces
Anaeromyces
0.02

Table 1-1. Substrate utilization pattern for *Pecoramyces ruminantium* strains C1A and S4B.

Substrate		Substrate utilization pattern
Polysaccharides	Cellulose	++
	Xylan	++
	Starch	++
	Inulin	++
	Raffinose	++
	Polygalacturonate	+
	Chitin	-
	Alginate	-
	Pectin	-
Disaccharides	Cellobiose	++
	Sucrose	++
	Maltose	++
	Trehalose	+
	Lactose	-
Monosaccharides	Glucose	++
	Xylose	++
	Mannose	++
	Fructose	++
	Fucose	+
	Arabinose	-
	Ribose	-
	Glucuronic acid	-
	Galactose	-
Peptides	Peptone	-
	Tryptone	-
Alkali-treated plant material	Switchgrass	++
	Corn stover	++
	Alfalfa	++
	Sorghum	++
	Energy cane	++
	Mixed prairie grass	++

Growth patterns are shown for strains C1A and S4B when the substrates shown in column 1 were used as the sole carbon and energy source. Positive results are reported after four subcultures on the substrate, where '++', denotes excellent growth with biofilm formation; '+', denotes weak growth; '-', denotes no growth was observed.

Figure 1-3. (A) Percentage plant dry weight loss when strains C1A and S4B were grown on alkali-pretreated plant biomass as the sole carbon and energy source. (B) Cellulolytic (endoglucanase, exoglucanase, cellobiohydrolase, and β-glucosidase) and xylanolytic (xylanase, and β-xylosidase) enzyme activities in the cell-free (supernatant), as well as the cell-associated (pellet) fractions when strains C1A and S4B were grown on alkali-pretreated corn stover. Enzyme activities are shown in U/mg protein where 1U is defined as 1 µmole of product released per minute. Error bars are standard deviations from at least 3 measurements from at least two biological replicates.

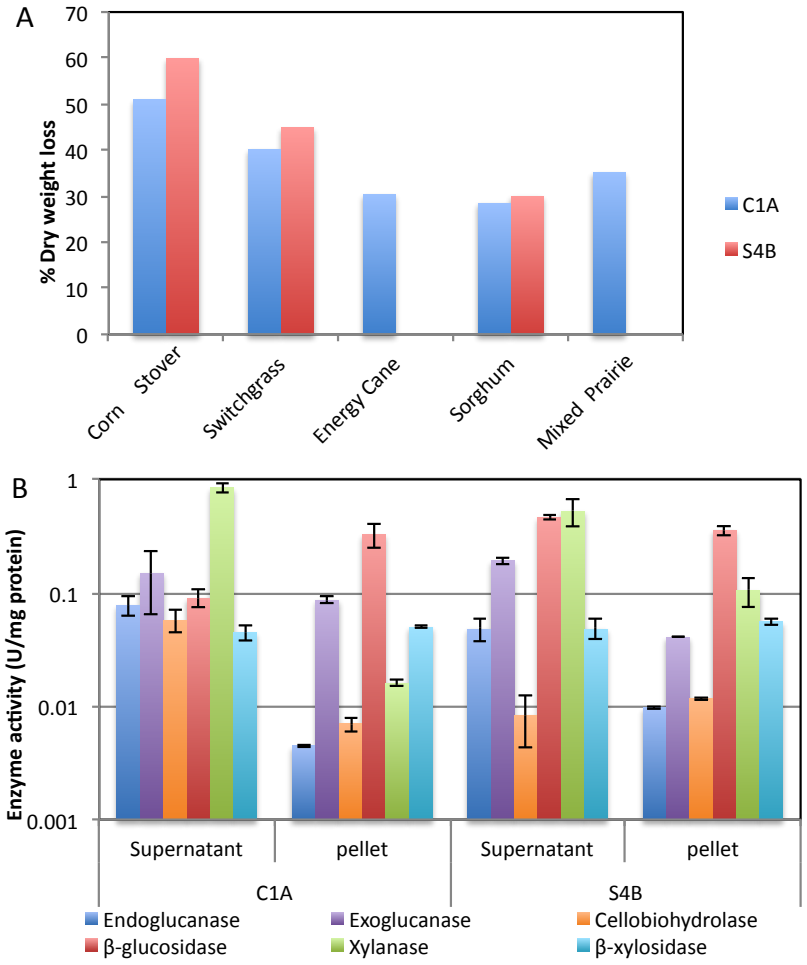


Table 1-2. Total enzymatic activity detected when *Pecoramyces ruminatum* strain S4B was grown on different alkali-pretreated plant material as opposed to alkali-pretreated corn stover.

Enzymatic activity detected	Alkali-pretreated plant material used[a]				
	Switch grass	Sorghum	Alfalfa	Sugar cane	Mixed Prairie
Endoglucanase	1.11	**3.21**	1.41	2.38	0.4
Exoglucanase	0.8	**3.66**	1.17	0.8	0.66
Cellobiohydrolase	**2**	1.13	1.30	1	1.33
β-glucosidase	1.79	**2.08**	1.19	0.63	1.71
Xylanase	**1.99**	1.41	1.04	0.69	1.21
β-xylosidase	**1.50**	0.81	0.44	0.69	0.63

a: Numbers are the fold differences in the total enzymatic activity (in the cell-pellet as well as the cell-free fractions) indicated in the first column when strain S4B was grown on alkali-pretreated plant material shown in the table header as opposed to alkali-pretreated corn stover. Highest activities are shown in boldface.

Figure 1-4. Phylogenetic affiliation of the *Pecoramyces* clade to other AGF genera based on the sequences of the 28 rRNA gene. The tree was obtained using a maximum likelihood approach with *Chytriomyces* sp. WB235A isolate AFTOL-ID 1536 as an outgroup (not shown). Bootstrap values (from 100 replicates) are shown for nodes with more than 50% bootstrap support. Analysis was conducted in MEGA6.

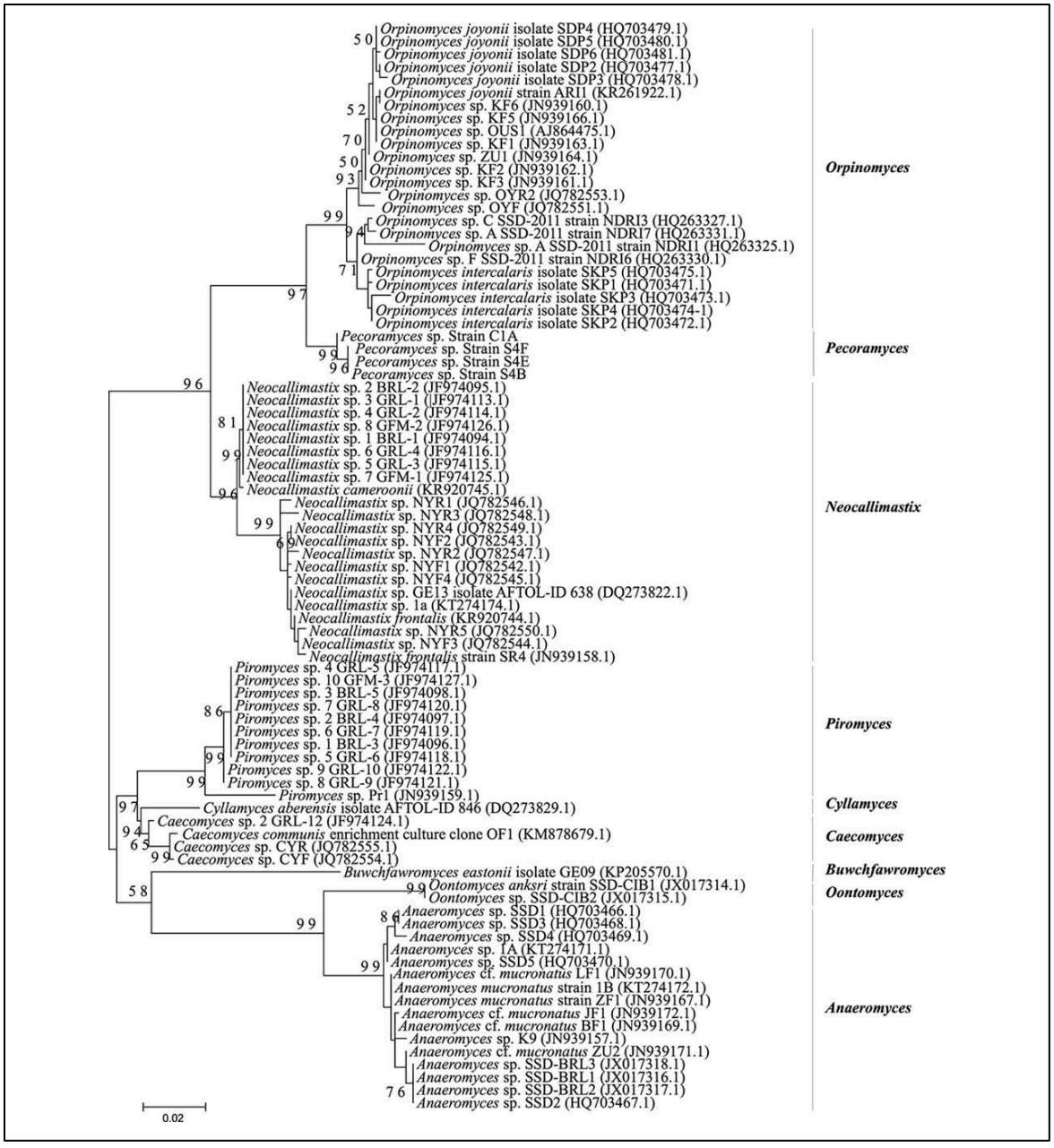
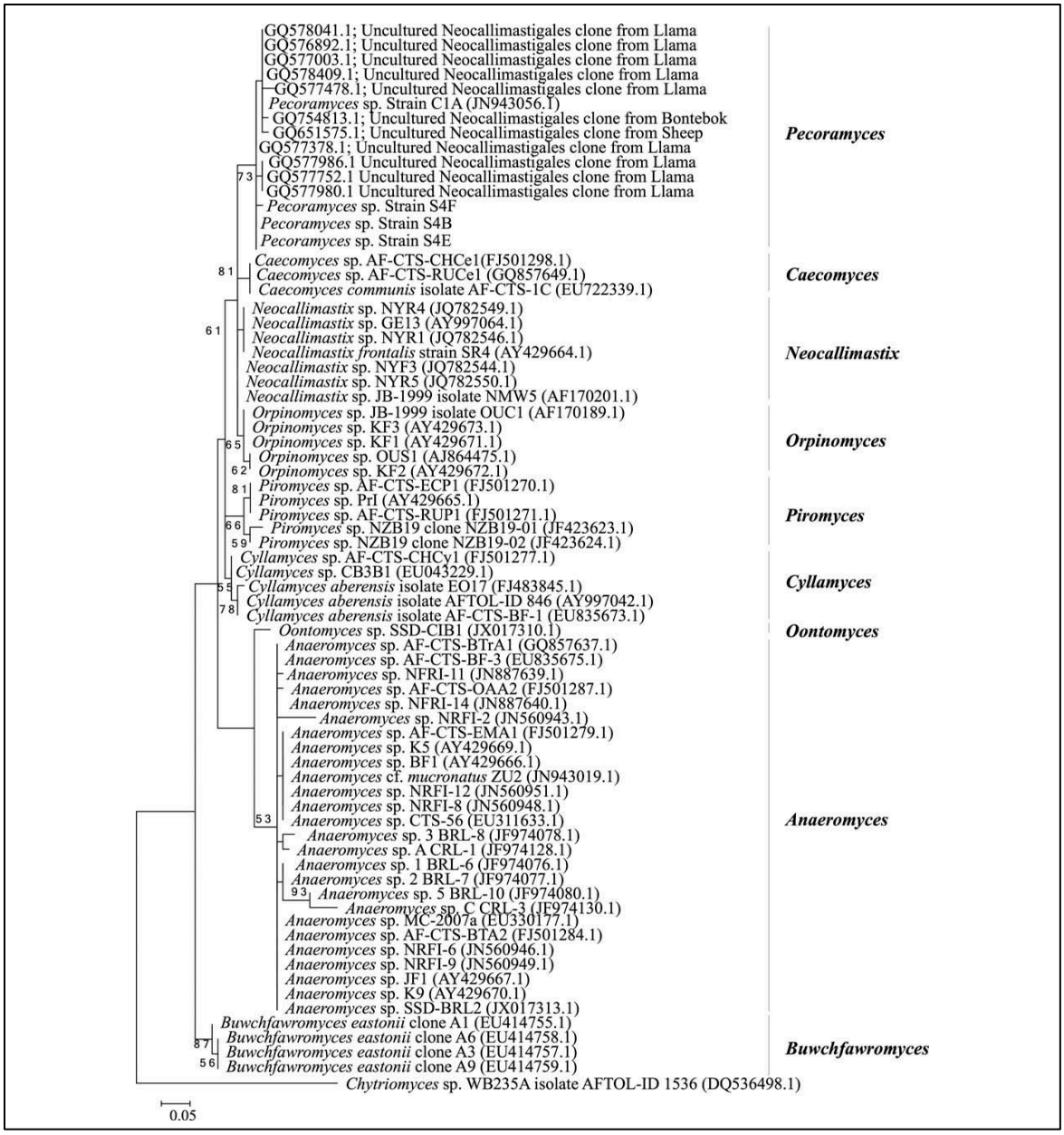


Figure 1-5. Phylogenetic affiliation of the *Pecoramyces* clade to other AGF genera based on partial ITS1 sequences of the rRNA locus. Eleven clones representing the 1090

sequences identified through BLASTn analysis to be potential members of the clade are added to the tree. The tree was obtained using a maximum likelihood approach with *Chytriomyces* sp. WB235A isolate AFTOL-ID 1536 as an outgroup. Bootstrap values (from 100 replicates) are shown for nodes with more than 50 bootstrap support. Analysis was conducted in MEGA6.



Discussion

The four isolates reported in this study formed a clearly monophyletic sister clade

to the genus Orpinomyces (FIG. 1-4, FIG. 1-2C). In addition, key morphological and microscopic differences between these strains and described members of the genus Orpinomyces justify their placement in a novel genus (FIG. 1-1, FIG. 1-2). These differences include (i) the production of monocentric thalli (FIG. 1-1F–I) and monoflagellated zoospores (FIG. 1-1A–C) by strains C1A and S4B, both of which are quite different from the wide rhizomycelia (FIG. 1-2B), polycentric thalli (FIG. 1-2B), and polyflagellated zoospores (Barr et al. 1989; Ho and Barr 1995; Sridhar et al. 2010) that define the genus Orpinomyces; (ii) the exhibition of a smooth biofilm- like growth in cellobiose-containing liquid media and the formation of small pinpoint circular colonies on agar roll tubes (FIG. 1-2A, left) by strains C1A and S4B, both of which are distinct from the cottony growth and large (usually >1 cm diam.) colonies formed by *Orpinomyces* spp. (e.g., *O. joyonii*) (FIG. 1-2A, right). Therefore, based on the above key morphological characteristics that differentiate strains C1A and S4B from *Orpinomyces* isolates, as well as the consistent phylogenetic position in ribosomal RNA gene–based trees, we propose a new anaerobic fungal genus (*Pecoramyces*) to accommodate strains C1A, S4B, S4E, and S4F.

Genera *Orpinomyces* and *Neocallimastix* are characterized by the formation of zoospores with multiple (4– 20) posterior flagella, a unique feature within the super-kingdom Ophisthokonta (James et al. 2006; Callaghan et al. 2015). The occurrence of genera with monoflagellate zoospores within the AGF suggests that more than one event of gene gain and/or gene loss (leading to the switch to/from formation of polyflagellate zoospores) may have occurred during the evolution of AGF.

Morphological characteristics of strains C1A and S4B are similar to members of the genus *Piromyces*, previously defined to accommodate AGF isolates with monoflagellated zoospores and monocentric thalli (Gold et al. 1988; Li et al. 1990; Ho et al. 1993a, 1993b; Breton et al. 1991; Ho and Barr 1995). Other morphological characteristics of strains C1A and S4B with similarities to *Piromyces* include discarded flagella exhibiting bead-like structure at the former point of attachment (FIG. 1-1D), which has previously been reported for *Piromyces minutus* and *Piromyces spiralis* (Ho et al. 1993a, 1993b), and the presence of apophysis, a distinct subsporangial swelling,

previously reported in *Piromyces* spp., e.g., *P. spiralis* (Ho et al. 1993b), *P. mae* (Li et al. 1990), and *P. rhizinflata* (Breton et al. 1991). It is interesting to note that, in addition to strains C1A and S4B, the two recently described genera *Buwchfawromyces* and *Oontomyces* (Callaghan et al. 2015; Dagar et al. 2015) are also morphologically similar to *Piromyces* spp., with monocentric thalli and monoflagellated zoospores. Only sequence data, which placed *Pecoramyces* as a sister clade to the *Orpinomyces*, and *Buwchfawromyces* and *Oontomyces* as sister clades to the *Anaeromyces*, revealed their phylogenetic distinction from the *Piromyces*.

The description of the novel genera *Buwchfawromyces*, *Oontomyces*, and *Pecoramyces* highlights the current challenges in genus-level assignment of monocentric mono- flagellated AGF isolates. The genus *Piromyces* was described and validated based on extensive morphological characterization of multiple isolates obtained from disparate sources: *P. mae* (Li et al. 1990), *P. minutus* (Ho et al. 1993a), *P. spiralis* (Ho et al. 1993b), *P. rhizinflata* (Breton et al. 1991), and *P. communis* (Gold et al. 1988). These isolates were extensively characterized in terms of microscopic features and growth patterns. Unfortunately, no sequence data are available, to our knowledge, for these strains, and most of these original Piromyces species appear to be extinct and/or are not readily accessible to the scientific community. Phylogenetic analyses of additional strains putatively assigned to the genus *Piromyces* based on morphological similarities suggest that the genus *Piromyces* is polyphyletic (Brookman et al. 2000; Fliegerova et al. 2004; Gruninger et al. 2014). As such, the taxonomic boundaries of the genus *Piromyces* and criteria that constitute membership to this genus are currently unclear. We believe that the current uncertainty associated with nomenclature and taxonomy of monoflagellated monocentric AGF strains is an impediment to Neocallimastigomycota taxonomy, and that it is necessary to redefine and reassess the taxonomic position of extant and extinct strains formally or putatively assigned to the genus Piromyces as part of a broader framework to resolve the genus-level taxonomic relationships within the Neocallimastigomycota.

Strain C1A was isolated in Apr 2009 and has been viable through at least ~730 subcultures so far. Strain S4B was isolated in Sep 2015 and has been viable through at least 104 subcultures so far. Strains C1A and S4B are actively subcultured on a biweekly

basis. Both strains are also maintained on anoxic solid media at 39º C, where they are regularly revived every 3 months as described previously (Calkins et al. 2016). In addition, frozen cultures, DNA, and RNA samples are long-term stored at −80º C. The difficulty in maintaining AGF cultures and senescence issues have long hampered not only taxonomic assessment of historic specimens using sequence data, as out- lined above for the *Piromyces*, but also the overall progress in AGF comparative genomics, physiology, and structural comparisons. The recent description of multiple storage approaches (Joblin 1981; Callaghan et al. 2015; Calkins et al. 2016; Solomon et al. 2016b), some of which require no cryopreservation and/or glycerol stock preparation, should help towards higher accessibility for all isolates, and a concerted effort should be made towards this goal. The genome and transcriptome of a *Pecoramyces* representative (strain C1A) have previously been sequenced (Youssef et al. 2013; Couger et al. 2015). Recently, the genomes and transcriptomes of three other AGF representatives were also made available (Solomon et al. 2016a). These studies showed the large repertoire of lignocellulolytic enzymes that AGF possess. Improved accessibility of isolates to the anaerobic fungal research community will certainly facilitate future genomic and transcriptomic studies. Clues from the genomes (Youssef et al. 2013) would undoubtedly aid with resolving the phylogenetic position of this basal fungal phylum in the fungal tree of life.

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CHAPTER II

ANAEROMYCES CONTORTUS SP. NOV., A NEW ANAEROBIC GUT FUNGAL SPECIES (NEOCALLIMASTOGOMYCOTA) ISOLATED FROM THE FECES OF COW AND GOAT

Abstract

Ten different anaerobic gut fungal strains were isolated from fecal samples of cow and goat in Oklahoma, USA. The isolates displayed polycentric thalli, uniflagellated zoospores, and
sausage-shaped hyphal constrictions, all characteristic of the genus *Anaeromyces,* and lobed or appressoria-like structures on the hyphae, previously reported in *Anaeromyces elegans* but not in *A. mucronatus* or *A. robustus*. Further, the strains exhibited highly coiled and entangled hyphae previously unreported in the Neocallimastigomycota, but resembling fungal traps previously observed in multiple nematophagous fungi. Scanning electron micrographs of isolates grown on switchgrass show entrapment of plant material by the hyphal coils and the penetration of plant fibers at various sites by the appressoria-like structures. Molecular analysis based on sequences of both partial nuc rDNA ITS1 spacer region and the D1-D2 domains of the nuc28S rDNA confirmed the classification of all isolates in *Anaeromyces* and confirmed their phylogenetic distance from the available *A. mucronatus* and *A. robustus* sequences. On the basis of morphological and phylogenetic distinctions, we conclude that these strains represent a new *Anaeromyces* species, for which *A. contortus* is proposed.

Introduction

Anaerobic gut fungi (AGF; Phylum Neocallimastigomycota) are primarily encountered in the alimentary tract of mammalian and some reptilian herbivores (Liggenstoffer et al. 2010). Adaptation of AGF to such environments is reflected in their anaerobic physiology, and the possession of multiple plant cell wall degrading enzymes that aid the host in the digestion of forage materials (Gruninger et al. 2014). Anaerobic fungi are fibrolytic microorganisms, producing a wide array of cell-bound and cell-free cellulolytic, hemicellulolytic, glycolytic, and proteolytic enzymes (Ljungdahl 2008; Youssef et al. 2013; Couger et al. 2015). The Neocallimastigomycota play an important role in plant biomass degradation in the herbivorous gut. By attaching themselves to plant materials, they colonize and excrete extracellular enzymes that mobilize the structural plant polymers for the broader microbial community. With their complete battery of enzymes that attack almost all plant polymers, anaerobic fungi represent a promising resource that could be exploited for biofuel and biogas production from lignocellulosic biomass (Gruninger et al. 2014).

Based on variations in morphological and microscopic characteristics, as well as phylogenetic analyses conducted on the nuc rDNA internal transcribed spacer 1 (ITS1) of the ribosomal RNA locus and the D1-D2 region of the nuc28S rDNA (D1-D2 28S rDNA) sequences, 9 genera and 29 species have been described (Gruninger et al. 2014; Ariyawansa et al. 2015; Callaghan et al. 2015; Dagar et al. 2015; Li et al. 2016; Hanafy et al. 2017). *Anaeromyces* was proposed in 1990 for isolates from the rumen contents of a Holstein cow (Breton et al. 1990) and a Hereford steer (Ho et al. 1990). Multiple studies reported morphologically similar isolates from the rumen and feces of cattle, elk, deer, and bison (Akin and Rigsby 1987; Phillips and Gordon 1989; Barr et al. 1995; Fliegerova et al. 2004). Further, culture-independent ITS1-based diversity surveys clearly demonstrated the occurrence of members of this clade in a broad range of host species (Liggenstoffer et al. 2010; Wang et al.2017).

Species of *Anaeromyces* are characterized by their polycentric thalli, and uniflagellated zoospores. Currently, three species, *A. mucronatus*, *A. elegans,* and *A. robustus*, are described (Breton et al. 1990; Ho et al. 1990; Ho et al. 1993; Li et al. 2016). *Anaeromyces mucronatus* and *A. elegans* share common morphological characters

including fusiform sporangia with a mucronate apex, spherical uniflagellated zoospores and constricted, sausage-shaped hyphae. Morphologically, the two species can be distinguished based by the presence of lobed or beadlike structures on the hyphae of *A. elegans* but not *A. mucronatus* (Breton et al. 1990; Ho et al. 1990; Ho et al. 1993; Ho and Barr 1995). However, the absence of ITS1 and D1-D2 28S rDNA reference sequences for *A. elegans* hinder the differentiation between the two species at the molecular level. *Anaeromyces robustus* is unique in possessing club-shaped sporangia that occasionally fuse to form whale tail-like structures (Li et al. 2016).

Here, we report the isolation and characterization of multiple *Anaeromyces* strains from cow and goat fecal material obtained in Oklahoma, USA. We highlight the distinction between these isolates and currently described *Anaeromyces* species based on microscopic characteristics and phylogenetic analysis, and describe a novel *Anaeromyces* species for these isolates, for which the name *A. contortus* is proposed.

Materials and methods

Isolation procedure. Ten anaerobic fungal strains were obtained from fecal samples of cow (*Bos taurus*) and goat (*Capra aegagrus hircus*) raised in pastures around Stillwater, OK. Both freshly collected and frozen fecal materials were used for isolation (TABLE 2-1). The isolation process was performed as described by Hanafy et al. (2017) using a rumen fluid medium reduced by cysteine-sulfide and dispensed under a stream of 100% CO2, as previously described (Youssef et al. 2013). The medium was prepared using the anaerobic Hungate technique (Bryant 1972), as modified by Balch and Wolfe (1976), used for the isolation and culturing of strict anaerobes. Cellulose (0.5%) or a mixture of switchgrass (0.5%) and cellobiose (0.5%) were used as the carbon source.

Fecal samples were serially diluted and incubated at 39°C for 24–48 h. The roll tube technique (Hungate 1969), employed for isolating strict anaerobes from environmental samples, was used to isolate strains from dilutions with visible growth. Single colonies were picked from roll tubes and used to start a pure culture. For zoospore induction, strains were grown in cellulose sloppy media (by the addition of 0.1% w/v agar). as previously described (Ho and Bauchop 1991). The cultures are publicly available as part of the Oklahoma State University AGF isolates collection. They are stored on agar media at 39ºC on RFC-agar medium as previously described (Calkins et al. 2016).

Morphological observations. The microscopic characters of *Anaeromyces* isolates were examined using both light and electron microscopy. For light microscopy, fungal biomass was collected from 3–5 d old cultures grown on rumenfluid- cellobiose medium (RFC) (Calkins et al. 2016) or cellulose sloppy medium (0.1% w/v). For visualization of fungal structures including thalli, sporangia, zoospores and other microscopic characters, lactophenol cotton blue was used to stain samples. To examine nuclei localization, samples were stained with 4', 6'-diamidino-2-phenylindole (DAPI, 10 µg/ml), then incubated for 10 min in the dark at RT as previously described (Hanafy et al. 2017). Treated samples were examined with an Olympus BX51 microscope (Olympus, Center Valley, PA), equipped with a Brightline DAPI high-contrast filter set for DAPI fluorescence. Pictures were obtained with a DP71 digital camera (Olympus Scientific Solutions Americas Inc., Massachusetts, USA).

For scanning electron microscopy (SEM), isolates were grown on RFC media containing cellobiose or a mixture of switchgrass and cellobiose as a carbon source. Sample fixation and preparation for SEM was described previously (Hanafy et al. 2017). The prepared samples were examined on a FEI Quanta 600 SEM (FEI Technologies Inc. (Oregon, United States).

DNA extraction and phylogenetic analysis. Biomass from 10 ml cultures (ca. 45 mg) was obtained by filtration. The biomass was crushed in liquid nitrogen in a sterile mortar using a sterile pestle, and DNA was extracted from crushed biomass using a modified CTAB protocol as previously described (Youssef et al. 2013). The D1-D2 domain was amplified using primer sets NL1 and NL4 (Dagar et al. 2011). PCR products were purified using PureLinkTM PCR Purification Kit (Life Technologies®, Carlsbad, California, USA) according to manufacturer's instructions and sequenced using capillary (Sanger) sequencing technology at the Oklahoma State University DNA/Protein core facility. Additionally, because of observed infra-isolate sequence divergence in the ITS1, the region encompassing the complete internal transcribed spacer (ITS1-5.8S-ITS2 = ITS) region and D1/D2 domains of 28S nuc-rDNA gene was also amplified using the primer pair ITS5-NL4 (Wang et al. 2017); the resulting ~1.3 Kb product was cloned into a TOPO-TA cloning vector following the manufacturer's instructions (Life Technologies®, Carlsbad, CA). At least six clones were randomly selected per isolate and used for Sanger sequencing. For 28S rDNA tree construction, sequences obtained the D1-D2 domain were aligned with reference AGF sequences using ClustalW and alignments were manually refined in MEGA7 (Kumar et al. 2016) and used to construct maximum likelihood trees. For the ITS1 trees, sequences spanning the complete ITS1-5.8S-ITS2-D1/D2 domains of 28S nuc-rDNA gene were first aligned using ClustalW, followed by trimming the ITS1 region. Reference *Anaeromyces* as well as other AGF genera ITS1 sequences were added to the alignment, which was then used to construct both maximum likelihood and maximum parsimony trees in MEGA7. *Chytriomyces* sp. WB235A (isolate AFTOL-ID 1536) was used as the outgroup (DQ536498 for ITS1, DQ536493 for 28S). Bootstrap values were calculated based on 100 replicates. The complete ITS region sequences are deposited in GenBank under accession numbers MG605677–MG605709, and D1-D2 sequences as MF121928–MF121943. Alignments

and phylogenetic trees are available through TreeBase under study accession URL http://purl.org/phylo/treebase/phylows/study/TB2:S22509.

Results

Isolations. Most isolates were obtained from fresh fecal material of cow (3) and goat (10), while only a few were obtained from frozen and thawed fecal material of cow (1) and goat (2). Thirteen isolates were obtained using cellulose, while three were obtained using a mixture of cellobiose and switchgrass as carbon sources (TABLE 2-1). On solid media, all strains exhibited small circular white colonies 4–6 mm diam. (FIG. 2-1 A). Clear discoloration zones were observed around the colonies when grown on cellulose-containing agar medium (FIG. 2-1 B). In liquid media, all strains had thick pearl-like growth (FIG. 2-1 C).

Microscopic characters. Preliminary microscopic observations demonstrated that all isolates have similar thalli growth patterns. In the absence of any morphological or microscopic differences among strains, one (strain O2) was randomly chosen as the type strain and was used for detailed microscopic analysis (FIGS. 2-2, 2-3, 2-4).

Strain O2 produced polycentric thalli with nucleated rhizomycelia (FIG. 2-2 A, B). The rhizomycelia of all isolates displayed extensively branched hyphae of indeterminate length. Both narrow hyphae, 0.5–1 μm wide, and broad hyphae 1.5–7 μm wide were observed (FIG. 2-2 C). The broad hyphae had frequent constrictions resulting in a characteristic sausage-like appearance (FIG. 2-2 D) observed in prior *Anaeromyces* isolates (Ho and Barr 1995).

In addition, the hyphae of strain O2 also produced lobed or bead-like structures (FIG.2-2 E), similar to the appressoria-like structures previously reported in *A. elegans* (Table 2-2), and postulated to aid in the mechanical penetration and access of AGF hyphae to plant biomass (Ho et al. 1988a). The appressoria-like structures developed either as lateral outgrowth arising from sites of hyphal constrictions (FIG. 2-2 E, F), or directly from the surface of the hyphae (FIG.2-2 G, H). Scanning electron micrographs showed that these structures start first as small protrusions 1.5– 15 μm x 1.5–12 μm (FIG. 2-2 H) that eventually enlarge into multi-lobed vesicles (FIG. 2-2 I). Several smaller penetration pegs 0.5–8 μm x 0.1–2 μm appeared to develop on individual lobes; and such pegs often protruded directly from the lobe (FIG. 2-2 J), and occasionally forked at their tips (FIG. 2-2 K). Finally, the hyphae exhibited an interesting feature hitherto unobserved in Neocallimastigomycota, where extensive hyphal entanglement and coiling occurred when

grown on a soluble carbon source (FIG. 2-3 A-B, TABLE 2-2). We suggest that appressoria-like structure formation and entangled hyphae could serve as attachment mechanisms to maximize the contact area between the plant surface and the fungal hyphae. Indeed, when grown on switchgrass, strain O2 wrapped and coiled its hyphae around the plant fibers (FIG. 2-3 C-D), rather than coiling around their own cells, which they do when grown on a soluble carbon source (FIG. 2-3 A-B). Further, after entrapment of the plant fiber, the appressorium-like structures developed, and appeared to penetrate the plant fiber at different sites (FIG. 2-3 E). Strain O2 produced both intercalary and terminal sporangia. The majority were intercalary globose sporangia, i.e. sporangia that developed from expansion or swelling of hyphae, 8–20 μm diam. (FIG. 2-4 A-B). Terminal fusiform sporangia with pointed or mucronate apex, 12–20 μm x 5–8 μm at the end of a short sporangiophore 5–10 μm long (FIG. 2-4 C) were rarely encountered. All isolates rapidly lost their ability to produce both types of sporangia after repeated sub-culturing, as previously observed (Ho and Barr 1995), although sporangiogenesis of intercalary sporangia could be partly and transiently restored after sub-culturing on cellulose sloppy media as previously suggested (Ho and Bauchop 1991). Zoospores were small, 5–8.5 μm, circular, uniflagellated (flagellum length 16–20 μm), with a rough, uneven surface (FIG. 2-4 D-E), similar to prior descriptions of *Anaeromyces* zoospores (Ho and Barr 1995).

Phylogenetic analysis. Phylogenetic analysis using D1-D2 28S rDNA gene or ITS1 placed all obtained isolates in a single, monophyletic, and bootstrap-supported group within the genus *Anaeromyces* (FIG. 2-5, 2-6). The obtained isolates exhibited 0–1.8% inter-sequence divergence in D1-D2 and 0–8.8% inter-sequence divergence in ITS1. While the D1-D2 sequence within the same isolate was identical for different rRNA clones, the ITS1 clones from the same isolate exhibited some variation ranging from 0–7.3% (average intra-isolate variation ranged from 0.5- 4.5%). Strain O2, the proposed type species, displayed 3.3% sequence divergence from *A*. cf. *mucronatus* strain ZU2 ITS1 gene sequence (GenBank JN943019.1), and 1.89% sequence divergence from *A*. cf. *mucronatus* strain ZU2 D1-D2 gene sequence (JN939171.1), and 6.6% sequence divergence from A. robustus strain S4 ITS1 sequence (NR_148182.1). The lack of *A.*

elegans or *A*. cf. *elegans* sequence data rendered assessing phylogenetic relationships of *A*. *elegans* to our strains unfeasible.

TAXONOMY

Anaeromyces contortus Radwa A. Hanafy, Britny Johnson, Mostafa S. Elshahed, and Noha H. Youssef, sp. nov. (FIGS. 2-2, 2-3, 2-4).

MycoBank: MB821369.

Typification: The holotype is Fig. 1E in this manuscript, derived from the following: USA.

OKLAHOMA, Stillwater, 36.12′ N, 97.06′ W, ˜800 m ASL, 3 d old culture of isolate O2, originally isolated from frozen then thawed feces of domesticated cow (*Bos taurus*), Sept. 2015, Radwa A. Hanafy. Ex-type strain: O2. GenBank: ITS1 MG605693; D1-D2 MF121931.

Etymology: From contortum (Latin), meaning coiled or tangled, reflecting the fact that this fungus forms hyphae that entangle and coil on themselves.

An obligate anaerobic fungus with polycentric thalli, highly branched nucleated rhizoidal system, with both narrow hyphae (0.5–1 μm wide and broad hyphae 1.5–7 μm wide, with extensive coiling and constrictions imparting a sausage-like appearance. Lobed or appressoria-like structures developing as lateral outgrowth arising either from the sites of constrictions or directly from the surface of the hyphae. Penetration pegs, 0.5–8 μm x 0.5–2 μm arising on individual lobes, protruding directly from the lobe, occasionally forked at their tips. Mature sporangia globose, 8–20 μm diam., mainly intercalary, terminal sporangia 12–20 μm x 5–8 μm few, with a mucronate apex produced on short sporangiophores 5–10 μm long. Zoospores 5–8.5 μm, with a single flagellum 16–20 μm long. Cultural characters: Producing a pearl-like growth in liquid rumen fluid-cellobiose media. Producing small circular colonies, 0.4–0.6 cm diam. on rumen fluid-cellobiose-agar roll tubes.

The clade is defined by the sequences MG605677–MG605709 (ITS1), and MF121928–MF121943 (D1-D2).

Additional specimens examined: U.S.A. OKLAHOMA: Stillwater, 36.12′ N, 97.06′ W at

~800 m above sea level. Freshly deposited as well as frozen and thawed feces of cow (*Bos taurus*) and goat (*Capra aegagrus hircus*), Sep 2015, Radwa A. Hanafy and Britny L. Johnson strains C3G, C3J, Na, G3A, G3B, G3C, G3G, X4, X5 (isotype).

Figure 2-1. Macroscopic features of *Anaeromyces contortus* type strain O2 in liquid culture and on agar roll tubes. (A) Circular white colonies on cellobiose agar medium. (B) Clear discoloration zones around the fungal colonies when grown on cellulose agar medium. (C) Thick pearl-like growth in liquid medium.

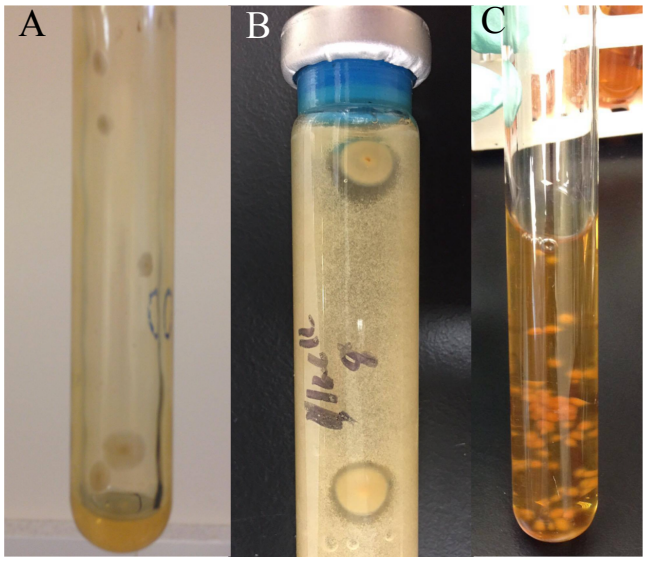
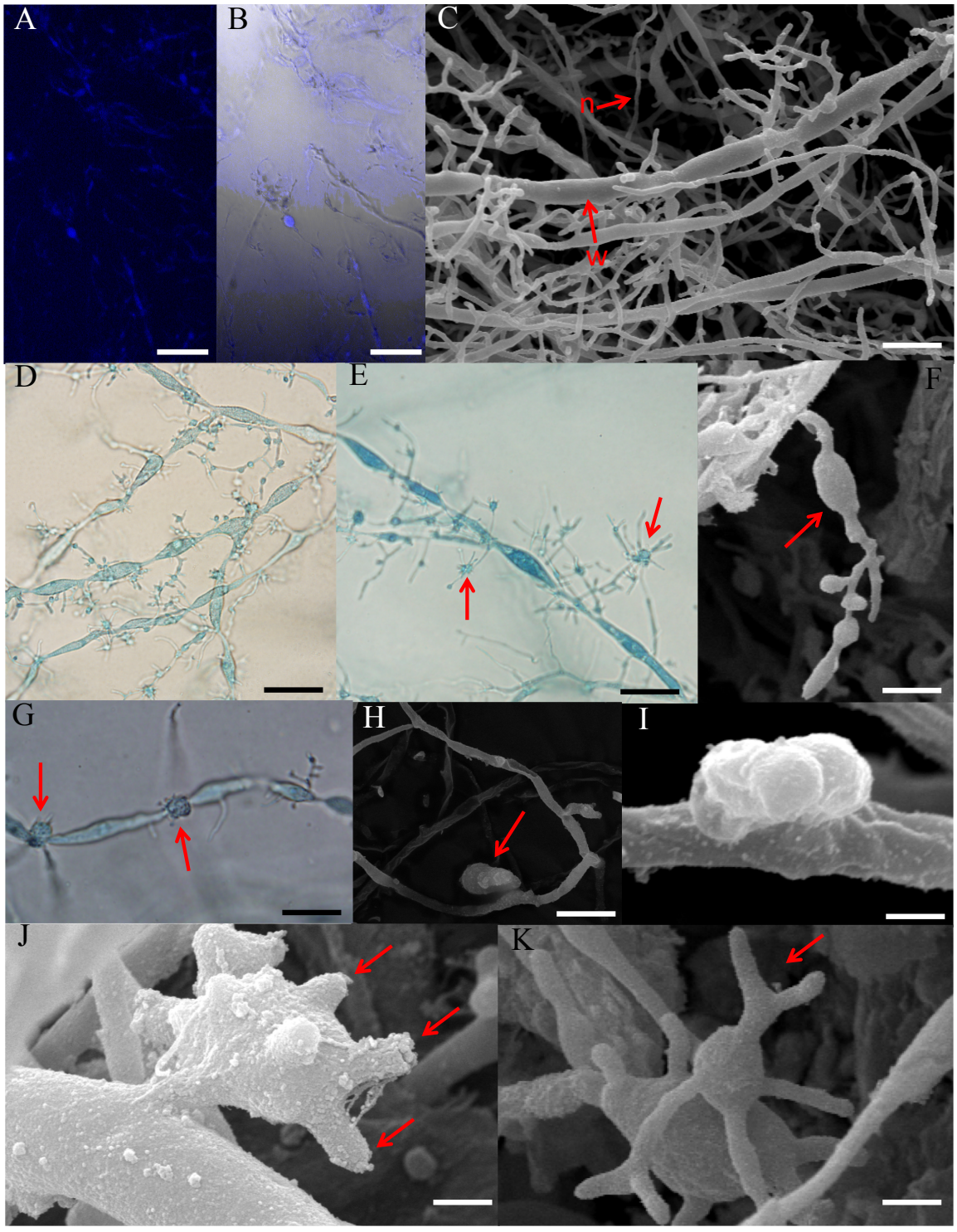


Figure 2-2. Thalli and rhizomycelia of *Anaeromyces contortus* (ex-type strain). Light (A, B, D, E and G) and scanning electron (C, F, H–P) micrographs. Light microscopy images were examined after staining with lactophenol cotton blue (D, E, and G), as well as following nuclei staining with DAPI (A). The overlay image is shown in (B). (A–B) Polycentric thalli. (C-D) Hyphal structures (C), with constrictions in wide hypha imparting the sausage-like appearance (arrows in D). (E-K) Lobed or appressoria-like structures, arising at the sites of hyphal constriction (arrows in E), developing as a lateral hyphal outgrowth (F), developing on the hyphal surface (G-H), defined into multiple lobes (I), with several penetration pegs (arrows in J), or with forked or branched penetration pegs (arrow in K). Abbreviations: (n), narrow hyphae; (w) wide hyphae. Bars: A-E, G = 20 μm; F, H-K = 5 μm.


A
B
C
n
w
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E
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G
H
I
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K

Figure 2-3. Entangled and coiled hyphae of *Anaeromyces contortus* (ex-type strain). Scanning electron micrographs. (A-B) Hyphae wrapping upon themselves, (C-D) coiling and wrapping around switchgrass fibers, and with complete entrapment of the switchgrass fibers within the fungal hyphae (E). Abbreviations: (s), intercalary sporangia. Bars: A, and E = 20 µm; B-D = 5 µm.

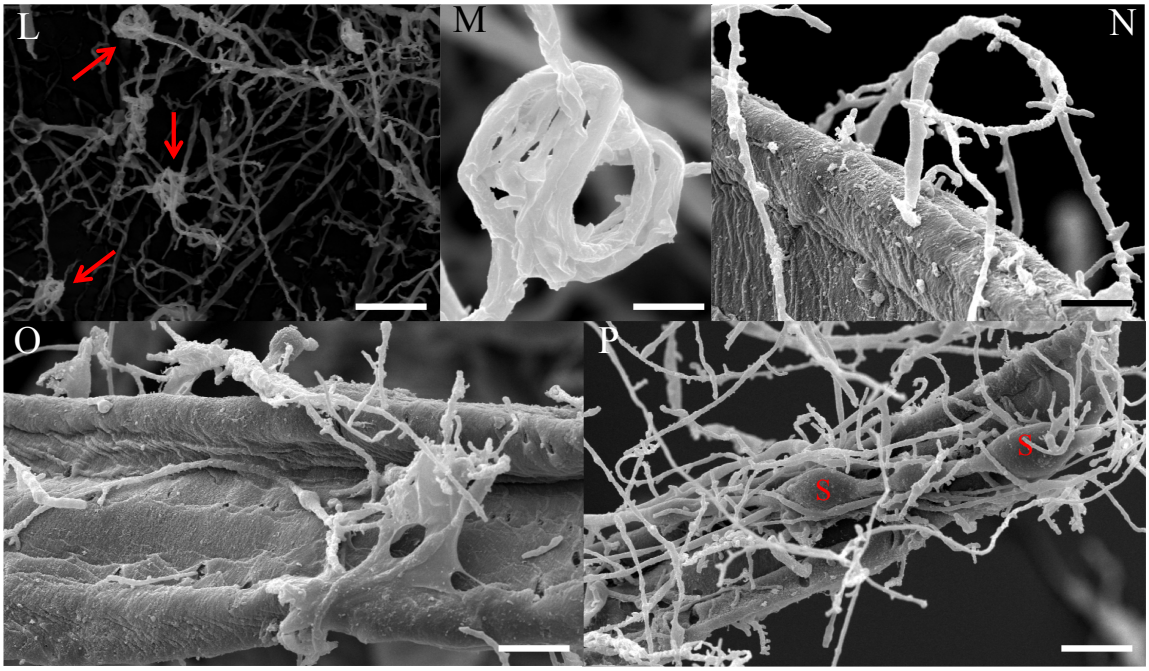

Figure 2-4. Sporangia and zoospores of *Anaeromyces contortus* (ex-type strain). (A and B) globose intercalary sporangia (arrows). (C) Terminal fusiform sporangia with pointed or mucronated apex. (D and E) Uni-flagellated zoospore. (F) A zoospore after losing its flagellum. (G) A zoospore cyst attached to switchgrass fibers. Bars: A-E = 20 µm; F-G = 5 µm.

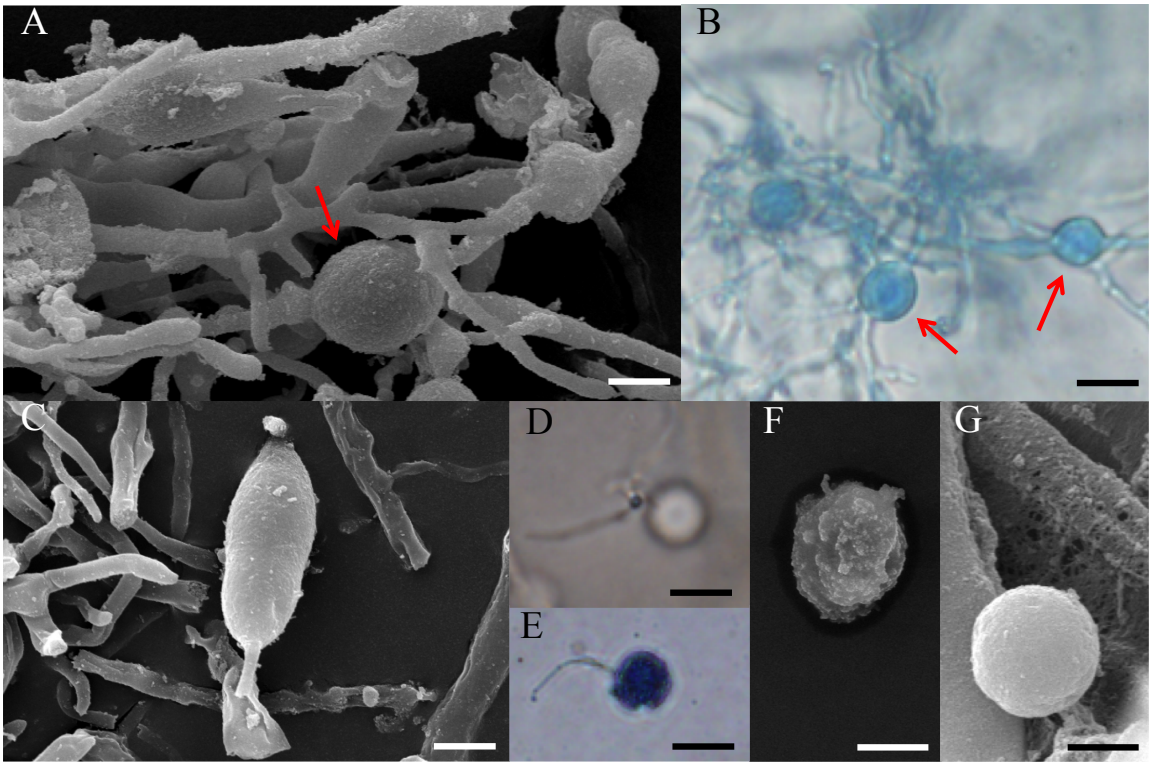

Figure 2-5. Phylogenetic affiliation of the *Anaeromyces contortus* clade to other *Anaeromyces* species as well as other AGF genera based on the sequences of the partial 28S rDNA. *Anaeromyces contortus* type strain O2 is shown in boldface. GenBank accession numbers are shown between parentheses. Genera and *Anaeromyces* species are depicted with lines on the right-hand side of the trees. The tree was obtained using a maximum likelihood approach based on the Tamura-Nei model. A maximum parsimony tree was also constructed using the Subtree- Pruning-Regrafting (SPR) algorithm but is not shown. Bootstrap values from 100 replicates are shown for nodes with more than 80% bootstrap support (as ML-BS/MP-BS). Analysis was conducted in MEGA7.

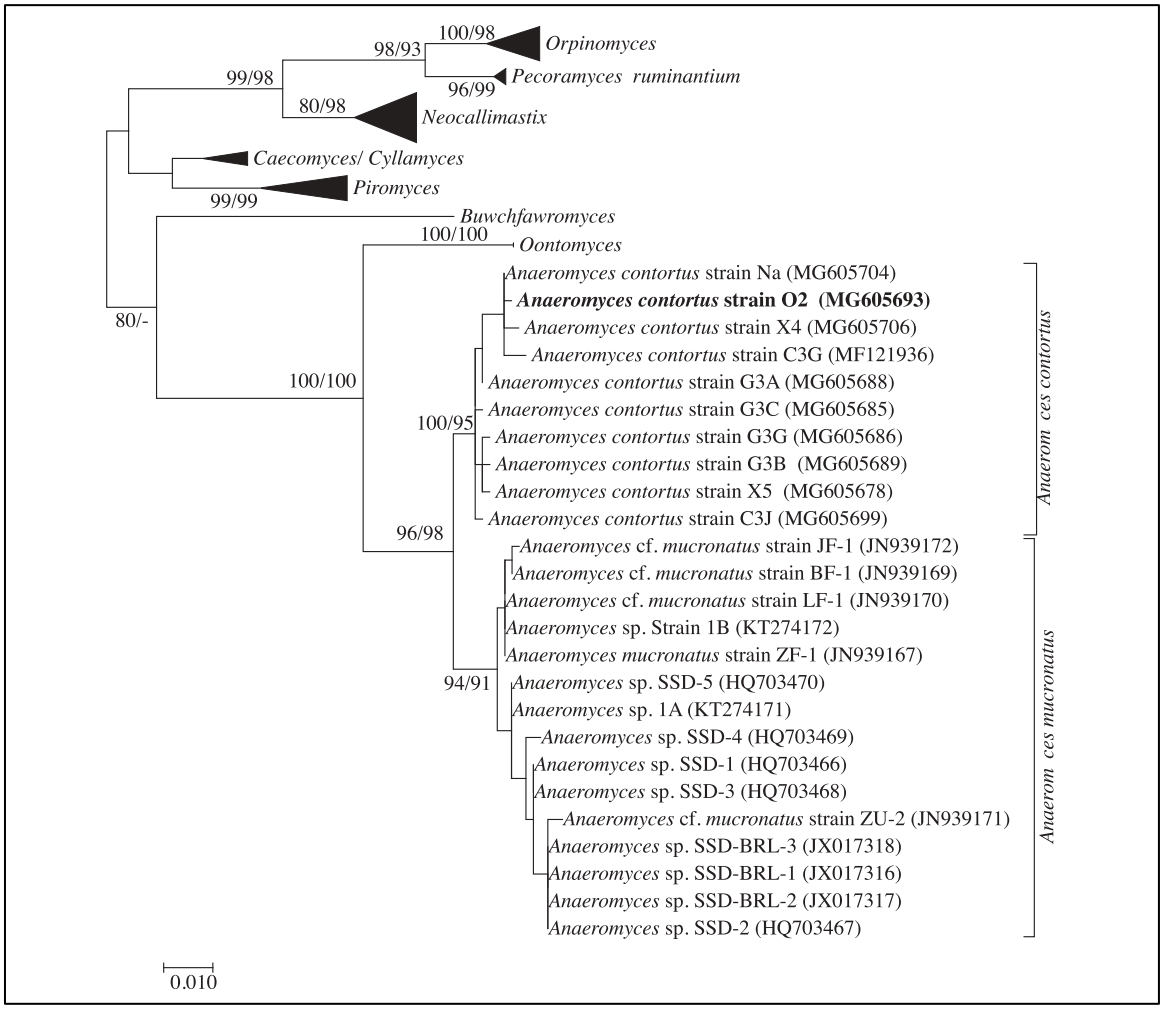

Figure 2-6. Phylogenetic affiliation of the *Anaeromyces contortus* clade to other *Anaeromyces* species as well as other AGF genera based on the sequences of the ITS1 regions of the rDNA locus. *Anaeromyces contortus* type strain O2 is shown in boldface. GenBank accession numbers are shown between parentheses. Genera and *Anaeromyces* species are depicted with lines on the right-hand side of the trees. The tree was obtained using a maximum likelihood approach based on the Tamura-Nei model. A maximum parsimony tree was also constructed using the Subtree- Pruning-Regrafting (SPR) algorithm but is not shown. Bootstrap values from 100 replicates are shown for nodes with more than 80% bootstrap support (as ML-BS/MP-BS). Analysis was conducted in MEGA7.

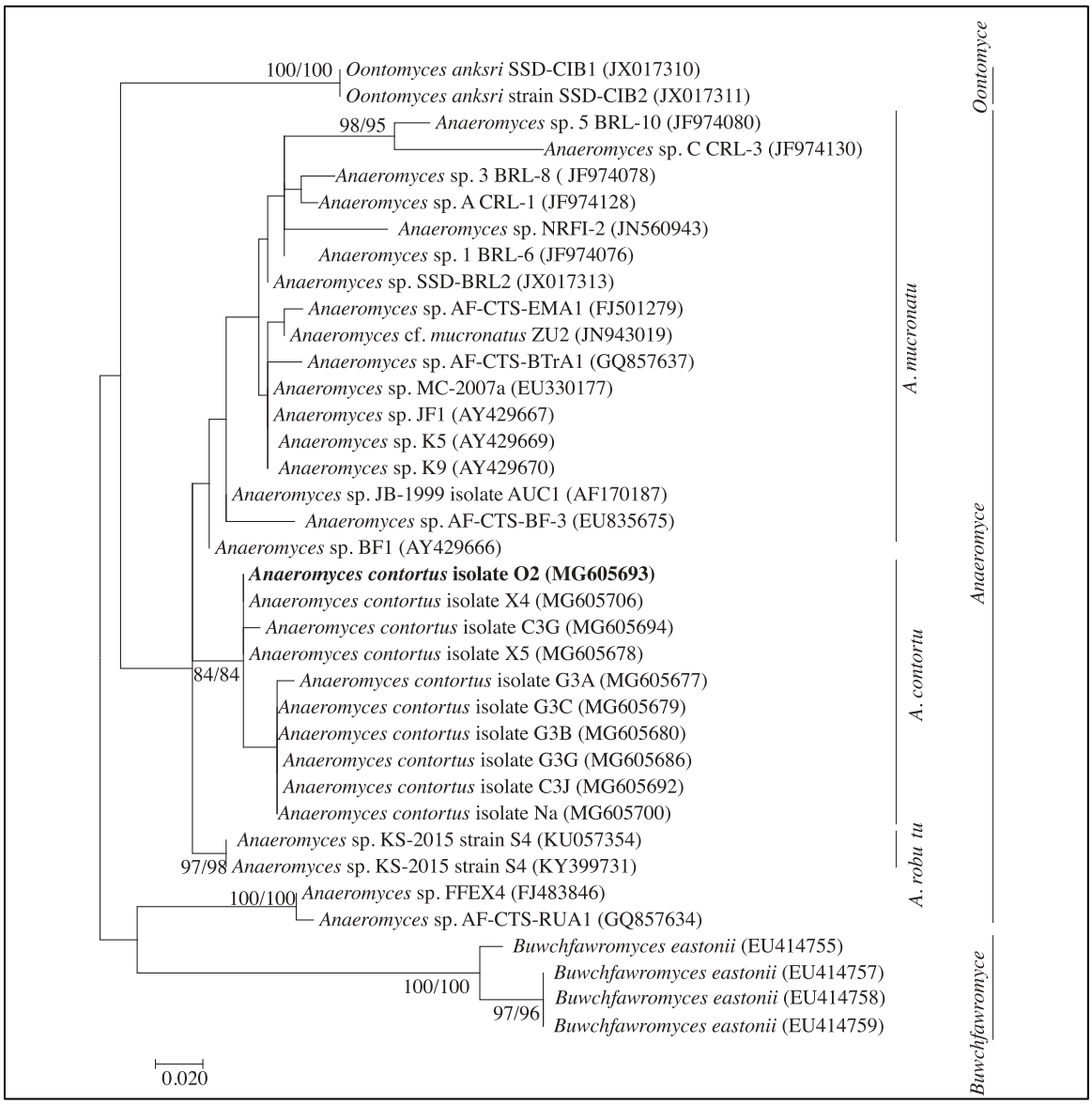

Table 2.1. Isolation criteria and GenBank accession numbers of the strains obtained in this study.

Strain	Host	Sample condition at the time of isolation	Carbon source	GenBank accession numbers	
				ITS1	28S rDNA
O2	Cow	Frozen	Cellobiose + switchgrass	MG605693, MG605698, MG605708	MF121931
C3G	Cow	Fresh	Cellulose	MG605694, MG605696, MG606702, MG605703, MG605705, MG605707	MF121936
C3J	Cow	Fresh	Cellulose	MG605692, MG605699	MF121942
Na	Cow	Fresh	Cellulose	MG605700, MG605701, MG605704	MF121943
G3A	Goat	Fresh	Cellulose	MG605681, MG605684, MG605688, MG605697	MF121930
G3B	Goat	Fresh	Cellulose	MG605680, MG605689	MF121938
G3C	Goat	Fresh	Cellulose	MG605679, MG605683, MG605685, MG605687	MF121941
G3G	Goat	Fresh	Cellulose	MG605686, MG605690, MG605691, MG605695	MF121935
X4	Goat	Frozen	Cellobiose + switchgrass	MG605706	MF121929
X5	Goat	Frozen	Cellobiose + switchgrass	MG605678, MG605682, MG605709	MF121928

Table 2.2. Distinguishing characters between *A. contortus* and previously described species of *Anaeromyces**.

Species	Thallus	Zoospore flagellation	Hyphal constriction	Sporangium shape	Appressorium-like structures	Hyphal coils
A. contortus	Polycentric	Uniflagellate	Present	Terminal fusiform with pointed apex or globose intercalary	Present	Present
A. mucronatus	Polycentric	Uniflagellate	Present	Ellipsoidal or fusiform with pointed apex	Absent	Absent
A. elegans	Polycentric	Uniflagellate	Present	Ellipsoidal or fusiform with pointed apex	Present	Absent
A. robustus	Polycentric	Multiflagellate	Absent	Club-shaped, often fuse to form a whale tail–like shape	Absent	Absent

*Information on *A. mucronatus*, *A. elegans*, and *A. robustus* was obtained from Breton et al. (1990), Ho et al. (1990, 1993), and Li et al. (2016).

Discussion

Comparison between the ex-type microscopic characters and those of published *A. mucronatus,* and *A. elegans* lectotypes. The ex-type strain of *Anaeromyces contortus* exhibited many microscopic characters observed in other species of *Anaeromyces*, e.g. sausage shaped hyphae, polycentric thalli, uniflagellated zoospores, and acuminate fusiform sporangia. The ex-type is similar to *A. elegans* in producing appressorium-like or lobed structures. While such character was not reported in the *A. mucronatus* lectotype (Breton et al. 1990), *A.* cf. *mucronatus* (Fliegerova et al. 2004), or A. robustus (Li et al. 2016) (TABLE 2-2), the production of appressorium-like structures in Neocallimastigomycota has been observed microscopically in colonized plant tissues directly recovered from the rumen (Ho et al. 1988a; Ho et al. 1988b). The production of appressoria has been extensively studied in plant pathogens, e.g. *Magnaporthe oryzae* (Ryder and Talbot 2015). In these organisms, true appressorium structures originate from germ tubes in response to pressure, although direct production of appressorium-like structures from hyphae has also been observed in *M. oryzae* (Kong et al. 2013). Because of the paucity of zoospores in *Anaeromyces* cultures, it is difficult to ascertain if true appressoria could be formed from germ tubes in the ex-type. However, direct formation of appressoria from zoospores in anaerobic fungi was previously reported in direct observations of rumen contents (Ho et al. 1988a; Ho et al. 1988b). Appressoria exhibit strong turgor pressure for puncturing the epidermis of plant hosts and thus aid plant pathogens by enabling development of intracellular mycelia in the host cells (Ryder and Talbot 2015). We reason that such abilities could also be beneficial for ensuring efficient plant biomass degradation in the non-pathogenic Neocallimastigomycota. Because of the polycentric and polynucleate nature of *Anaeromyces* hyphae, it is possible that following plant biomass penetration at the appressoria-like sites, hyphal nuclei are directly transferred from the hyphae into the plant cells, allowing for intracellular hyphal development, as previously observed (Ho et al. 1988a; Ho et al. 1988b).

Another interesting hyphal feature observed in the ex-type strain is the formation of intercalary sporangia. This type of sporangium has been observed in *Orpinomyces*, the other polycentric genus in phylum Neocallimastigomycota (Ho et al. 1994). Intercalary sporangia were not observed in *A. mucronatus*, *A. elegans*, or *A. robustus* (TABLE 2-2).

However, it is interesting to note that *Anaeromyces* strains BF1 and JF1, which are phylogenetically affiliated with *A. mucronatus*, produce such structures (Fliegerova et al. 2004). Therefore, it appears that intercalary sporangia formation is not a taxonomically informative character for differentiating among *Anaeromyces* species.

A third feature in the ex-type strain of *A. contortus* is the characteristic formation of entangled and coiled hyphae that appear to maximize its attachment to plant substrates (FIG.2-3 C-E). Such a feature has not been described previously in the AGF to our knowledge. The production of entangled hyphae in strain O2 appears to occur constitutively, regardless of the presence of plant substrate (FIG. 2-3A-B), possibly an adaptation to life in the herbivorous gut where an abundance of plant substrates is encountered.

Phylogenetic relationships between *A. contortus* and other *Anaeromyces* spp.

Phylogenetically, the ex-type strain of *A. contortus* and related strains are distinct from *A.* cf. *mucronatus* and all related isolates (FIG. 2-5-2-6). The absence of sequence data from the *A. elegans* lectotype (Breton et al. 1990), or any *A.* cf. *elegans* strains, renders any genetic evaluation of its relationship to our isolates or to *A. mucronatus* unfeasible.

In addition to the *A. mucronatus* and *A. contortus* clades, *A. robustus* constitutes a third *Anaeromyces* cade in ITS1 trees (ITS1 KY399731, KU057354). In addition to the relatively high-level of sequence divergence compared to other *Anaeromyces* species, *A. robustus* exhibits distinct microscopic features that deviate from the typical *Anaeromyces* morphology (Li et al. 2016). Such features include the production of polyflagellated rather than a uniflagellated spores, club-shaped sporangia that often fuse rather than fusiform sporangia, and the apparent lack of constricted sausage-shaped hyphae (Li et al. 2016). Finally, two additional isolates designated as FFEX4 (FJ483846) and AF-CTS-RUA1 (GQ857634) form a fourth distinct monophyletic clade distantly related to the *A. mucronatus* and *A. contortus* in ITS1 trees (Figure 5). These two latter isolates were designated as *Anaeromyces* sp., but the lack of microscopy data renders it difficult to assess whether they represent a new *Anaeromyces* species, or whether they might be different enough in morphology to warrant the proposal of a new genus as suggested by Wang et al. (2017).

Collectively, the distinct microscopic characteristics, i.e. hyphal coils and mostly intercalary sporangia, as well as ITS1 and D1-D2 28S rDNA sequence divergence between the ex-type strain and other *Anaeromyces* isolates justifies proposing a new species for these strains, described here as *Anaeromyces contortus.*

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CHAPTER III

FERAMYCES AUSTINII, GEN. NOV., SP. NOV., AN ANAEROBIC GUT FUNGUS FROM RUMEN AND FECAL SAMPLES OF WILD BARBARY SHEEP AND FALLOW DEER

Abstract

Anaerobic gut fungi are common inhabitants of the alimentary tracts of herbivorous animals. Nine different Neocallimastigomycota genera have been described so far. However, culture-independent diversity surveys suggest the presence of numerous, yet-uncultured Neocallimastigomycota genera. Here, we report on the isolation and characterization of the first cultured representatives of Neocallimastigomycota clade AL6, originally identified in culture-independent surveys of fecal samples from captive wild animals. Six strains were isolated from rumen and fecal samples of a wild Barbary sheep (*Ammotragus lervia*) and a wild fallow deer (*Dama dama*) in Texas, USA. The isolates displayed medium-sized (3–7 mm), circular, beige colonies with filamentous edges and a dark center on agar roll tubes. Microscopic analysis revealed monocentric thalli with both endogenous and exogenous sporangial development patterns. Zoospores were spherical, with a diameter of 9.6 ± 1.9 μm, and polyflagellated, with 7–16 flagella. Phylogenetic analysis based on nuc rDNA ITS1 region and D1–D2 domains of nuc 28S rDNA revealed that the isolated strains formed a single monophyletic and bootstrap-supported clade distinct from all currently described Neocallimastigomycota genera. Substrate utilization experiments using the type strain (F3a) demonstrated robust and fast growth on sugars and plant biomass, as well as the capability to metabolize a wide range of mono-, oligo-, and polysaccharides, including galactose, arabinose, alginate, and pectin. On the basis of the morphological, physiological, and phylogenetic analyses, we propose to accommodate these isolates in a new genus, *Feramyces* (derived from the Latin word for "wild" to reflect their isolation and apparent distribution in undomesticated herbivores), and a new species, *F. austinii*. The type strain is *Feramyces austinii* F3a.

Introduction

The anaerobic gut fungi (AGF) represent a distinct basal phylum lineage (Neocallimastigomycota) commonly encountered in the rumen and alimentary tracts of herbivores (Gruninger et al. 2014). Several unique metabolic and structural adaptations have enabled the AGF to survive and successfully colonize their strictly anaerobic, plant bio- mass–rich, and prokaryote-dominated environments. These include dependence on fermentative pathways as the sole mechanism for energy production (Boxma 2004), possession of an impressive arsenal of lignocellulolytic enzymes for efficient plant biomass degradation (Ljungdahl 2008; Couger et al. 2015), substitution of ergos- terol with tetrahymanol in their cell membrane (Youssef et al. 2013), and the reductive evolution of their mitochon- dria to hydrogenosomes (Yarlett et al. 1986).

Since their discovery in 1975 (Orpin 1975), multiple strains of AGF have been isolated and characterized (Ho and Barr 1995; Gruninger et al. 2014), and a taxonomic scheme that relies on microscopic features and phylogenetic analysis of marker genes such as nuc rDNA ITS1 (hereafter ITS1) region and more recently D1–D2 domains of nuc 28S rDNA (hereafter 28S rDNA) has been utilized to delineate genus and species boundaries. As of writing, nine different genera and ~29 different species have been described (Heath et al. 1983; Gold et al. 1988; Barr et al. 1989; Breton et al. 1990; Ozkose et al. 2001; Ariyawansa et al. 2015; Callaghan et al. 2015; Dagar et al. 2015; Li et al. 2016; Hanafy et al. 2017).

Anaerobic fungi appear to be present in all foregut fermenters, hindgut fermenters, some large herbivorous rodents, e.g., Mara (genus *Dolichotis*) (Teunissen et al. 1991), and some reptilian herbivores, e.g., genus Iguana (Gruninger et al. 2014). Although the occurrence of AGF has been documented in at least 24 host genera belonging to eight different families of animals (Gruninger et al. 2014), the majority of AGF isolation and characterization efforts has been conducted on samples obtained from a relatively few domesticated animal hosts, e.g., horses, mules, cows, goats, sheep, and water buffalos (Orpin 1975; Bauchop and Mountfort 1981; Lowe et al. 1987; Ho et al. 1993b; Dagar et al. 2011), with few exceptions, e.g., *Piromyces rhizinflata*, and *P. minutis* (Breton et al. 1991; Ho et al. 1993a). This is due to the relative ease of obtaining samples from herds reared in research facilities or animal farms. This restricted sampling pattern is unfortunate, since culture-independent diversity surveys suggest that animal host phylogeny plays an important role in shaping AGF community in the herbivorous

gut (Liggenstoffer et al. 2010). Efforts to enrich and isolate AGF from undomesticated animals that either are kept in captivity in zoos (Orpin et al. 1985; Milne et al. 1989; Teunissen et al. 1991; Paul et al. 2010, 2011; Nagpal et al. 2011) or are truly free-living (Orpin et al. 1985; Tuckwell et al. 2005) have been sparse. Further, isolates obtained from such studies were often only morphologically described, but seldom fully characterized or preserved.

Animal husbandry involves providing a monotonous diet at fixed intervals to captive animals. Such regimens can reduce overall microbiome diversity by selecting for a single or a few strains ideally suited to a specific feeding pattern. On the other hand, wild animals usually graze on a wider range of plants with more diverse chemical compositions and experience significant fluctuations in feeding frequencies. Therefore, we hypothesized that free-living mammalian herbivores could harbor novel, yet-uncultured AGF taxa. Here, we report on the isolation of multiple AGF strains from rumen and fecal samples of Barbary sheep and fallow deer from Texas, USA. The morphological, physiological, and phylogenetic distinct characteristics of the obtained isolates justify their placement in a new genus, *Feramyces* (derived from the Latin word for "wild" to reflect their isolation and apparent distribution in undomesticated herbivores), and a new species, *F. austinii* (in recognition of Mr. Jim Austin who provided the feces and rumen samples for this study).

Materials and Methods

Samples. Fresh rumen contents and fecal samples were obtained from a wild female Barbary sheep (*Ammoragus lervia*), and fresh fecal samples were obtained from a wild female fallow deer (*Dama dama*) in two separate hunting expeditions in Val Verde and Coke counties, Texas, respectively. Samples were transferred on ice to the laboratory within 24 h of collection, where they were immediately used as an inoculum for subsequent enrichment and isolation procedures. All hunters had the appropriate licenses, and the animals were shot either on private land with the owner's consent or on public land during the hunting season.

Isolation procedure. Isolation of anaerobic fungal strains was conducted as previously described (Hanafy et al. 2017). Samples were serially diluted into antibiotic (50 μg/mL kanamycin, 50 μg/mL penicillin, 20 μg/mL streptomycin, and 50 μg/mL chloramphenicol, respectively)-supplemented rumen fluid cellobiose (RFC) media (Calkins et al. 2016) and incubated at 39 º C for 18 h. Roll tubes (Hungate 1969) of RFC agar media were prepared from dilutions displaying visible signs of growth (plant materials clumping and floating and production of gas bubbles) with 0.5 mL of inoculum. Single colonies were picked into liquid RFC media, and three rounds of colony picking and tube rolling were conducted to ensure purity. Strains were maintained by biweekly subculturing into RFC media. Long-term storage was conducted by surface inoculation of RFC agar media as described previously (Calkins et al. 2016).

Morphological characterization. Samples for light and scanning electron microscopy were obtained from liquid cultures at various stages of growth. An Olympus BX51 microscope (Olympus, Center Valley, Pennsylvania) equipped with a DP71 digital camera (Olympus) was used for visualization of fungal structures by first staining the samples with lactophenol cotton blue. For examination of localization of nuclei, samples were stained with 4,6′-diamidino-2- phenylindole (DAPI; 10 μg/mL), then incubated for 10 min in the dark at room temperature as previously described (Hanafy et al. 2017), and visualized with an Olympus IX81 spinning disc confocal microscope in the differential interference contrast (DIC) mode. Scanning electron microscopy was conducted with a FEI quanta scanning electron microscope (Hillsboro, Oregon) as described previously (Hanafy et al. 2017).

Substrate utilization. Growth of the type strain (strain F3a) on 25 different substrates was assessed in rumen fluid (RF) media with 0.5% w/v of each substrate. Tubes were scored positive if they continued to exhibit visible growth after five subculturings with 10% inoculum. Glucose utilization was quantified in RF media amended with 0.5%

glucose using the PGO (glucose oxidase/peroxidase) assay kit (Sigma-Aldrich, St. Louis, Missouri). Growth on untreated switchgrass and corn stover was examined using 0.5 g as the sole C source in 45 mL RF media serum bottles. After 7 d, the remaining dry weight of switchgrass and corn stover was quantified as described previously (Ranganathan et al. 2017). A fungal uninoculated control was included to account for abiotic plant biomass loss due to solubilization in liquid media.

Phylogenetic analysis. DNA extraction was performed with a modified cetyltrimethylammonium bromide (CTAB) protocol as previously described (Youssef et al. 2013). The region encompassing the complete ITS1-5.8S-ITS2 (ITS) and the D1–D2 domains of 28S nuc rDNA gene was amplified with the primer pair ITS5-NL4 (Wang et al. 2017) using the following protocol: initial denaturation at 94 C for 4 min followed by 8 cycles of denaturation at 94 C for 2 min, annealing at 52 C for 1 min, and elongation at 72 C for 2 min, then 30 cycles of denaturation at 94 C for 1 min, annealing at 60 C for 1 min, and elongation at 72 C for 2 min. A final elongation step at 72 C for 10 min was added. The resulting polymerase chain reaction (PCR) product (~1.3 kb) was cloned into a TOPO-TA cloning vector according to the manufacturer's instructions (Life Technologies, Carlsbad, California). At least six clones were randomly selected per isolate and used for Sanger sequencing at the Oklahoma State University DNA/Protein core facility. Sequences were aligned with Clustal W and manually refined in MEGA7 (Kumar et al. 2016). Regions encompassing the ITS1 and the D1–D2 28S rDNA regions were trimmed and used for respective maximum likelihood and maximum parsimony tree construction in MEGA7 with Chytriomyces sp. WB235A isolate AFTOL-ID 1536 as the outgroup (accession numbers DQ536498.1 for ITS1 and DQ536493.1 for the 28S rDNA). Bootstrap values were calculated on the basis of 100 replicates.

Data and culture accession. Sequences generated in this study have been deposited in GenBank under accession numbers MG584190–MG584229 and MG605673–MG605676. Alignments and phylogenetic trees are available through TreeBASE under study accession URL http://purl.org/phylo/treebase/phylows/ study/TB2:S22503. The cultures are publicly available as part of the Department of Microbiology and Molecular Genetics at Oklahoma State University AGF isolates collection. The cultures are stored on agar media by the procedure previously described (Calkins et al. 2016a).

Results

Isolation. Four isolates (F2a, F2c, F3a, F3b) were obtained from fecal samples, and one isolate (R4a) was obtained from the rumen content of a female Barbary sheep. One additional isolate (DS10) was obtained from the feces of a female fallow deer. Colony and liquid growth patterns, microscopic observation, and phylogenetic analysis revealed no distinct differences among all six strains, and one isolate (strain F3a) was chosen as type strain and described in detail.

Colony and liquid growth patterns. On solid media, strain F3a formed beige, circular, filamentous colonies with a dark brown central core of dense sporangial structures; a smaller, light brown ring of younger, less dense sporangia; and an outer ring of light gray hyphal growth. The relative size of each ring was dependent on the colony age, with the sporangial core decreasing in size and the outer sporangia and hyphae increasing in size in older colonies (FIG. 3-1A). Colony size ranged between 3 and 7 mm. In liquid media, strain F3a exhibited thick growth on tube surfaces (FIG. 3-1B).

Microscopic features. Zoospores produced by strain F3a were spherical (FIG. 3-2a), with an average diameter of 9.6 ± 1.9 μm (standard deviation for 65 zoospores; range: 6.5–13 μm). In instances where motility was observed, zoospores appeared more oval (FIG. 3-2b). We speculate that this is due to contortions associated with motility, as previously suggested (Barr et al. 1989; Ho and Barr 1995). All zoospores were polyflagellated, with 7–16 flagella (FIG. 3-2a–b) and an average flagellum length of 29 ± 5.6 μm (standard deviation for 65 zoospores; range: 17–37 μm).

Zoospore encystment was associated with shedding of the flagella (FIG. 3-2c–d). Zoospore cysts germinate and produce either one or two (FIG. 3-2e) germ tubes. Germ tubes branch to develop a highly branched (FIG. 3-2f) anucleated rhizoidal system with both wide and narrow hyphae. The wide hyphae displayed multiple constrictions at irregular intervals (FIG. 3-2g), a characteristic previously reported in hyphal structures of *Orpinomyces joyonii, O. intercalaris, Neocallimastix frontalis, Piromyces communis, P. minutis, Aneromyces mucronatus, and A. elegans* (Barr et al. 1989; Breton et al. 1990; Ho and Jalaludin 1994; Ho et al. 1990, 1993a; Li et al. 1991).

Strain F3a displayed both endogenous (zoospores enlarging into sporangia; FIG. 3-2h–i), and exogenous (sporangia developing at the apex of sporangiophores opposite to the rhizoidal system; FIG. 3-2f and m–v) monocentric thallus development patterns. A typical endogenous sporangium is shown in FIG. 3-2h. Endogenous sporangia were either globose (10–70 μm diam.; FIG. 3-2h, k–l) or pyriform (25–110 μm L × 15–45 μm W; FIG. 3-2i–j) in shape. Occasionally, an endogenous thallus development pattern

where encysted zoospores produce two germ tubes was observed. These two germ tubes eventually expanded, forming two main rhizoidal systems (FIG. 3-2i–k). In some instances, this pattern results in a sporangium positioned in the middle of the two rhizoids (FIG. 3-2k), with an apparent morphological similarity to intercalary sporangia previously observed in *Orpinomyces intercalaris* (Ho 1994). However, the monocentric nature of strain F3a precludes labeling such structures as true intercalary sporangia; the formation of which is dependent on the occurrence of a polycentric thallus nucleation pattern. It is worth noting that similar structures were also observed in some monocentric fungi, e.g., Neocallimastix sp. and *Oontomyces anksri* (Barr et al. 1989; Ho et al. 1993c; Dagar et al. 2015), although the localization of nuclei in such structures (to differentiate sporangia from mere hyphal swellings) was not ascertained by DAPI staining. Finally, endogenous sessile lateral sporangia, similar to those previously reported in *Orpinomyces intercalaris* (Ho 1994), were occasionally observed (FIG. 3-2l), although they were far less common than typical endogenous and pseudo-intercalary sporangial structures.

Exogenous sporangia were typically observed at the end of sporangiophores that ranged in length between 15 and 600 μm. A typical exogenous sporangium in strain F3a is shown in FIG. 3-2m. Long, coiled sporangiophores were commonly observed (FIG. 3-2n). Many of the long sporangiophores ended in sub-sporangial swelings (apophysis) (FIG. 3-2q–r, v). Wide, flattened sporangiophores were also frequently encountered (FIG. 3-2o). Exogenous sporangia ranged in size between (40 and185μmL×20 and 80μmW) and displayed a variety of shapes, including pyriform (FIG. 3-2n), triangular (FIG. 3-2p), spherical (FIG. 3-2q), ovoid (FIG. 3-2r), heart-shaped (FIG. 3-2s), ellipsoidal (FIG. 3-2t), constricted ellipsoidal (FIG. 3-2u), and egg-shaped (FIG. 3-2v).

Zoospores were released through a wide apical pore of the sporangia (FIG. 3-2w), and the sporangial wall stayed intact after zoospores discharged (FIG. 3-2x). In aged cultures, sporangia were often observed to break off and detach from the hyphae or sporangiophores (FIG. 3-2y–z). Such behavior could conceivably aid in fungal dispersal.

Substrate utilization patterns. Strain F3a displayed a wide substrate utilization pattern, being able to grow on the majority of sugar, sugar acid, and polysaccharide substrates examined (TABLE 3-1). Notably, strain F3a was able to metabolize substrates often reported not to support growth of multiple AGF isolates, e.g., galactose, fucose, arabinose, and glucuronic acids (Breton et al. 1990; Teunissen et al. 1993; Hanafy et al. 2017), a phenomenon that we corroborated with reference strains from our culture collection.

Preliminary visual inspection suggested that strain F3a grew faster than representatives of other AGF genera recently isolated in our laboratory. Therefore, we quantified F3a growth on a soluble substrate (glucose) against multiple representatives of AGF genera. Strain F3a displayed faster glucose utilization (0.43 mg glucose/h) and growth (0.34 μg biomass/h) rates when compared with the absolute majority of reference strains examined in parallel in this study (TABLE 3-2). Further, when grown on plant biomass (untreated switchgrass and corn stover), the extent of substrate utilization (i.e., % plant biomass utilized) by strain F3a after 7 d of incubation was significantly higher (56% on switchgrass, 72% on corn stover) compared with other genera (11–52% on switchgrass, 18–65% on corn stover), attesting to its robust growth pattern compared with reference isolates (TABLE 3-3).

Phylogenetic analysis. Analysis of multiple clones per strain (spanning the ITS1-5.8S rDNA-ITS2-D1– D2 domains of 28S rDNA region) revealed almost identical rRNA locus sequences. Intersequence divergence between different isolates showed 0–0.29% difference at the ITS1 level and 0–2.5% (average 0.6%) at the D1–D2 28S rDNA level.

In contrast, the closest named AGF isolates to strain F3a based on ITS1 sequence similarity were Piromyces sp. JB-1999 isolate PLA1 (87% similar; accession number AF170207.1) and *Neocallimastix frontalis* isolate CHN2 (86% similar; accession number GQ355330.1). Similarly, strain F3a 28S rDNA sequence was 94% similar to those of *Neocallimastix cameroonii* (accession number KR920745.1) and 93% similar to *Neocallimastix frontalis* (accession number KR920744.1). Phylogenetic analysis (FIG. 3-3) using both gene markers consistently placed all six strains into a single, monophyletic, and bootstrap- supported group unaffiliated with all currently described nine AGF genera. In D1–D2 28S rDNA–based phylogeny (FIG. 3-3a), the new clade formed a distinct group basal to the superclade of the *Orpinomyces-Neocallimastix- Pecoramyces* genera. In ITS1-based phylogeny (FIG. 3-3b), all six strains clustered with *Neocallimastix, Caecomyces, Orpinomyces*, and *Pecoramyces*.

Ecological distribution. We queried GenBank database to identify closely related ITS1 sequences deposited as part of AGF culture-independent diversity surveys. The phylogenetic position of all closely related sequences (>93% sequence similarity) was evaluated. A collection of sequences (n = 1913) clustering into seven distinct operational taxonomic units (OTUs) based on 5% sequence divergence were phylogenetically monophyletic with isolates obtained in this study. These sequences were obtained during a survey of AGF in fecal samples obtained from greater kudu, Rothschild giraffe, and okapi at the Oklahoma City Zoo and previously referred to as uncultured anaerobic fungal group AL6 (Liggenstoffer et al. 2010).

TAXONOMY

Feramyces Radwa Hanafy, Mostafa Elshahed & Noha Youssef, gen. nov.

MycoBank MB823650

Typification: *Feramyces austinii* Radwa Hanafy, Mostafa Elshahed & Noha Youssef.

Etymology: Fera = derived from the Latin word for wild; myces = the Greek name for fungus. Obligate anaerobic fungus with determinate, monocentric thallus. The majority of thalli forming a single terminal sporangium, with the occasional formation of pseudo-intercalary sporangia (an endogenous thallus development pattern where encysted zoospores produce two germ tubes that eventually expand forming two main rhizoidal systems), as well as sessile lateral sporangia. Zoospores are polyflagellated (7–16 flagella). Zoospores may germinate either endogenously or exo- genously. The clade is defined by the sequence MG584193 (ITS1, 5.8S rDNA, ITS2, D1–D2 28S rDNA).

Feramyces austinii Radwa Hanafy, Mostafa Elshahed & Noha Youssef, sp. nov. FIGS. 3-1–3-2 MycoBank MB823651

Typification: The holotype is shown in FIG. 3-2h in this paper, derived from the following: USA. TEXAS: Val Verde, 29°53′N, 101°09′W, ~300 m above sea level (ASL), 3-d-old culture of isolate F3a, originally isolated from freshly deposited feces and rumen content of female Barbary sheep (*Ammotragus lervia*), Apr 2017, Radwa Hanafy. Ex-type strain: F3a.

GenBank: ITS + 28S = MG584193.

Etymology: The species epithet honors Mr. Jim Austin, who sampled the wild animals used for isolation of the fungus.

An obligate anaerobic fungus with a determinate monocentric thallus with the majority of thalli forming a single terminal sporangium, with the occasional formation of pseudo-intercalary sporangia, as well as sessile lateral sporangia. Extensive highly branched, anucleate rhizoidal system with both wide and narrow hyphae. The wide hyphae displayed multiple constrictions at irregular intervals. Mature endogenous sporangia were globose (10–70 μm) or pyriform (25–110 μm L × 15–45 μm W), whereas mature exogenous sporangia displayed a variety of shapes, including pyriform, triangular,

spherical, ovoid, heart-shaped, ellipsoidal, constricted ellipsoidal, and egg-shaped. Sporangiophores, variable in length (15–600 μm), frequently coiled or wide and flattened, often form an apophysis-like or eggcup-like swelling below the sporangium. Zoospores formed abundantly, spherical (6.5–13 μm diam.) with 7–16 flagella (17–37 μm long). Colonies grown on cellobiose liquid media exhibit a thick, biofilm-like growth on tube surfaces and form beige, circular, filamentous colonies with a darker central core (3–7 mm diam.) on agar roll tubes. The clade is defined by the sequence MG584193 (for ITS1, 5.8S rDNA, ITS2, D1–D2 28S rDNA).

Additional specimens examined: USA. TEXAS: Val Verde, 29°53′N, 101°09′W ~300 m above sea level. Freshly deposited feces of a female Barbary sheep (*Ammotragus lervia*), Apr 2017, Radwa Hanafy, strains F2a, F2c, F3b (isotype); ibid., rumen content of a female Barbary sheep (*Ammotragus lervia*), Apr 2017, Radwa Hanafy, strain R4a (isotype); ibid., freshly deposited feces of a female fallow deer (*Dama dama*), Sep 2017, Radwa Hanafy, strain DS10 (isotype).

Figure 3-1. Macroscopic features of *Feramyces austinii* (ex-type strain) in liquid culture and on agar roll tubes. A. Circular, beige, filamentous colonies with a darker central core of sporangia on cellobiose agar medium. Arrows depict a 2-d-old colony at the bottom and a 3-d-old colony at the top of the roll tube. B. Thick growth in liquid medium.

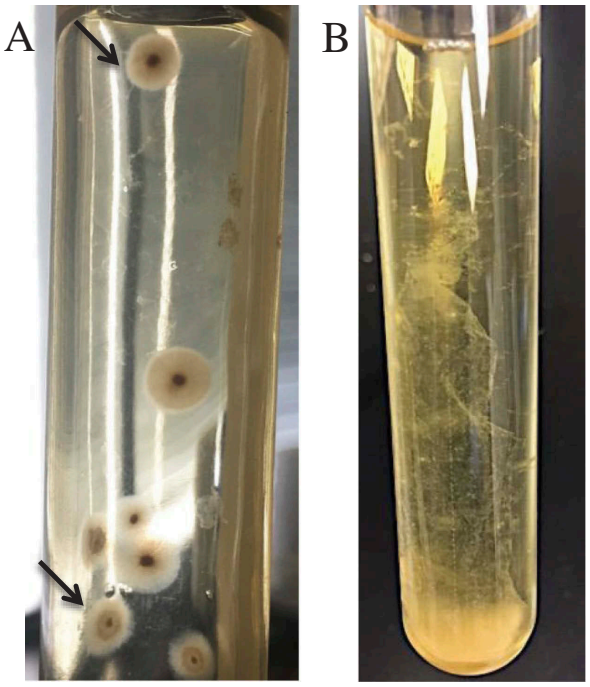
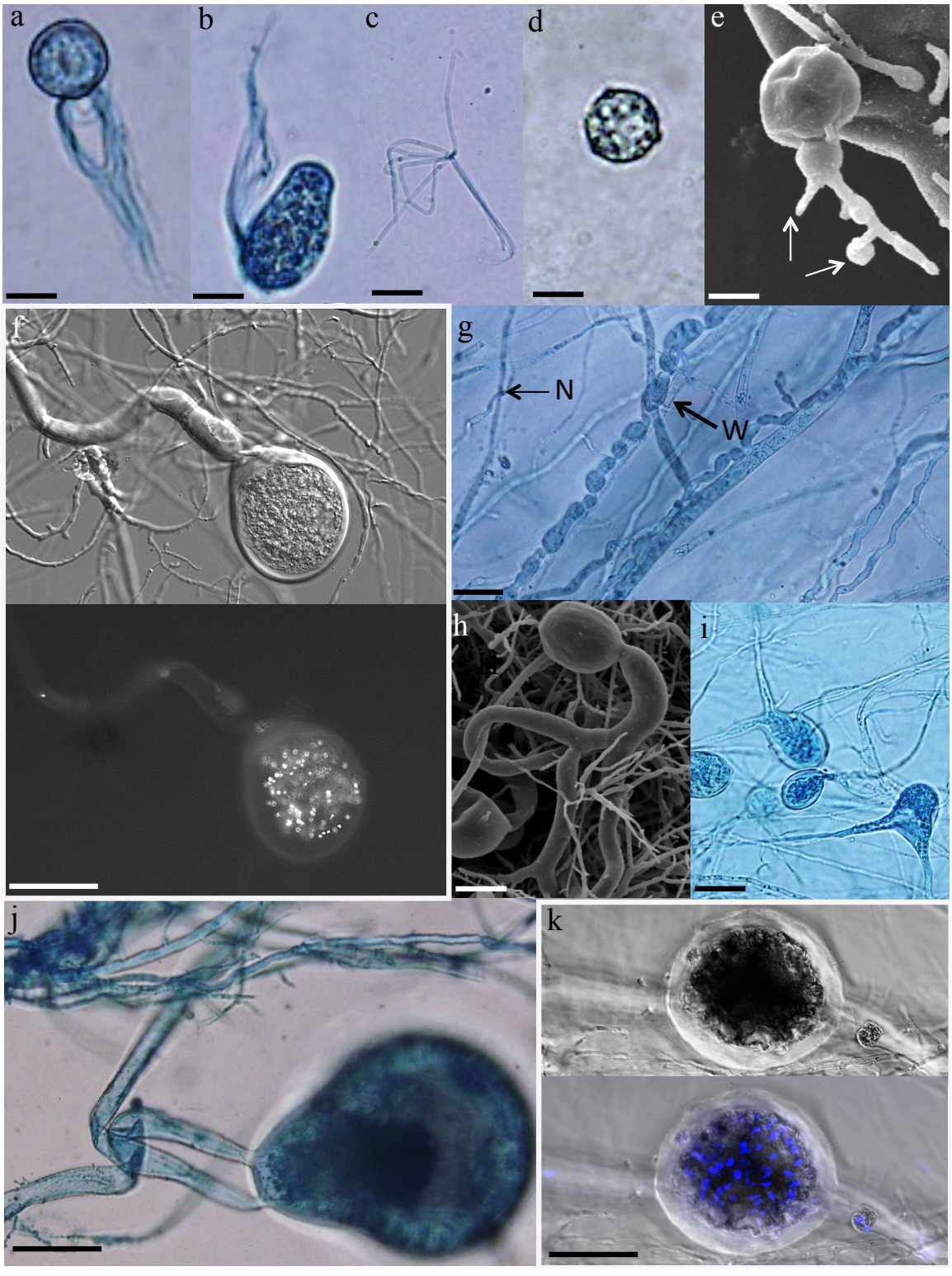


Figure 3-2. Microscopic features of *Feramyces austinii* (ex-type strain). Light (a–d, f, g, i–v, and x–z) and scanning electron (e, h, and w) micrographs are shown. a–e. Zoospores: a spherical polyflagellated zoospore (a), a motile polyflagellated zoospore (b), shed flagella (c), a zoospore cyst (d), and a germinating zoospore cyst with two germ tubes (arrows) (e) (note that one of the germ tubes is branching [arrows]). f. Monocentric thallus (DAPI stained; DIC). g. Rhizoidal system with narrow and wide hyphae. h–l. Endogenous sporangial development: h. Young globose sporangium with single rhizoidal system. i. Young pyriform sporangium with two main rhizoidal systems. j. Mature pyriform sporangium with two main rhizoidal systems. k. Globose pseudo-intercalary sporangium, between two main rhizoidal systems with nuclei located in the sporangium, but not the rhizoids (DAPI stained; CLSM). l. Mature sessile lateral globose sporangium. m–v. Exogenous sporangial development: m. Young globose sporangium on a short sporangiophore, arrows point to the zoospore cyst and the rhizoid. n. Pyriform sporangium on a long, coiled sporangiophore. o. Young ovoid sporangium on a wide flattened sporangiophore. p. Triangular-shaped sporangium. q. Spherical sporangium on a long sporangiophore ending with an apophysis, subsporangial swelling (arrow). r. Large ovoid sporangium exhibiting subsporangial swelling (arrow). s. Heart-shaped sporangium. t. Ellipsoidal sporangium. u. Constricted ellipsoidal sporangium. v. Egg-shaped sporangium exhibiting a subsporangial swelling. w–z. Zoospore release: w. A sporangium releasing its zoospores through a wide apical pore (arrow). x. An empty sporangium with partially intact wall after zoospore release. y. Sporangium broken off and detached from the sporangiophore. z. Shed sporangium (z). Abbreviations: N, narrow hyphae; W, wide hyphae; Sp, sporangiophore; Zc, zoospore cyst; R, rhizoid; DIC, differential interference contrast; CLSM, confocal laser scanning microscope. Bars: a–e, g–i, l, o, p, r, t, w = 20 µm; f, j,k,m,n,q,s,v,x–z=100µm.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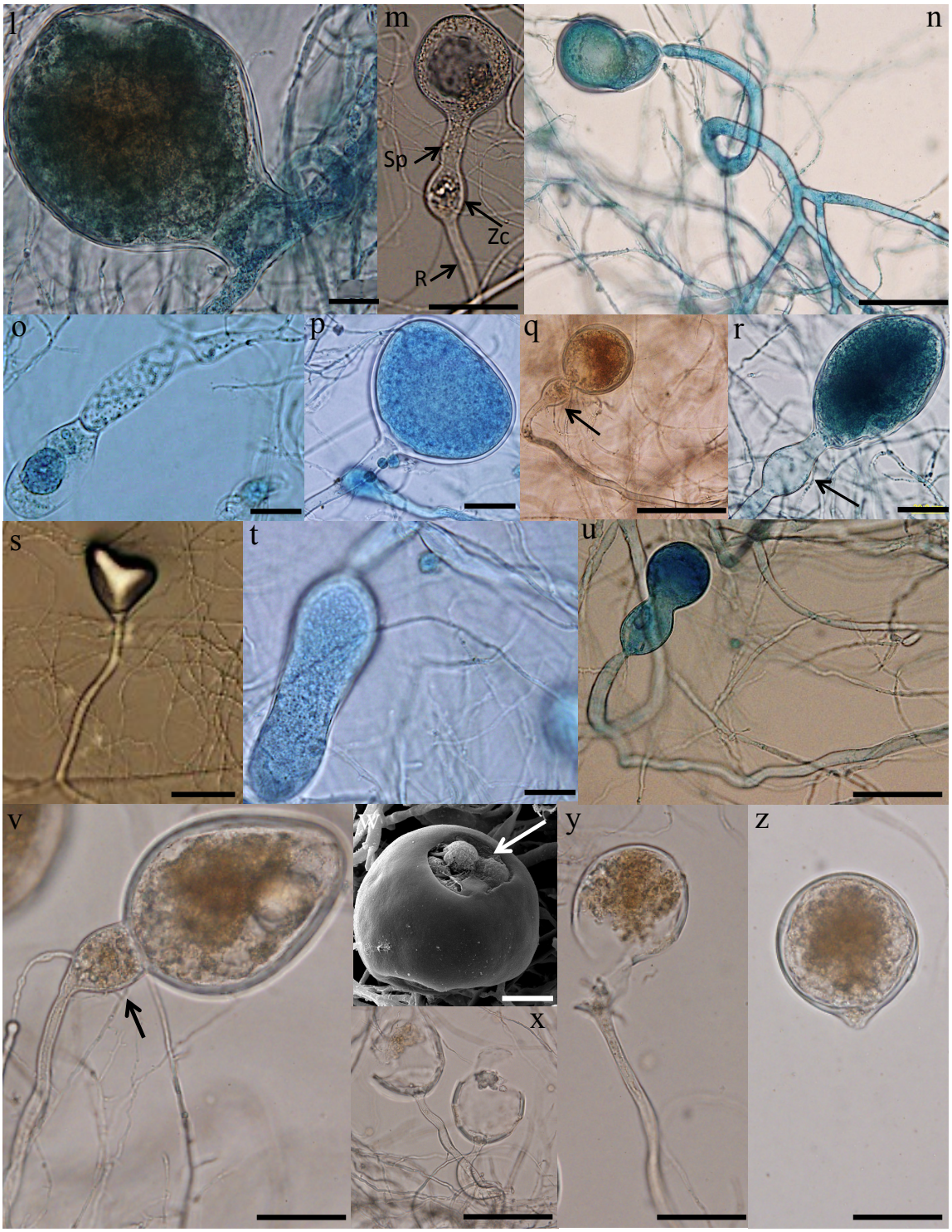
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Figure 3-3. A. Phylogenetic affiliation of the *Feramyces* clade to other AGF genera based on the sequences of the D1–D2 domains of nuc 28S rDNA gene. *Chytriomyces* sp. WB235A isolate AFTOL-ID 1536 was used as the outgroup (not shown). B. Phylogenetic affiliation of the *Feramyces* clade to other AGF genera based on partial ITS1 sequences. Five clones representing the 1913 sequences identified through BLASTn analysis to be potential members of the clade are added to the tree. *Chytriomyces* sp. WB235A isolate AFTOL-ID 1536 was used as the outgroup. The trees were obtained using both a maximum likelihood approach based on the Tamura-Nei model and a maximum parsimony method using the Subtree-Pruning-Regrafting (SPR) algorithm. Bootstrap values from 100 replicates are shown for nodes with more than 80% bootstrap support (as ML-BS/MP-BS). Analysis was conducted in MEGA7.

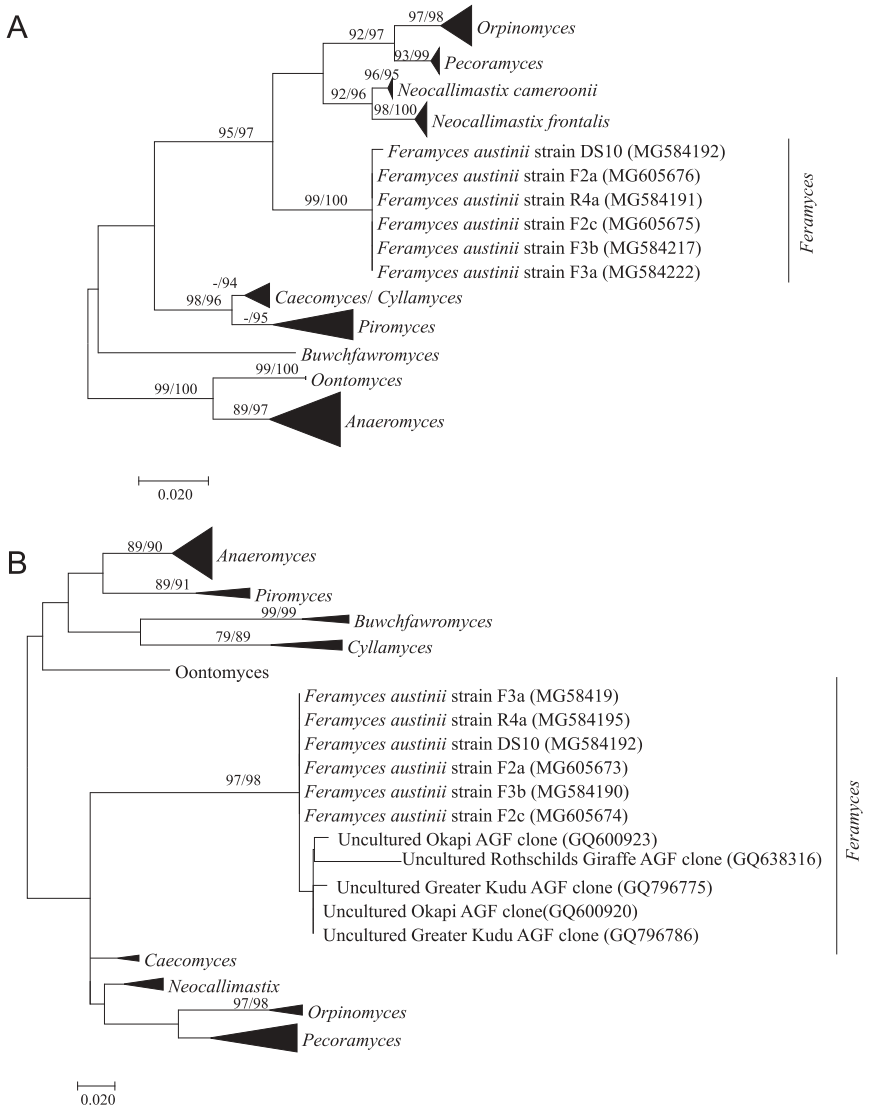

Table 3-1. Substrate utilization patterns by *Feramyces austinii* strain F3a.

Substrate		Growth[a]
Polysaccharides	Cellulose	+
	Xylan	+
	Starch	+
	Inulin	+
	Raffinose	+
	Poly-galacturonate	-
	Chitin	-
	Alginate	+
	Pectin[b]	+
Disaccharides	Cellobiose	+
	Sucrose	+
	Maltose	+
	Trehalose	+
	Lactose	+
Monosaccharides	Glucose	+
	Xylose	+
	Mannose	+
	Fructose	+
	Fucose	+
	Arabinose	+
	Ribose	-
	Glucuronic acid	+
	Galactose	+
Peptides	Peptone	-
	Tryptone	-

[a]Positive results are reported after five subcultures on the substrate.

[b]Growth on pectin was comparatively slow: visible biomass was usually obtained after ≈10 days post inoculation.

Table 3-2. Glucose utilization and growth rates of *Feramyces austinii* strain F3a compared with other anaerobic gut fungi when grown on glucose.

Genus	Glucose utilization rate ($mg.h^{-1}$)	Growth rate (µg biomass.h^{-1})
Feramyces austenii strain F3a	0.43	0.34
Pecoramyces ruminantium strain C1A	0.29	0.22
Neocallimastix frontalis strain Hef5	0.38	0.26
Anaeromyces contortus strain O2	0.10	0.29
Piromyces communis strain Jen1	0.29	0.16
Caecomyces sp. strain Brit4	0.18	0.30
Orpinomyces joyonii strain D4A	0.21	0.20

Table 3-3. Percentage plant dry weight loss when *Feramyces austinii* strain F3a, *Pecoramyces ruminantium* strain C1A, *Neocallimastix frontails* strain Hef5, *Anaeromyces contortous* strain O2, *Piromyces communis* strain Jen1, *Caecomyces* sp. strain Brit 4, and *Orpinomyces* sp. strain D4A were grown on untreated plant biomass as the sole carbon and energy source.

Genus	Percentage dry weight loss*	
	Corn stover	Switchgrass
Feramyces austenii strain F3a	72	56
Pecoramyces ruminantium strain C1A	65	52
Neocallimastix frontalis strain Hef5	55	36
Anaeromyces contortus strain O2	30	11
Piromyces communis strain Jen1	37	38
Caecomyces sp. strain Brit4	18	18
Orpinomyces joyonii strain D4A	30	43

*Plant biomass remaining at the end of incubation (7 days) was centrifuged and dried overnight at 40oC. Final dry weight remaining (DWf) was used to calculate the percentage dry weight loss using the equation: % dry weight loss = (DW0 – DWf)/DW0 x 100, where DW0 is the starting dry weight of plant biomass corrected for abiotic plant solubilization (calculated using uninoculated control).

Discussion

All isolated strains exhibited filamentous monocentric thallus developmental pattern (similar tothe genera *Neocalimastix, Pecoramyces, Piromyces, Bwchfawromyces, and Oontomyces*) and produced polyflagellated spores (similar to the genera *Orpinomyces* Neocallimastix). The genus *Feramyces* hence represents the second genus after *Neocallimastix* that exhibits a filamentous rhizoidal system, a monocentric thallus, and polyflagellated zoospores. However, several macroscopic and microscopic distinctions were observed between *Feramyces* strains and previously reported *Neocallimastix* spp. (*N. frontalis, N. hurleyensis* and *N. cameroonii*). Macroscopically, *Feramyces* formed larger colonies on agar roll tubes than those observed in *Neocallimastix frontalis* (Orpin 1994). In liquid medium, F3a developed a denser biofilm than that developed by *Neocallimastix* frontalis strain Hef5 from our culture collection. Microscopically, multiple sporangial shapes were observed in *Feramyces* strain F3a, and such sporangial morphological diversity has not been reported in *N. cameroonii* or *N. hurleyensis*, where the majority of their sporangia were spherical, columnar, or ovoid (Jill Webb and 1991).

Moreover, *Feramyces* differs from N. frontalis in their zoospores release mechanism. In *Feramyces*, zoospores were released either through a defined apical pore with the sporangial wall staying intact after zoospore release or through detachment of the whole sporangium. On the other hand, in N. frontalis, zoospores are released following lysis of the sporangial wall (Ho and Barr 1995). In addition to distinct microscopic differences, phylogenetic analysis also supported proposing a new Neocallimastigomycota genus to accommodate the isolated strains (FIG. 3-3).

ITS1-based phylogeny placed *Feramyces* as a sister genus to the genera *Neocallimastix*, *Caecomyces, Orpinomyces*, and *Pecoramyces* with a high bootstrap support. However, the value of using ITS1 to resolve supra-genus relationships in anaerobic fungi has been called into question due to difficulties associated with aligning this polymorphic and seemingly homoplasious region (Edwards and others 2017). 28S rDNA gene has been proposed as an alternative marker in AGF phylogeny (Dagar and others 2011; Edwards and others 2017) due to its lack of indels and uniform size across taxa, as well as sequence divergence levels that allow for phylogenetic resolution at the supra-genus,

genus, and species levels. D1-D2 28S rDNA gene-based phylogeny placed *Feramyces* as a distinct lineage basal to the *Neocalliamstix- Pecoramyces-Orpinomyces* clade (FIG. 3-3a). In addition to *Neocallimastix*, comparison of *Feramyces* strains to other members of the *Neocallimastix-Pecoramyces-Orpinomyces-Feramyces* supragenus clade suggested by D1-D2 28S rDNA analysis reveals multiple similarities and differences. When compared to members of the genus *Pecoramyces*, isolates from both genera develop a biofilm-like growth in liquid media.

Feramyces colonies on agar roll tubes are considerably larger than *Pecoramyces* colonies. Additionally, the spore flagellation pattern differs, with monoflagellation observed in *Pecoramyces* (Hanfay et al. 2017) strains and polyflagellation in *Feramyces* strains. When compared to members of the genus *Orpinomyces*, isolates from both genera produce polyflagellated zoospores. In addition, wide hyphae with constrictions are commonly observed in strains from both genera. Differences, on the other hand, include pattern of thallus development (monocentric in *Feramyces* versus polycentric in *Orpinomyces*, Ho and Jalaludin 1994; Li et al. 1991), and colony sizes, where *Orpinomyces* strains produce much larger colonies that could reach 1 cm in diameter (Orpin 1994).

All strains representing the novel genus *Feramyces* were isolated from fecal samples and rumen digesta of wild, undomesticated animals (Barbary sheep and fallow deer). Further, analysis of ITS1 AGF datasets obtained from prior culture-independent AGF diversity surveys suggested the occurrence of *Feramyces* in fecal samples from multiple additional wild animals (greater kudu, giraffe, and okapi) that were held in captivity at the Oklahoma City Zoo. Two different, but not mutually exclusive, hypotheses could describe the observed relatively restricted ecological distribution pattern for the genus *Feramyces*. The first posits that members of the genus *Feramyces* have an intrinsically limited phylogenetic distribution in a relatively restricted number of animal hosts, and that the animals' lifestyle (free, captive, or domesticated) play little role, if any, in its occurrence in the herbivorous gut. The role of animal host phylogeny in shaping anaerobic fungal community structure has been previously demonstrated (Liggenstoffer and others 2010). Under this scenario, the lack of prior reports describing members of this genus would be due to the paucity of isolation and

characterization efforts conducted on their few animal hosts. Further, the few prior studies reporting AGF isolates from *Feramyces*-hosting animals, e.g. deer (Nagpal and others 2011; Paul and others 2010), and Kudu (Tuckwell and others 2005) focused on assessing the fibrolytic activity of the obtained isolates, with only cursory morphological observations of isolates provided; hence possibly overlooking phylogenetically novel, but morphologically inconspicuous taxa.

The second possibility is that the abundance, colonization, and propagation of members of the genus *Feramyces* depends on the animal hosts' feeding patterns. Under this scenario, members of the genus *Feramyces* would be widely distributed in undomesticated and foraging animals, but would be outcompeted in domesticated herds provided with ample and monotonous substrates. The success of *Feramyces* in colonizing undomesticated and foraging animals could be due to their observed broad substrate utilization range (TABLE 3-1), allowing for more efficient utilization of sugars present even as minor components of plant biomass, e.g. arabinose, galactose, and fucose. Under this scenario, acquisition of wild animals into zoo settings and implementation of ample and monotonous feeding regimens is expected to result in the progressive decrease in the proportion of *Feramyces* in the host's gut, and its replacement with other AGF genera. As such, the identification of *Feramyces* in only a fraction of sampled zoo animals (3 out of 27) in a prior study (Liggenstoffer and others 2010) represents a gross underestimation of its actual distribution in nature. Targeting wild animals for isolation and culture-independent characterization efforts is crucial for testing these two hypotheses.

In conclusion, our efforts resulted in the isolation of members of a hitherto uncultured genus of AGF genus with distinct microscopic characteristics, substrate preferences, and ecological distribution patterns. We posit that undomesticated animals represent a relatively untapped reservoir of novel AGF genera, and that similar efforts could lead to the identification and isolation of multiple yet-unrecognized novel fungal taxa.

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CHAPTER IV

SEVEN NEW NEOCALLIMASTIGOMYCOTA GENERA FROM WILD, ZOO-HOUSED, AND DOMISTICATED HERBIVORES GREATLY EXPAND THE TAXONOMIC DIVERSITY OF THE PHYLUM

Abstract

We isolated and characterized 65 anaerobic gut fungal (AGF; Neocallimastigomycota) strains from fecal samples of five wild (W, axis deer, white-tailed deer, Boer goat, mouflon, and Nilgiri tahr), one zoo-housed (Z, zebra), and three domesticated (D, horse, sheep, and goat) herbivores in the US states of Texas (TX) and Oklahoma (OK), Wales (WA), and the Indian states of Kerala (KE) and Haryana (HA). Phylogenetic assessment using the D1–D2 regions of the large subunit (28S) rDNA and internal transcribed spacer 1 (ITS1) identified seven monophyletic clades that are distinct from all currently recognized AGF genera. All strains displayed monocentric thalli and produced exclusively or predominantly monoflagellate zoospores, with the exception of axis deer strains, which produced polyflagellate zoospores. Analysis of amplicon-based AGF diversity surveys indicated that zebra and horse strains are representatives of uncultured AL1 group, whereas domesticated goat and sheep strains are representatives of uncultured AL5 group, previously encountered in fecal and rumen samples of multiple herbivores. The other five lineages, all of which were isolated from wild herbivores, have not been previously encountered in such surveys. Our results significantly expand the genus-level diversity within the Neocallimastigomycota and strongly suggest that wild herbivores represent a yet-untapped reservoir of AGF diversity. We propose seven novel genera and eight novel Neocallimastigomycota species to comprise these strains, for which we propose the names *Agriosomyces longus* (mouflon and wild Boer goat), *Aklioshbomyces papillarum* (white-tailed deer), *Capellomyces foraminis* (wild Boar goat), and *C. elongatus* (domesticated goat), *Ghazallomyces constrictus* (axis deer), *Joblinomyces apicalis* (domesticated goat and sheep), *Khoyollomyces ramosus* (zebra-horse), and *Tahromyces munnarensis* (Nilgiri tahr).

Introduction

Members of the anaerobic gut fungi (AGF; phylum Neocallimastigomycota) colonize the alimentary tract of mammalian herbivores (Gruninger et al. 2014). Within the herbivorous gut, motile flagellated zoospores of AGF are released from sporangia and attach to ingested plant material. Zoospore encystment and germination results in the production of rhizoidal growth that penetrates and digests plant biomass through the production of a wide array of cellulolytic and hemicellulolytic enzymes (Gruninger et al. 2014). With their anaerobic fermentative lifestyle (Youssef et al. 2013), wide array of lignocellulolytic enzymes (Youssef et al. 2013; Couger et al. 2015; Solomon et al. 2016; Henske et al. 2017), and superior biomass degradation capacity (Youssef et al. 2013), anaerobic gut fungi are regarded as a promising platform for biofuel and biogas production from lignocellulosic biomass (Ranganathan et al. 2017; Young et al. 2018).

Currently, 11 different AGF genera have been described (Heath et al. 1983; Gold et al. 1988; Barr et al. 1989; Breton et al. 1990; Ozkose et al. 2001; Ariyawansa et al. 2015; Callaghan et al. 2015; Dagar et al. 2015; Li et al. 2016; Hanafy et al. 2017, 2018; Joshi et al. 2018). The inherent difficulty in isolating and maintaining these strictly anaerobic and senescence-prone organisms severely hampers isolation and characterization efforts and limits the number of research groups dedicated to uncovering AGF diversity. Further, it is entirely plausible that multiple AGF taxa are extremely fastidious, with complex nutritional requirements that are not satisfied in current isolation protocols. Indeed, culture-independent diversity surveys utilizing the internal transcribed spacer 1 (ITS1) as a phylogenetic marker demonstrated that multiple novel AGF lineages remain to be isolated and characterized (Liggenstoffer et al. 2010; Kittelmann et al. 2012; Mura et al. 2019).

On the other hand, culturing efforts have occasionally recovered novel AGF strains that bear no clear similarities to clades identified in culture-independent studies (Callaghan et al. 2015; Joshi et al. 2018). This surprising observation could be attributed to mismatches in the isolates' ITS1 region to commonly utilized ITS1 AGF primers, e.g., in case of *Buwchfawromyces eastonii* strain GE09 (Callaghan et al. 2015), as well as the extremely narrow host range of some AGF taxa (Callaghan et al. 2015).

Moreover, it is important to note that although both culture-based and culture-independent surveys have reported the presence of AGF communities in a relatively wide range of animal hosts, such studies by no means represent an exhaustive catalog of global AGF

diversity in nature. For example, due to logistical considerations, the great majority of studies have utilized samples from domesticated herbivores, with efforts to isolate AGF strains from wild herbivores being extremely rare (Tuckwell et al. 2005; Paul et al. 2010; Nagpal et al. 2011).

In an effort to broaden the current Neocallimastigomycota global culture collection, we conducted a multiyear isolation effort targeting novel AGF taxa in fecal samples from a wide range of wild (W), zoo-housed (Z), and domesticated (D) herbivorous mammals. Here, we report on the isolation and characterization of seven novel AGF genera, including the first cultured representative of the hitherto uncultured AGF lineages AL1 and AL5 (Liggenstoffer et al. 2010). The results expand the known AGF genus-level diversity by over 50% (from 11 to 18) and strongly suggest that wild, undomesticated herbivores represent a yet-untapped reservoir of novel AGF taxa.

Materials and Methods

Samples. In the USA, fecal samples were obtained from axis deer (*Axis axis*), white-tailed deer (*Odocoileus virginianus*), mouflon sheep (*Ovis orientalis*), and Boer goat (*Capra aegagrus*) in two separate hunting expeditions in Sutton and Val Verde counties, Texas (TX), and Payne County, Oklahoma (OK), in Oct 2017 and Apr 2018 (TABLE 4-1). The hunting parties had all appropriate licenses, and the animals were shot either on private land with the owner's consent or on public land during the hunting season. Samples were also obtained from a Grevy's zebra (*Equus grevyi*) housed in the Oklahoma City Zoo in May 2018, with the sampling protocol approved by the Oklahoma City Zoo and Botanical Garden's Scientific Review Committee. All fecal samples were placed on ice on site, transferred to the laboratory within 24 h of collection, where they were immediately used as an inoculum for subsequent enrichment and isolation procedures.

In India, dried fecal samples were obtained from Nilgiri tahr (*Nilgiritragus hylocrius*) and domesticated but forest grazing goat (*Capra aegagrus hircus*) in Munnar in the state of Kerala. Fresh fecal samples were also obtained from domesticated goats and sheep (*Ovis aries*) in Sonipat in the state of Haryana. Fresh fecal samples were transferred to the laboratory within 24 h of collection, whereas dried fecal samples were transferred within 72 h of collection. In Wales, samples were obtained from two domesticated horses in Llanbadarn Fawr, Ceredigion. Samples were transferred to the laboratory for processing within 2 h of sampling.

Isolation procedures. In the USA, isolation of anaerobic fungal strains was conducted as previously described (Dagar et al. 2015; Hanafy et al. 2017). Fecal samples were suspended in rumen-fluid (RF) medium (Calkins et al. 2016; Hanafy et al. 2018), with either cellobiose or 0.5% cellobiose plus 0.5% switchgrass used as a substrate. Antibiotics (50 µg/mL kanamycin, 50 µg/mL penicillin, 20 µg/mL streptomycin, and 50 µg/ mL chloramphenicol) were added to inhibit growth of bacteria and methanogenic archaea. Samples were serially diluted by adding approximately 1 g of fecal sample to 9 mL of RF medium, shaking, and subsequently transferring 1 mL of this suspension to 9 mL RF medium up to a 10^{-5} dilution. Serial dilutions were incubated at 39 ºC for 24–48 h. Dilutions showing visible signs of growth (clumping or floating plant materials and production of gas bubbles) were then used for the preparation of roll tubes (Hungate 1969) on RF-cellobiose agar medium. Single colonies were picked into liquid RF-cellobiose medium, and at least three rounds of tube rolling and colony picking were conducted to

ensure purity of the obtained colonies. Isolates were maintained by biweekly subculturing into RF-cellobiose medium. In India, the fecal samples were homogenized in anaerobic diluent (McSweeney et al. 2005) using BagMixer (Interscience, Paris, France). One milliliter of homogenate was inoculated into 9 mL of fungal culture medium (Joshi et al. 2018) containing rice straw neutral detergent fiber (NDF) as the sole carbon source, and serially diluted up to 10^{-3} dilution. The antibiotics benzylpenicillin and streptomycin sulfate (final concentration 2 mg/mL) were used to inhibit the bacterial growth. Following incubation at 39 ° ± 1 C for 5–10 d, the tubes showing visible colonization of NDF were used to isolate pure cultures of anaerobic fungi using serum roll bottle method as described previously (Joshi et al. 2018). The colonies differing in morphology were picked, grown in liquid culture medium, and the roll bottle procedures were repeated until pure cultures were established.

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

Morphological characterization. The colony morphology of 3d-old cultures on roll bottles was examined using a stereomicroscope (Leica M205 FA; Buffalo Grove, IL) equipped with a digital camera (Leica DFC450 C) or directly from the roll tube. Samples for light and scanning electron microscopy were obtained from liquid cultures at various stages of growth. For visualization of fungal structures, fungal biomass was suspended in a drop of lactophenol cotton blue stain for 5 min and subsequently examined using an Olympus BX51 microscope (Olympus, Center Valley, Pennsylvania) equipped with a DP71 digital camera (Olympus) or a phase- contrast microscope equipped with a Canon DS126191 digital camera (Leica). For examination of localization of nuclei, samples were stained with 4′,6-diamidino-2-phenylindole (DAPI; 10 μg/mL) as previously described (Callaghan et al. 2015; Hanafy et al. 2018; Joshi et al. 2018) and examined using a Olympus BX51 fluorescence microscope equipped with a Brightline (Olympus) DAPI high-contrast filter set for DAPI fluorescence and a DP71 digital camera (Olympus), or an Olympus BX53 differential interference contrast (DIC) microscope equipped with a DP73 digital camera (Olympus). Sample preparation and scanning electron microscopy were conducted with a FEI

quanta (Hillsboro, Oregon) or a Carl Zeiss EVO MA15 (White Plains, NY) scanning electron microscope (Hanafy et al. 2017; Joshi et al. 2018).

Phylogenetic analysis. Biomass was harvested and crushed in liquid nitrogen. DNA was extracted from the ground fungal biomass using DNeasy PowerPlant Pro Kit (Qiagen, Germantown, Maryland) according to the manufacturer's instructions, or using the cetyltrimethylammonium bromide (CTAB) DNA extraction protocol (Joshi et al. 2018). To assess phylogenetic relationships, the ITS1 region and the D1– D2 regions of the 28S rDNA were amplified using the primer pairs MN100 (5′-TCCTACCCTTTGTGAA TTTG-3′)/MNGM2 (5′-CTGCGTTCTTCATCGTTGC G-3′) and NL1 (5′-GCATATCAATAAGCGGAGGAA AAG-3′)/NL4 (5′-GGTCCGTGTTTCAAGACGG-3′), respectively, as previously described (Tuckwell et al. 2005; Dagar et al. 2011). The resulting polymerase chain reaction (PCR) amplicon for the ITS1 region was cloned into a TOPO-TA cloning vector according to the manufacturer's instructions (Life Technologies, Carlsbad, California) and several clones were Sanger sequenced, whereas the purified 28S rDNA PCR amplicons were directly Sanger sequenced using the services of the Oklahoma State University DNA Core Facility or a commercial provider (1st BASE, Singapore). The obtained sequences were aligned to anaerobic fungal reference ITS1 and 28S rDNA sequences downloaded from the National Center for Biotechnology Information (NCBI) GenBank nr database using MAFFT aligner (Nakamura et al. 2018), and the alignments were used to construct maximum likelihood phylogenetic trees in MEGA7 (Kumar et al. 2016), using Chytriomyces sp. JEL176 (ITS1) or Chytriomyces sp. WB235A (28S rDNA) as the outgroup. Bootstrap values were calculated on the basis of 100 replicates.

Ecological distribution. We queried GenBank and ITS1 data sets (Liggenstoffer et al. 2010; Kittelmann et al. 2012; Paul et al. 2018) using BLAST with reference ITS1 sequences from isolates recovered in this study. The phylogenetic position of all closely related sequences (>87% sequence similarity) was evaluated by insertion into maximum likelihood trees. Taxonomy of uncultured taxa followed the schemes outlined in prior publications (Liggenstoffer et al. 2010; Kittelmann et al. 2012; Paul et al. 2018).

Accession numbers. Sequences generated in this study have been deposited in GenBank under accession numbers MK881965–MK882046, MK775304, MK775310–MK775313, MK775315, MK775321–MK775324, MK755326–MK755327, MK755330, and MK755333. Alignments and phylogenetic trees are available through TreeBASE under study accession URL http://purl.org/phylo/tree base/phylows/study/TB2:S24394.

Results

Isolation summary. Sixty-five different isolates were obtained and characterized in this study (TABLE 4-1). These isolates were obtained from fecal samples of five wild, undomesticated herbivores: axis deer (W-TX), white-tailed deer (W-OK), mouflon (W-TX), Boer goat (W-TX), and Nilgiri tahr (W-KE), one zoo- housed Grevy's zebra (Z-OK), two domesticated horses (D-WA), two domesticated goats (D-HA and D-KE), and a domesticated sheep (D-HA) (TABLE 4-1). Morphological, microscopic, and phylogenetic analysis described below grouped these isolates into seven distinct clades (labeled clades 1–7 in TABLE 4-1 according to alphabetical order of suggested genus names). Three clades were each obtained from one host animal only: clade 2 isolates from white-tailed deer, clade 4 isolates from axis deer, and clade 7 isolates from Nilgiri tahr. On the other hand, representatives of four clades were identified in more than one animal: mouflon–Boer goat strains (clade 1), Boer goat–domesticated goat strains (clade 3), domesticated sheep-goat strains (clade 5), and zebra- horse strains (clade 6). Within each clade, no specific morphological or microscopic decipherable differences were identified between different strains belonging to most of these clades, and one strain from each clade was chosen for detailed analysis (TABLE 4-1). The only two exceptions were (i) strains belonging to clade 3 (Boer goat–domesticated goat), where strains from wild Boer goat (W-TX) displayed distinct microscopic and phylogenetic differences from those obtained from the domesticated goat (D-KE) to warrant the detailed characterization and eventual description of two different strains (TABLE 1 and detailed descriptions below); and (ii) strains belonging to clade 6 (zebra- horse), where few microscopic, but negligible phylogenetic differences were observed between the 16 zebra strains (Z-OK) and the 5 horse strains (D-WA) identified. These differences are highlighted below, but we do not believe that they warrant the description of a new species, given the negligible sequence divergences between these strains. Below, we provide a detailed characterization of the type species for each of the novel seven genera.

Colony morphology and macroscopic growth characteristics. Clade 1 (mouflon–Boer goat) strain MS-2 (W-TX) produced small, light brown, circular colonies (0.2–1 mm diam.) on agar, and a thin biofilm- like growth in liquid media (FIG. 4-1 a). Clade 2 (white- tailed deer) strain WT-2 (W-OK) produced beige, circular colonies (from 0.5 to 2.5 mm diam.) with a brown central core of dense sporangial structures and an outer ring of light gray hyphal growth. In liquid media, it produced heavy growth of thick biofilms that firmly attached to the tube's glass surface (FIG. 4-1 b). Clade 3 (Boar goat–domesticated goat) strain BGB-11 (W-TX) produced small, brown, circular colonies (0.1–0.5 mm diam.), with dark center of sporangia structures and a thin fungal

biofilm in liquid media (FIG. 4-1 c), whereas Boar goat– domesticated goat strain GFKJa1916 (D-KE) produced compact, cottony, off-white colonies ranging in size from 2 to 3 mm, with a fluffy center of thick sporangial structures and surrounded by radiating rhizoids (FIG. 4-1 d). In liquid media, strain GFKJa1916 produced numerous fungal thalli attached to the glass bottles on initial days of growth, which later developed into thin, mat-like structures. Clade 4 (axis deer) strain Axs-31 (W-TX) produced small, white, circular colonies (from 1 to 44 mm diam.) with a white to light brown central core of dense sporangial structures on agar, and a thick fungal biofilm-like growth in liquid media (FIG. 4-1 e). Clade 5 (domesticated goat-sheep) strain GFH683 (D-HA) produced colonies ranging in size from 1 to 2 mm, having a dense, dark central core of abundant sporangial growth, surrounded by long and thin radiating rhizoids (FIG. 4-1 f). In liquid media, strain GFH683 produced numerous fungal thalli attached to the glass bottles on initial days of growth, which later developed into thin, mat-like structures. Clade 6 (zebra-horse) zebra strain ZS-33 (Z-OK) produced small yellow to yellowish brown irregularly shaped colonies (FIG. 4-1 g). In liquid media, the fungal thalli were loose and exhibited a sand-like appearance resembling liquid growth patterns generally observed with isolates belonging to the bulbous genera *Caecomyces* and *Cyllamyces* (personal observation) (FIG. 4-1g). Finally, clade 7 (Nilgiri tahr) strain TDFKJa193 (W-KE) colonies were smaller, approximately 1 mm in size, white in color with a compact and fluffy center, and surrounded by dotted circles of fungal thalli. In liquid media, the strain produced numerous fungal thalli attached to the glass bottles on initial days of growth, which later developed into thin, mat-like structures (FIG. 4-1 h).

Microscopic features. *Clade 1: mouflon–Boer goat strains (W-TX).* Strain MS2 produced small, globose zoospores, (2.7–)4.0 ± 1.1(–7.5) µm (n = 29). Zoospores were mainly monoflagellate, with a flagellum length of (16.6–)22 ± 3.8(–30) µm (n = 29), approximately 5–6 times longer than the zoospore body (FIG. 4-2 a). Biflagellate zoospores (FIG. 4-2 b) were rarely encountered. Zoospores germinated into monocentric thalli with filamentous anucleate rhizoidal systems (FIG. 4-2 c–d). Both endogenous and exogenous sporangia were observed, which were very homogenous and displayed no pleomorphism. Endogenous sporangia were globose, with a diameter range of 15–65 µm (FIG. 4-2 e–f). The rhizoid was swollen below the sporangial neck, which was tightly constricted (FIG. 4-2 e–f). Exogenous sporangia were also consistently globose and developed at the end of swollen sporangiophores (30–80 µm L × 5–10 µm W) (FIG. 2 g–h). The sporangial neck was constricted with a narrow neck port. Zoospores were released through dissolution and rupturing of the sporangial wall (FIG. 4-2 i).

Clade 2: white-tailed deer strains (W-OK). Strain WT-2 produced globose zoospores, (4.5–)7.4 ± 2.4(–13) µm (n = 35). Zoospores were mostly monoflagellate, with a flagellum length of (12–)22.8 ± 6.3(–35) µm (n = 35) (FIG. 4-3 a). Zoospores with two (FIG. 4-3 b) to three (FIG. 4-3 c) flagella were less frequently observed. Fungal thalli were consistently monocentric with filamentous anucleate rhizoids (FIG. 4-3 d). Germination of zoospores produced two types of monocentric thalli, endogenous and exogenous. Endogenous sporangia with single (FIG. 4-3e) and two adjacent (FIG. 4-3 f) rhizoidal systems were observed. Occasionally, pseudointercalary endogenous sporangia (sporangia present in the middle of two main rhizoids) were encountered (FIG. 4-3 g), similar to what have previously been observed with the genera *Oontomyces* (Dagar et al. 2015) and *Feramyces* (Hanafy et al. 2017). Exogenous sporangia developed at the end of unbranched sporangiophores of varying lengths from a few microns to 230 µm (FIG. 4-3 h–j). No morphological differences were noticed between endogenous and exogenous sporangia, and their shapes varied between ovoid (FIG. 4-3 e–f), globose (FIG. 4-3 g–h), obpyriform (FIG. 4-3 j–k and n–o), and ellipsoidal (FIG. 4-3 i and l). Many, but not all, sporangia were papillated, with one (FIG. 4-3 m–p) or two (FIG. 4-3 q) papillae. These papillated sporangia are similar to those previously observed in *Piromyces mae* (Li et al. 1990). It is believed that these papillae disintegrate to facilitate zoospore release. However, we were unable to observe zoospore discharge through papillae in strain WT-2.

Clade 3: Boer goat–domesticated goat strains (W-TX and D-KE). Boer goat strain BGB-11 produced globose zoospores, (4–)5.5 ± 0.97(–7) µm (n = 40). The major- ity of zoospores were monoflagellate, with a flagellum length of (15–)19.6 ± 3.2(–25) µm (n = 40) (FIG. 4-4 a). Occasionally, biflagellate zoospores were observed (FIG. 4-4 b). Zoospore encystment followed flagellar shedding (FIG. 4-4 c). Zoospore cyst germinated, producing germ tube (FIG. 4-4 d) that subsequently branched (FIG. 4-4e) into monocentric thalli with filamentous anucleate rhizoidal systems (FIG. 4-4f–g). The expansion of the zoospore cysts resulted in the formation of endogenous sporangia that were ellipsoidal (FIG. 4-4 h) and ovoid (FIG. 4-4 i). In addition to endogenous sporangia, exogenous sporangia were also observed at the end of unbranched sporangiophores, ranging in length between 20 and 150 µm (FIG. 4-4 j–p). Some of the sporangiophores ended with subsporangial swellings (FIG. 4-4 l–m). Exogenous sporangia varied in shape between ovoid (FIG. 4-4 k–l), ellipsoidal with a single constriction (FIG. 4-4 n), and globose (FIG. 4-4 o–p). Zoospores were liberated through a wide apical pore at the top of the sporangia followed by sporangial wall collapse (FIG. 4-4 m, q–r). Domesticated goat strain GFKJa1916 on the other hand produced globose zoospores (FIG. 4-5 a), ranging in average

diameter between 4 and 5 µm. The majority of zoospores were monoflagellate, with a flagellum length ranging between 15 and 20 µm. Bi- and triflagellate zoospores were also observed. Strain GFKJa1916 zoospores germinated either endogenously or exogenously into a single monocentric thallus, which was also confirmed by the presence of nuclei only in sporangia and their absence in rhizoids (FIG. 4-5 b–c). Endogenous sporangia varied in shape between cylindrical, elongate, globose, subglobose, ellipsoid, and obovoid, with size ranging between 8 and 10 and 140µmL × 60µmW (FIG. 4-5d–g). Unlike Boer goat strain BGB-11, exogenous sporangia in the domesticated goat strain GFKJa1916 developed at the end of long thick sporangiophores (up to 300 µm in some cases) (FIG. 4-5 h–l), and multisporangiate thalli were commonly observed with two sporangia of either the same (FIG. 4-5 j) or different (FIG. 4-5 K–l) shapes, similar to *Piromyces rhizinflatus* (Ho and Barr 1995) and *Neocallimastix frontalis* (Barr et al. 1995).

Clade 4: axis deer strains (W-TX). Strain Axs-31 produced globose zoospores, (6–)8.1 ± 1.3(–10.5) µm (n = 35). All zoospores were polyflagellate, ranging between 7 and 14 flagella and a flagellum length of (16–)23.5 ± 4.9(–31) µm (n = 35) (FIG. 4-6 a). Zoospores germinated into monocentric thalli with highly branched anucleate rhizoidal systems (FIG. 4-6 b–c). Strain Axs-31 exhibited both endogenous and exogenous monocentric thallus development. In endogenous thalli, zoospore cysts enlarged into new sporangia of different shapes, including globose (FIG. 4-6 d), tubular (FIG. 4-6 e), clavate (FIG. 4-6 f), and ellipsoidal (FIG. 4-6 g). Endogenous sporangia displayed tightly constricted necks with narrow ports (arrows in FIG. 4-6 d–g). During exogenous thallus development, zoospore cysts germinated from both ends. Rhizoids developed on one side, whereas sporangiophores developed on the opposite side. The empty zoospore cyst remained as a persistent swollen structure at the base of sporangiophore (FIG. 4-6 h). Exogenous sporangia developed at the end of unbranched sporangiophores of varied lengths. Short sporangiophores had an average length ranging between 6 and 20 µm (FIG. 4-6 h–i), whereas long sporangiophores extended up to 200 µm (FIG. 4-6 j). Some of the short sporangiophores had eggcup-shaped appearance (FIG. 4-6 k). Exogenous sporangia were ellipsoidal (FIG. 4-6 j), ovoid (FIG. 4-6 k), globose (FIG. 4-6 l), constricted ellipsoidal (FIG. 4-6 m), pyriform (FIG. 4-6 n), bowling pin-shaped (FIG. 4-6 o), and rhomboidal (FIG. 4-6 p). Sporangial necks were constricted with narrow port (FIG. 4-6 m–p). At maturity, a fine septum developed at the base of the sporangium (white arrow in FIG. 4-6 n and black arrow in FIG. 4-6 p). Zoospores were released through an apical pore followed by collapse of the sporangial wall (FIG. 4-6 q).

Clade 5: domesticated goat and sheep strains (D-HA). Strain GFH683 produced globose zoospores (FIG. 4-7 a–b), with an average diameter ranging between 5 to 6 µm. The majority of

zoospores were monoflagellate, although, biflagellate zoospores were occasionally observed (FIG. 4-7 a–b). Flagellum length ranged between 20 and 22 µm. Zoospores germinated to produce both endogenous and exogenous monocentric thalli (FIG. 4-7 c– f), as evidenced by the presence of a single sporangium per thallus, nucleated sporangia, but anucleate rhizoids. Endogenous sporangia were globose, subglobose, ovoid, and obovoid (FIG. 4-7 g), with sizes ranging between 8 and 10 and 40 µm L × 40 µm W. Exogenous sporangia were terminal and varied in shape between globose, ovoid, and obovoid with sporangiophores that varied in length from 20 to 80 µm (FIG. 4-7 h–i). Zoospores discharge occurred through gradual dissolution of a wide apical portion of sporangial wall, resulting in formation of an empty cup-shaped sporangium (FIG. 4-7 j–l). Such zoospore liberation patterns and empty cup-shaped sporangia were earlier documented for *Piromyces minutus* (Ho and Barr 1995). Heavy colonization of rice straw fibers by fungal rhizoids was observed (FIG. 4-7m).

Clade 6: zebra-horse strains (Z-OK and D-WA). Strain ZS-33 produced spherical zoospores, (6–)10.8 ± 3(–17) µm (n = 54). All zoospores were monoflagellate, with a flagellum length of (18–)26 ± 6.5(–40) µm (n = 54) (FIG. 4-8 a). After shedding their flagella, zoospores started to encyst (FIG. 4-8 b) and germinate, producing germ tube (FIG. 4-8 c). Germ tube branched and developed a highly branched anucleate rhizoidal system (FIG. 4-8 d–e). Both narrow, from 0.5 to 2.5 µm wide, and broad, from 3 to 12.5 µm wide, hyphae were observed; intercalary swellings were frequently encountered in the broad hyphae (arrow in FIG. 4-8 f). Both endogenous and exogenous sporangia were observed. Endogenous sporangia varied in shape and size. Small endogenous sporangia were mainly subglobose (from 20 to 60 µm diam.) (FIG. 4-8 g). Large endogenous (from 80 to160µmL×from 35 to 65µmW) sporangia were mainly ellipsoidal (FIG. 4-8 h). Exogenous sporangial size ranged between 80 and 270 µm L × 35 and 85 µm W and displayed a wider range of morphologies, e.g., heart-shaped (FIG. 4-8 k), ovoid (FIG. 4-8 l), and pyriform (FIG. 4-8 m). Sporangiophores ranged in length between 20 and 400 µm. Characteristically, strain ZS-33 displayed a multisporangiate thallus: the majority of sporangiophores were branched and bore two to four sporangia (FIG. 4-8 i–j). Similar sporangial morphology has previously been observed in members of the genera *Piromyces* (e.g., *P. rhizinflatus*) and *Caecomyces* (e.g., *C. communis*) (Akin et al. 1988, 1989; Breton et al. 1991). Unbranched sporangiophores with single sporangia were less frequently encountered (approximately 30% of observed sporangiophores, n = 50; Fig. 4-8 k–m). Zoospores were liberated through a wide apical pore at the top of the sporangia. The sporangial wall stayed intact after the discharge (FIG. 4-8 n–p). Further, mature sporangia frequently detached from hyphae or

sporangiophores, probably serving as an additional mean of fungal dispersal (FIG. 4-8 q). The type strain ZS-33 was obtained from zebra fecal samples collected at the Oklahoma City Zoo. No noticeable differences were observed between ZS-33 and all other strains (n = 15) obtained from zebra fecal samples from the Oklahoma City Zoo. On the other hand, two distinct microscopic differences were identified in strains from domesticated horses in Llanbadarn Fawr, Ceredigion, Wales. First, multisporangiate thalli, copiously observed in ZS-33, were extremely rare in Welsh horse strains; and second, distinct resting stages (FIC. 4-8 r) were often observed in Welsh horse strains, but never in Oklahoma City zebra strains. Whether these differences are distinct characteristics of each group of strains or induced by variations in medium composition as well as growth and incubation procedures remains to be seen.

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

Phylogenetic analysis. Phylogenetic analysis using 28S rDNA (FIG. 4-10 A) placed the isolated strains into seven monophyletic and bootstrap-supported lineages that were distinct from all currently described AGF genera. 28S rDNA sequence divergence estimates between various strains within a single clade ranged between 0% and 1%. Within the 28S rDNA taxonomic framework, clade 4 strains recovered from axis deer (W-TX) clustered within the *Orpinomyces*-

Neocallimastix-Pecoramyces-Feramyces suprageneric clade, whereas clade 3 strains recovered from Boer goat (W-TX) and domesticated goat (D-KE) clustered within the *Oontomyces-Anaeromyces- Liebetanzomyces* supragenus clade. On the other hand, clade 7 strains recovered from Nilgiri tahr (W-KE) and clade 5 strains recovered from domesticated goat and sheep (D-HA) formed two distinct new clades associated with the genus *Buwchfawromyces*. In contrast, clade 6 strains recovered from zebra-horse (Z-OK and D-WA) and clade 1 strain from mouflon–Boer goat (W-TX) formed two distinct clades associated together but with no specific affiliation to any suprageneric clade. The remaining clade represented by clade 2 strains isolated from white-tailed deer (W-OK) displayed no specific affiliation to any currently characterized genera or supragenus clades within the Neocallimastigomycota.

To investigate ITS1 sequence variability often reported within a single AGF strain, the ITS1 region was amplified, cloned, and sequenced from all type strains. ITS1 sequence divergence within type strains ranged between 0% for strain TDFKJa193 representative of the Nilgiri tahr clade and 0–8.4% (average 3.4%) for strain MS-2 representative of the mouflon–Boer goat clade. ITS1-based analysis confirmed the mono- phyletic and distinct nature of all seven lineages but yielded a different topology (FIG. 4-10 B), as consistently observed in prior studies (Hanafy et al. 2017; Wang et al. 2017). Of special note was the surprisingly high ITS1 sequence similarity of the Boer goat–domesticated goat clade represented by strains BGB-11 and GFKJa1916 (FIG. 10B) to an *Anaeromyces* sp. isolate GA-04 (GenBank accession number FJ912851.1, unpublished) and to *Anaeromyces* robustus (GenBank accession number NR_148182.1; Li et al. 2016). Average ITS1 sequence divergence between various clones of the Boer goat–domesticated goat clade and *Anaeromyces* sp. GA-04 was 1.2%, and that between various clones of the Boer goat–domesticated goat clade and A. robustus was 4.2%. However, stark morphological differences exist between Boer goat–domesticated goat strains and all members of the genus Anaeromyces, e.g., monocentric thalli as opposed to polycentric thalli, absence of hyphal constrictions as opposed to sausage-shaped hyphae with multiple constrictions, and occasional identification of biflagellate and triflagellate zoospores versus exclusively monoflagellate zoospores (TABLE 4-1; FIGS. 4-4 – 4-5). Such differences, in addition to the high ITS1 sequence divergence values between the Boer goat–domesticated goat clade and other members of the genus *Anaeromyces* (from 7.1 to 13.1% to *A. mucronatus* and from 8.5 to 18.6% to *A. contortus*), strongly sup- port the distinction between these strains and the genus *Anaeromyces*.

Ecological distribution. We queried the GenBank nr database to determine whether representatives of these seven novel clades were encountered in prior ITS1- based culture-

independent AGF diversity surveys. Multiple sequences with high (from 95.3 to 100%) sequence similarity to the ITS1 sequence of clade 6 zebra-horse strains (type strain ZS-33 and additional strains listed below; Z-OK and D-WA) were identified. These sequences were recovered from fecal samples obtained from multiple animals housed in the Oklahoma City Zoo and the environs of the city of Stillwater, Oklahoma, USA (Liggenstoffer et al. 2010), as well as from various locations (left and right dorsal colon, cecum, and right ventral colon) within the digestive tract of horses (Mura et al. 2019). This clade has previously been assigned the alphanumeric designation AL1 (Liggenstoffer et al. 2010; Kittelmann et al. 2012). As well, multiple sequences with high (96– 98.02%) sequence similarity to the ITS1 sequence of clade 5 domesticated goat and sheep strains (type strain GFH683 ITS1 and additional strains listed below; D-HA) were identified. These sequences were also identified in 14/30 samples examined in Liggenstoffer et al. (2010) and given the alphanumeric designation AL5 (Liggenstoffer et al. 2010; Kittelmann et al. 2012). AL5 sequences were encountered in foregut fermenters and typically represented a minor fraction of the overall AGF community within a specific sample. Interestingly, the highest reported relative abundance of AL5 (19.6%) was observed in fecal samples of a domesticated goat, the same animal from which clade 5 isolates were obtained.

Surprisingly, ITS1 sequences from the remaining five lineages (axis deer, white-tailed deer, mouflon–Boer goat, Boer goat–domesticated goat, and Nilgiri tahr) bore no close resemblance to all currently available ITS1 sequence data (TABLE 4-1), with highest similarity being 83% in white-tailed deer to sequences from bontebok, 84% in mouflon–Boer goat to sequences from bontebok, 88% in domesticated goat–domesticated sheep to sequences from horse, 89% in Nilgiri tahr strains to sequences from Okapi, 91% in axis deer to sequences from Llama, and 91–92% in Boer goat– domesticated goat strains to sequences from cow. As such, representatives of these novel lineages, all of which have been recovered from fecal samples of wild, nondomesticated herbivores, do not appear to correspond to any of the alphanumerically designated uncultured clades previously identified in prior culture- independent efforts.

TAXONOMY

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

MycoBank MB830737.

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

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

Obligate anaerobic fungus that produces small, sphe- rical, and mostly monoflagellate zoospores with an extremely long flagellum (22 ± 3.8 μm). Biflagellate zoospores are rarely encountered. Zoospores germinate into monocentric thalli with filamentous anucleate rhizoidal systems. Both endogenous and exogenous glo- bose sporangia are observed, which are very homogenous and display no pleomorphism. Rhizoids are swollen below the sporangial neck, which is tightly constricted. Zoospores are released through dissolution and rupturing of the sporangial wall. The clade is defined by the sequences MK882010–MK882013 (ITS1) and MK881996 (D1–D2 28S rDNA).
Agriosomyces longus Hanafy, Vikram B. Lanjekar, Prashant K. Dhakephalkar, T.M. Callaghan, Dagar, G. W. Griff., Elshahed & N.H. Youssef, sp. nov. MycoBank MB830738

Typification: The holotype (FIG. 4-2g) was derived from the following: USA. TEXAS: Val Verde County, 29.369° N, 100.829°W, ~300 m above sea level (asl), 3-d-old culture of isolate MS-2, originally isolated from freshly deposited feces of male mouflon sheep (*Ovis orientalis*), Apr 2018, Radwa Hanafy. Ex-type strain: MS-2.
GenBank: MK881996 (D1–D2 28S rDNA).

Etymology: The species epithet “longus” refers to the extremely long flagellum observed in zoospores of strain MS-2 (FIG. 4-2a).

Obligate anaerobic fungus that produces small, globose monoflagellate zoospores with an average diameter of 4 ± 1.1 μm. Zoospores are mainly monoflagellate, with rare occurrence of biflagellate spores. The average flagellum length was 22 ± 3.8 μm, approximately 5 to 6 times longer than the zoospore body. Zoospores germinate into monocentric thalli with filamentous anucleate rhizoidal systems. Both endogenous and exogenous sporangia are observed, which display no pleomorphism and both show

globose morphology. In endogenous sporangia, the rhizoids are swollen below the sporangial neck, which is tightly constricted. Exogenous sporangia develop at the end of swollen sporangiophores, and the sporangial neck is constricted with a narrow neck port. Zoospores are released through dissolution and rupturing of the sporangial wall. Produces small, brown spherical colonies on agar, and a thin biofilm-like growth in liquid media. The clade is defined by the sequences MK882010– MK882013 (ITS1) and MK881996 (D1–D2 28S rDNA).

Additional specimens examined: Strain MS-2: USA. TEXAS: Val Verde County, 29.369°N, 100.829°W ~300 m asl, 3-d-old culture of isolate MS-4, originally isolated from freshly deposited feces of male mouflon sheep (Ovis orientalis), Apr 2018, Radwa Hanafy, GenBank: MK881997 (D1–D2 28S rDNA). Strain BGS-13: USA. TEXAS: Val Verde County, 29.369°N, 100.829°W ~300 m asl, originally isolated from freshly deposited feces of a female Boer goat (*Capra aegagrus*), Apr 2018, Radwa Hanafy, GenBank: MK881995 (D1– D2 28S rDNA amplicon).

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

MycoBank MB830735

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

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

Obligate anaerobic fungus that produces globose monoflagellate zoospores. Zoospores with two to three flagella were less frequently observed. Zoospores germinate into monocentric thalli with filamentous anucleate rhizoids. Exhibits both endogenous and exogenous monocentric thallus development. Exogenous sporangia develop at the end of unbranched sporangiophores of varying lengths. No morphological differences are observed between endogenous and exogenous sporangia, with ovoid, globose, and obpyriform sporangial shapes noted. The clade is defined by the sequences MK882038–

MK882042 (ITS1) and MK882001 (D1–D2 28S rDNA). Aklioshbomyces papillarum Hanafy, Vikram B. Lanjekar, Prashant K. Dhakephalkar, T.M. Callaghan, Dagar, G.W. Griff., Elshahed & N.H. Youssef, sp. nov.

MycoBank MB830736

Typification: The holotype (FIG. 4-3m) was derived from the following: USA. OKLAHOMA: Payne County, 36.145°N, 97.007°W, ~300 m asl, 3-d-old culture of isolate WT-2, originally isolated from freshly deposited feces of female white-tailed deer (*Odocoileus virginianus*), Oct 2017, Radwa Hanafy. Ex-type strain: WT-2.

GenBank: MK882001 (D1–D2 28S rDNA).

Etymology: The species epithet "papillarum" refers to the papillae observed on the majority of strain WT-2 sporangia (FIG. 4-3 m–q).

Obligate anaerobic fungus that produces globose monoflagellate zoospores with an average diameter of 7.4 ± 2.4 μm. The majority of zoospores are monoflagellate, with zoospores with two to three flagella less frequently observed. Fungal thalli are consistently monocentric with filamentous anucleate rhizoids. Germination of zoospores produces two types of monocentric thalli, endogenous and exogenous. Endogenous sporangia with single and two adjacent rhizoidal systems are observed. Pseudointercalary endogenous sporangia are occasionally observed. Sporangiophores carrying exogenous sporangia exhibit varying lengths from a few microns to 230 μm. Endogenous and exogenous sporangia are ovoid, globose, obpyriform, and ellipsoidal. Sporangia are mostly papillated, with one or two papillae. Produces beige, circular colonies with a brown central core of dense sporangial structures and an outer ring of light gray hyphal growth on agar, and heavy growth of thick biofilms that firmly attached to the tube's glass surface in liquid media. The clade is defined by the sequences MK882038–MK882042 (ITS1) and MK882001 (D1–D2 28S rDNA).

Additional specimens examined: USA. OKLAHOMA: Payne County, 36.145°N, 97.007°W, ~300 m asl, 3-d-old culture of isolates WT-1, WT-3, WT-4, WT- 41, WTS-51, WTS-52, WTS-53, and WTS-54, originally isolated from freshly deposited feces of

female white- tailed deer (*Odocoileus virginianus*), Oct 2017, Radwa Hanafy. GenBank (D1–D2 28S rDNA): WT-1 (MK882000), WT-3 (MK881998), WT-4 (MK881999), WT-41 (MK882002), WTS-51 (MK882006), WTS-52 (MK882003), WTS-53 (MK882004), and WTS-54 (MK882005).

***Capellomyces* Hanafy, Vikram B. Lanjekar, Prashant K. Dhakephalkar, T.M. Callaghan, Dagar, G.W. Griff., Elshahed & N.H. Youssef, gen. nov.**
MycoBank MB830739.

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

Etymology: Capello = derived from the Latin word for goat; myces = the Greek name for fungus. Obligate anaerobic fungus that produces mostly monoflagellate zoospores. Occasionally, biflagellate zoospores are also observed. Zoospores germinate into monocentric thalli with filamentous anucleate rhizoidal systems. Both endogenous and exogenous sporangia are observed, with varying shapes and sizes. The clade is defined by the sequences MK882007–MK882009 (ITS1) and MK881975 (D1–D2 28S rDNA). *Capellomyces foraminis* Hanafy, Vikram B. Lanjekar, Prashant K. Dhakephalkar, T.M. Callaghan, Dagar, G. W. Griff., Elshahed & N.H. Youssef, sp. nov. MycoBank MB830740 Typification: The holotype (FIG. 4-4 n) was derived from the following: USA. TEXAS: Val Verde County, 29.369°N, 100.829°W, ~300 m asl, 3-d-old culture of isolate BGB-11, originally isolated from freshly deposited feces of a female Boer goat (Capra aegagrus), Apr 2018, Radwa Hanafy. Ex-type strain: BGB-11.

GenBank: MK881975 (D1–D2 28S rDNA).

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

Obligate anaerobic fungus that produces spherical monoflagellate zoospores. Biflagellate zoospores are occasionally observed. Zoospores start to encyst after shedding their flagella. Zoospore cyst germinates, producing germ tube that subsequently branches into monocentric thalli with filamentous anucleate rhizoidal systems. Endogenous and

exogenous sporangia are produced. Endogenous sporangia are ellipsoidal or ovoid. Exogenous sporangia are formed at the end of unbranched sporangiophores (ranging from 20 to 150 μm). Some of the sporangiophores exhibit subsporangial swellings. Exogenous sporangia are ovoid, ellipsoidal with a single constriction, and globose. Zoospores are liberated through a wide apical pore at the top of the sporangia followed by sporangial wall collapse. Colonies are small (0.1–0.5 mm diam), circular, and brown, with dark center of sporangia structures on agar. Produces thin fungal biofilm in liquid media. The clade is defined by the sequences MK882007–MK882009 (ITS1) and MK881975 (D1–D2 28S rDNA).

Additional specimens examined: USA. TEXAS: Val Verde County, 29.369°N, 100.829°W, ~300 m asl, 3-d-old culture of isolates BGB-2, BGC-12, BGS-11, and BGS-12, originally isolated from freshly deposited feces of a female Boer goat (*Capra aegagrus*), Apr 2018, Radwa Hanafy.

GenBank (D1–D2 28S rDNA): BGB-2 (MK881974), BGC-12 (MK881976), BGS-11 (MK881977), and BGS-12 (MK881978).

Capellomyces elongatus Hanafy, Vikram B. Lanjekar, Prashant K. Dhakephalkar, T.M. Callaghan, Dagar, G. W. Griff., Elshahed & N.H. Youssef, sp. nov. MycoBank MB830869 Typification: The holotype (FIG. 5k) was derived from the following: INDIA. KERALA: Town of Munnar, 10.219°N, 77.106°E ~2100 m asl, 3-d-old culture of isolate GFKJa1916, originally isolated from freshly deposited feces of a domesticated but forest grazing goat (*Capra aegagrus*), Sumit Dagar. Ex-type strain: GFKJa1916. GenBank: MK775315 (ITS1); MK775304 (D1–D2 28S rDNA).

Etymology: The species epithet “elongatus” refers to the characteristic long sporangiophore of exogenous sporangia. Obligate anaerobic fungus that produces globose monoflagellate zoospores. Biflagellate zoospores are occasionally observed. Zoospore cyst germinate both endogenously and exogenously to produce monocentric thalli with filamentous anucleate rhizoidal systems. Endogenous sporangia are cylindrical, elongate, globose, subglobose, ellipsoid, and obovoid, with sizes ranging between 10 and 140 μm L × 8 and 60 μm W. Exogenous sporangia developed at the end of long thick

sporangiophores (up to 300 μm). Multisporangiate thalli are commonly observed with two sporangia of either the same or different shapes. Colonies are compact of 2 to 3 mm size, cottony, and off-white in color with a compact and fluffy center made up of thick sporangia type structures, and surrounded by radiating rhizoids. Produces numerous fungal thalli that attach to the glass bottles on initial days of growth, which later develop into thin, mat-like structures in liquid media. The clade is defined by the sequence MK775304 (D1– D2 28S rDNA). Additional specimens examined: None.

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

MycoBank MB830733

Typification: Ghazallomyces constrictus Hanafy, Vikram B. Lanjekar, Prashant K. Dhakephalkar, T.M. Callaghan, Dagar, G.W. Griff., Elshahed & N.H. Youssef.
Etymology: Ghazallo = derived from the Arabic word for deer (Ghazalla); myces = the Greek name for fungus.

Obligate anaerobic fungus that produces polyflagellate zoospores. Zoospores germinate into monocentric thalli with highly branched anucleate rhizoidal systems. Exhibits both endogenous and exogenous monocentric thallus development. Sporangia produced from endogenous and exogenous thallus development are pleomorphic, exhibiting a wide range of sporangial shapes. During exogenous thallus development, zoospore cysts germinate from both ends, with rhizoids developing from one side and sporangiophore developing from the opposite side. The empty zoospore cyst remains as a persistent swollen structure at the base of unbranched sporangiophore that exhibits wide variations in length. Zoospores are released through an apical pore followed by collapse of the sporangial wall. The clade is defined by the sequences MK882043 (ITS1) and MK881971 (D1–D2 28S rDNA).

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

MycoBank MB830734

Typification: The holotype (FIG. 4-6 h) was derived from the following: USA. TEXAS: Sutton County, 30.591°N, 100.138°W, ~300 m asl, 3-d-old culture of isolate Axs-31, originally isolated from freshly depos- ited feces content of female axis deer (Axis axis), Apr 2018, Radwa Hanafy. Ex-type strain: Axs-31. GenBank: MK881971 (D1–D2 28S rDNA).

Etymology: The species epithet “constrictus” refers to the observed constricted necks (point between sporangia and rhizoids) in the species endogenous sporangia (FIG. 4-6 d–g).

Obligate anaerobic fungus that produces globose polyflagellate zoospores with 7 to 14 flagella. Zoospores germinate into monocentric thalli with highly branched anucleate rhizoidal systems. Exhibits both endogenous and exogenous monocentric thallus development. Endogenous sporangia produced from zoospore cyst enlargement develop into different shapes, including globose, tubular, clavate, and ellipsoidal. Endogenous sporangia display tightly constricted necks (point between sporangia and rhizoids) with narrow ports. Exogenous sporangia develop at the end of unbranched sporangiophores of varied lengths. Both short (from 6 to 20 μm) and long (up to 200 μm) sporangiophores are observed. The exogenous sporangia display ellipsoidal, ovoid, globose, constricted ellipsoidal, pyriform, bowling pin-like, and rhomboidal shapes. Sporangial necks are constricted with narrow port. A fine septum develops at the base of the sporangium at maturity. Zoospores are released through an apical pore followed by collapse of the sporangial wall. Produces small, white, circular colonies (1 to 4 mm diam) with a brown central core of dense sporangial structures on agar, and a thick fungal biofilm growth in liquid media. The clade is defined by the sequences MK882043 (ITS1) and MK881971 (D1–D2 28S rDNA). Additional specimens examined: USA. TEXAS: Sutton County, 30.591°N, 100.138°W, ~300 m asl, 3-d-old culture of isolate ADC-2, ADS-14, AXS-33, AXS-34, ADS-12, AXS-32, ADS-11, and ADS-21, ori- ginally isolated from freshly deposited feces content of female axis deer (Axis axis), Apr 2018, Radwa Hanafy. GenBank (D1–D2 28S rDNA): ADC-2 (MK881965), ADS-14 (MK881966), AXS-33

(MK881967), AXS-34 (MK1881968), ADS-12 (MK881969), AXS-32 (MK881970), ADS-11 (MK881972), and ADS-21 (MK881973).

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

MycoBank MB830867

Typification: *Joblinomyces apicalis* Hanafy, Vikram B. Lanjekar, Prashant K. Dhakephalkar, T.M. Callaghan, Dagar, G.W. Griff., Elshahed & N.H. Youssef.

Etymology: Joblino = honoring Keith N. Joblin for his contributions to the field of anaerobic fungi; myces = the Greek name for fungus.

Obligate anaerobic fungus that produces globose monoflagellate zoospores. Biflagellate zoospores were occasionally observed. Both endogenous and exogenous sporangia are observed, with varying shapes and sizes. Sporangiophores of exogenous sporangia vary in length. Exogenous sporangia have short and frequently swollen sporangiophores. Zoospores discharge occurs through gra- dual dissolution of a wide apical portion of sporangial wall, resulting in formation of an empty cup-shaped sporangium. The clade is defined by the sequences MK910278 (ITS1) and MK910268 (D1–D2 28S rDNA).

Joblinomyces apicalis Hanafy, Vikram B. Lanjekar, Prashant K. Dhakephalkar, T.M. Callaghan, Dagar, G. W. Griff., Elshahed & N.H. Youssef, sp. nov. MycoBank MB830868

Typification: The holotype (FIG. 4-7c) was derived from the following: INDIA. HARYANA: City of Sonipat, 28.988°N, 76.941°E ~220 m asl, 3-d-old culture of isolate GFH683, originally isolated from freshly deposited feces of a domesticated goat (*Capra aegagrus hircus*), Sumit Dagar. Ex-type strain: GFH683. GenBank: MK910268 (D1–D2 28S rDNA).

Etymology: The species epithet “apicalis” refers to the zoospore discharge through the dissolution of a wide apical portion of the sporangial wall.

Obligate anaerobic fungus that produces globose monoflagellate zoospores. Biflagellate zoospores were occasionally observed. Zoospores germinate to produce both endogenous and exogenous monocentric thalli. Endogenous sporangia vary in shape between globose, subglobose, ovoid, and obovoid, with sizes ranging between 8 and 10 and 40 μm L × 40 μm W. Exogenous sporangia are terminal and vary in shape between globose, ovoid, obovoid. Sporangiophores vary in length from 20 to 80 μm. Zoospores discharge occurs through gradual dissolution of a wide apical portion of sporangial wall, resulting in formation of an empty cup-shaped sporangium. Produces 1 to 2 mm-sized colonies with a dense, dark central core of abundant sporangial growth, surrounded by long and thin radiat- ing rhizoids. In liquid media, it produces numerous fungal thalli that attach to the glass bottles on initial days of growth and later develop into thin, mat-like structures. The clade is defined by the sequences MK882019 (ITS1) and MK881981 (D1–D2 28S rDNA).

***Khoyollomyces ramosus* Hanafy, Vikram B. Lanjekar, Prashant K. Dhakephalkar, T.M. Callaghan, Dagar, G. W. Griff., Elshahed & N.H. Youssef, sp. nov.**

MycoBank MB830742

Typification: The holotype (FIG. 4-8j) was derived from the following: USA. OKLAHOMA: Oklahoma City, 35.524°N, 97.472°W, ~300 m asl, 3-d-old culture of isolate ZS-33, originally isolated from freshly depos- ited feces of a Grevy's zebra (Equus grevyi), May 2018, Radwa Hanafy. Ex-type strain: ZS-33. GenBank: MK881981 (D1–D2 28S rDNA).

Etymology: The species epithet "ramosus" (Latin for branched) refers to the observed branched sporangio- phores bearing two to four sporangia in K. ramosus type strain ZS-33 (FIG. 4-8i–k).

Obligate anaerobic fungus that produces spherical monoflagellate zoospores. Zoospores encyst and germinate producing germ tube that develops into a highly branched anucleate rhizoidal system. Both narrow, 0.5 to 2.5 μm W, and broad hyphae, 3 to 12.5 μm W, are produced; intercalary swellings are frequently encountered in the broad hyphae. Both

endogenous and exogenous sporangia were observed. Endogenous sporangia vary in shape and size, with small endogenous sporangia mainly subglobose (ranging from 20 to 60 μm diam) and large endogenous (from 80 to 160 μm L × from 35 to 65 μm W) sporangia mainly ellipsoidal. Exogenous sporangia ranged in size between 80 and 270 μm L × 35 and 85 μm W and display a wide range of morphologies, e.g., heart-shaped, ovoid, and pyriform. Displays a multisporangiate thallus, with the majority of sporangiophores being branched and bearing two to four sporangia. Unbranched sporangiophores with single sporangia are less frequently encountered (approximately 30% of observed sporangiophores). Zoospores are liberated through a wide apical pore at the top of the sporangia. The sporangia stay intact after the discharge. Mature sporangia frequently detach from hyphae or sporangiophores. Produces small, yellow to yellowish brown, irregularly shaped colonies on agar. In liquid media, the fungal growth is loose and exhibited a sand-like appearance. The clade is defined by the sequences MK882019 (ITS1) and MK881981 (D1–D2 28S rDNA).

Additional specimens examined: USA. OKLAHOMA: Oklahoma City, 35.524°N, 97.472°W, ~300 m asl, 3-d-old culture of isolates ZC-31, ZC-32, ZC-33, ZC-41, ZC-42, ZC-43, ZC-51, ZC-53, ZS-21, ZS-22, ZS-31, ZS-32, ZS-41, ZS-42, and ZS-43, originally isolated from freshly deposited feces of a Grevy's zebra (Equus grevyi), May 2018, Radwa Hanafy, GenBank (D1–D2 28S rDNA): ZC-31 MK910278–MK910282 (ITS1) and MK910272 (D1–D2 28S rDNA). MK910268–

Additional specimens examined: INDIA. HARYANA: City of Sonipat, 28.988°N, 76.941°E, ~220 m asl, 3-d-old culture of isolates strains GFH681 and GFH682, originally isolated from freshly deposited feces of a domesticated goat (*Capra aegagrus hircus*), Sumit Dagar. Genbank (D1–D2 28S rDNA): GFH681 (MK910263–MK910267) and GFH682 (MK775330).

Khoyollomyces Hanafy, Vikram B. Lanjekar, Prashant K. Dhakephalkar, T.M. Callaghan, Dagar, G.W. Griff., Elshahed & N.H. Youssef, gen. nov. MycoBank MB830741

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

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

Obligate anaerobic fungus that produces spherical monoflagellate zoospores. Zoospores encyst and develop a highly branched anucleate rhizoidal system. Both endogenous and exogenous sporangia are observed. Small endogenous sporangia are subglobose and large endogenous sporangia are ellipsoidal. Exogenous sporangia displayed a wider range of shapes. The majorities of sporangiophores are branched and bear two to four sporangia. Unbranched sporangiophores bearing a single sporangium are less frequently encountered. Zoospores are liberated through a wide apical pore at the top of the sporangia. Mainly found in the digestive tracts of equids. The clade is defined by the sequences (MK881979), ZC-32 (MK881980), ZC-33 (MK881981), ZC-41 (MK881982), ZC-42 (MK881983), ZC-43 (MK881984), ZC-51 (MK881985), ZC-53 (MK881986), ZS-21 (MK881987), ZS-22 (MK881988), ZS-31 (MK881989), ZS-32 (MK881990), ZS-41 (MK881992), ZS-42 (MK881993), and ZS-43 (MK881994). WALES: Aberystwyth; 52.4156°N, −3.8878°W, 3-d-old culture of isolates HoCal4.A2, HoCal4.A2.2, and HoCal4.A4, originally isolated from freshly deposited feces of one horse (*Equus caballus*), Aug 2013, and strains Tmc003.6a and TMC3.6b from freshly deposited feces of another host in the same location, Nov 2013, Tony Callaghan.

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

MycoBank MB830865

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

Etymology: Tahro = referring to the Nilgiri tahr from which the species was isolated; myces = the Greek name for fungus.

Obligate anaerobic fungus that produces globose monoflagellate zoospores. Biflagellate and triflagellate zoospores are rare. Both endogenous and exogenous sporangia are observed with varying shapes and sizes. Endogenous sporangia with one or two main

rhizoidal systems and with branched rhizoidal system are frequently observed. Exogenous sporangia have short and frequently swollen sporangiophores. Sporangial necks are frequently constricted. Septa often form at the base of mature exogenous sporangia. Zoospore liberation happens after irregular dissolution of the sporangial wall. The clade is defined by the sequences MK775321 (ITS1) and MK775310 (D1–D2 28S rDNA).

Tahromyces munnarensis Hanafy, Vikram B. Lanjekar, Prashant K. Dhakephalkar, T.M. Callaghan, Dagar, G. W. Griff., Elshahed & N.H. Youssef, sp. nov. MycoBank MB830866

Typification: The holotype (FIG. 4-9 h) was derived from the following: INDIA. KERALA: Town of Munnar, 10.219°N, 77.106°E, ~2100 m asl, 3-d-old culture of isolate TDFKJa193, originally isolated from freshly deposited feces of a Nilgiri tahr (Nilgiritragus hylocrius), Sumit Dagar. Ex-type strain: TDFKJa193. GenBank: MK775321 (ITS1); MK775310 (D1–D2 28S rDNA).

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

Obligate anaerobic fungus that produces globose monoflagellate zoospores. Biflagellate and triflagellate spores are rarely identified. Both endogenous and exogenous sporangia were observed. The sporangia vary in size between 12 and 100 μm in length and between 10 and 70 μm in width and display a wide range of morphologies such as globose, ovoid, and obovoid. Sporangiophores are short (12 to 20 μm) with frequent subsporangial swellings. Sporangial necks (1 to 8 μm W and 2 to 10 μm L) are frequently constricted. Septa often form at the base of mature exogenous sporangia. Zoospore liberation happens after irregular dissolution of the sporangial wall. Produces colonies that are small (1 mm), white in color with a compact and fluffy center, surrounded by dotted circles of fungal thalli. In liquid media, it produces numerous fungal thalli attaching to the glass bottles on initial days of growth and later developing into thin, mat-like structures. The clade is defined by the sequence MK775310 (D1–D2 28S rDNA).

Additional specimens examined: INDIA. KERALA: Town of Munnar, 10.219°N, 77.106°E, ~2100 m asl, 3-d-old culture of isolates TDFKJa1924, TDFKJa1926, and TDFKJa1927, originally isolated from freshly deposited feces of a Nilgiri tahr (Nilgiritragus hylocrius), Sumit Dagar. GenBank (D1–D2 28S rDNA): TDFKJa1924 (MK775312.1), TDFKJa1926 (MK775311.1), and TDFKJa1927 (MK775313.1).

Figure 4-1. Macroscopic features of colony morphology on agar media and fungal biomass in liquid media. a. *Agriosomyces longus* strain MS2 (W-TX). b. *Aklioshbomyces papillarum* strain WT-2 (W-OK). c. *Capellomyces foraminis* strain BGB-11 (W-TX). d. *Capellomyces elongatus* strain GFKJa1916 (D-KE). e. *Ghazallomyces constrictus* strain Axs-31 (W-TX) f. *Joblinomyces apicalis* strain GFH683 (D-HA). g. *Khyollomyces ramosus* strain ZS-33 (Z-OK). h. *Tahromyces munnarensis* strain TDFKJa193 (W-KE).

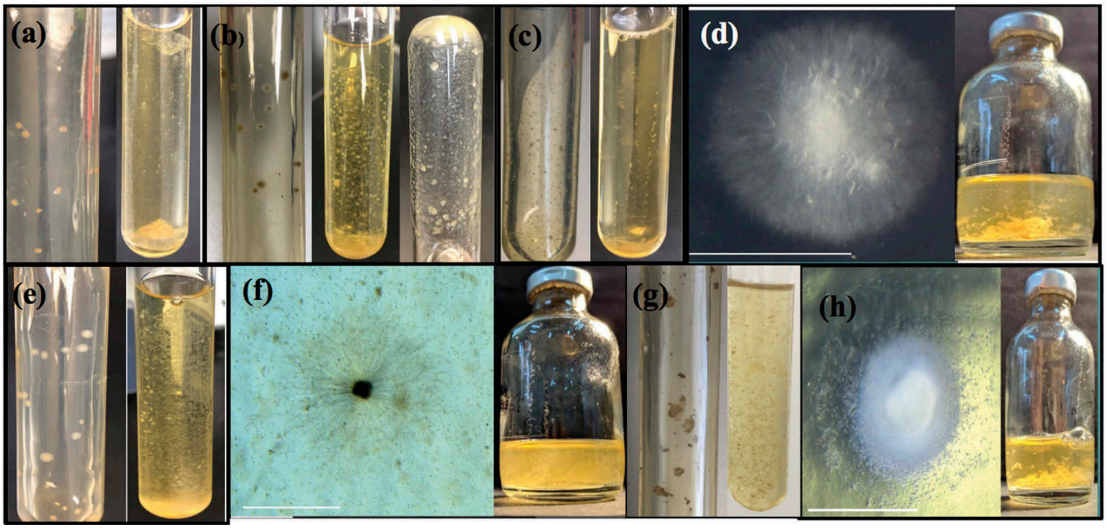

Figure 4-2. Microscopic features of *Agriosomyces longus* (clade 1, mouflon–Boer goat) strain MS2 (W-TX). Light (b, e–h) and scanning electron (a, i) micrographs. c. DAPI staining for visualizing nuclei using a fluorescence microscope equipped with a Brightline DAPI high-contrast filter set. d. Overlay image. a. A monoflagellate zoospore (Z). b. A biflagellate zoospore. c–d. Monocentric thalli, with nuclei occurring in sporangia, not in rhizoids or sporangiophores. e–f. Endogenous globose sporangia with tightly constricted necks and subsporangial swellings (arrows). g–h. Exogenous globose sporangia; note the swollen sporangiophores (arrows). i. An empty sporangium (S) after zoospore (Z) release and rupturing of the sporangial wall. Bars: a = 5 μm; b = 20 μm; c–i = 50 μm.

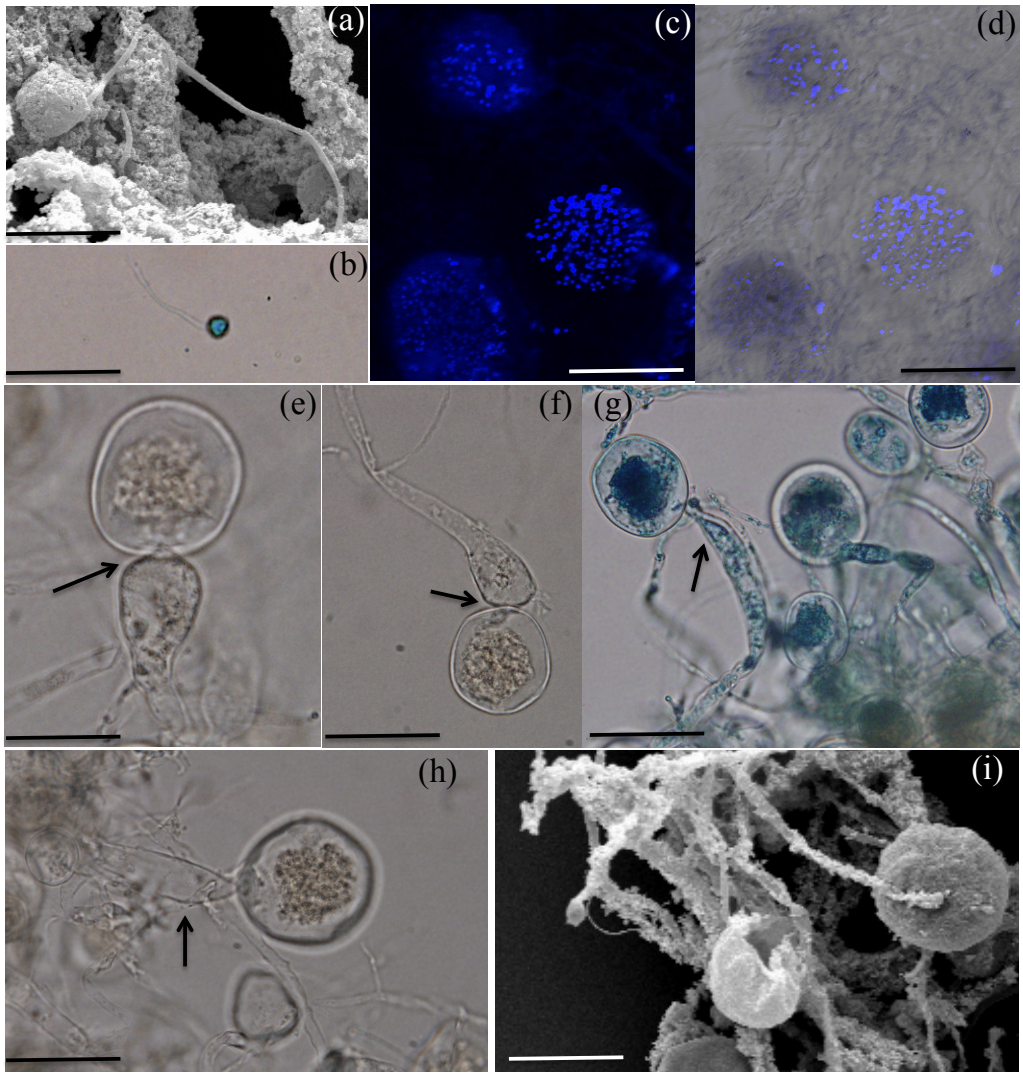

Figure 4-3. Microscopic features of *Aklioshbomyces papillarum* (clade 2, white-tailed deer) strain WT-2 (W-OK). Light (a–e, g–q) and scanning electron (f) micrographs. Light microscopy images were examined after staining with lactophenol cotton blue (a–c, e, g–q), as well as following staining of nuclei with DAPI (d). a. A monoflagellate zoospore. b. A biflagellate zoospore. c. A triflagellate zoospore. d. Monocentric thalli, with nuclei occurring in sporangia, not in rhizoids or sporangiophores. e–g. Endogenous sporangial development: e. Ovoid sporangium with single rhizoidal system. f. Ovoid sporangium with two main rhizoidal systems. g. Globose pseudointercalary sporangium, between two main rhizoidal systems. h–k. Exogenous sporangial development: h. Globose sporangium on a very short sporangiophore. i. Ellipsoidal sporangium. j. Obpyriform sporangium on a long sporangiophore. k. Obpyriform sporangium. l. Ellipsoidal sporangium. m. Sporangia with lateral single papilla. n–p. Sporangia with terminal single papilla. q. Sporangium with two papillae. Bars: a–c, e, f, n, o = 20 μm; g–k, p, q = 50 μm; d, l, m = 100 μm.

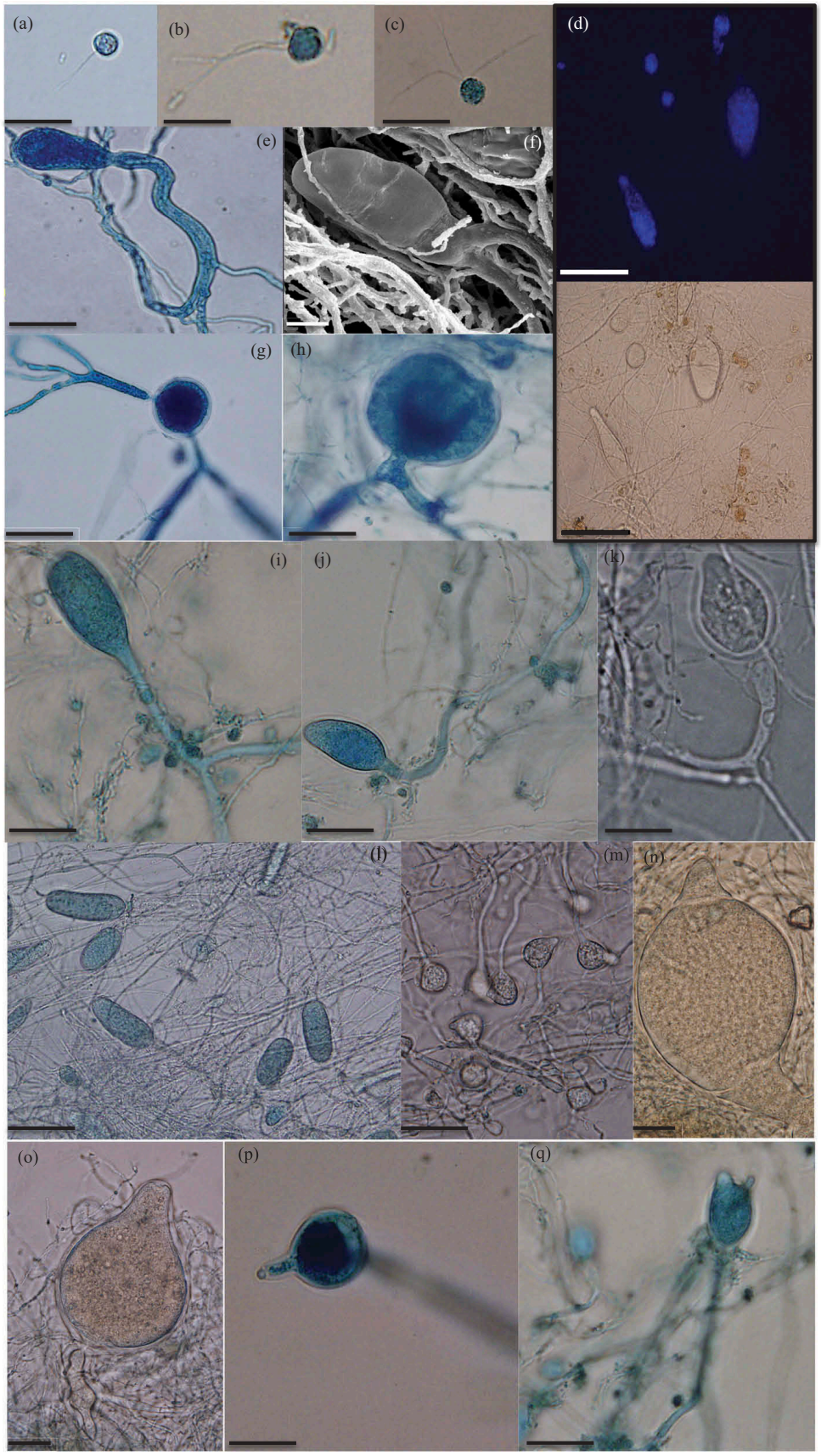
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Figure 4-4. Microscopic features of *Capellomyces foraminis* (clade 3, Boer goat) strain BGB-11 (W-TX). Light (a–i, l, o, p) and scanning electron (j, k, m, n, q, r) micrographs. Light microscopy images were examined after staining with lactophenol cotton blue (a–e, h, i, o), as well as following staining of nuclei with DAPI (f). g. Overlay image. a. A monoflagellate zoospore. b. A biflagellate zoospore. c. Zoospore cyst; arrow points to the shed flagellum. d. Germinating zoospore cyst producing a germ tube (arrow). e. Rhizoidal system development. f–g. Monocentric thalli with nuclei occurring in sporangia, not in rhizoids or sporangiophores. h–i. Endogenous sporangial development: h. Ellipsoidal sporangium with two main rhizoidal systems (arrows). i. Ovoid sporangium with single rhizoidal system. j–q. Exogenous sporangial development: j. Exogeous sporangium with a short sporangiophore (Sp); note the empty zoospore cyst (Zc). k. Ovoid sporangium with a long sporangiophore. l. Ovoid sporangium on a long sporangiophore ending with subsporangial swelling (arrow). m. Collapsed empty sporangium on a long sporangiophore ending with subsporangial swelling (arrow). n. Constricted ellipsoidal sporangium. o–p. Globose sporangia. q. Zoospores are released through apical pore. r. An empty sporangium following zoospores release. Sp = sporangiophore; Zc = zoospore cyst. Bars: a–j, l, o, p = 20 μm; k, m, n, q, r = 50 μm.

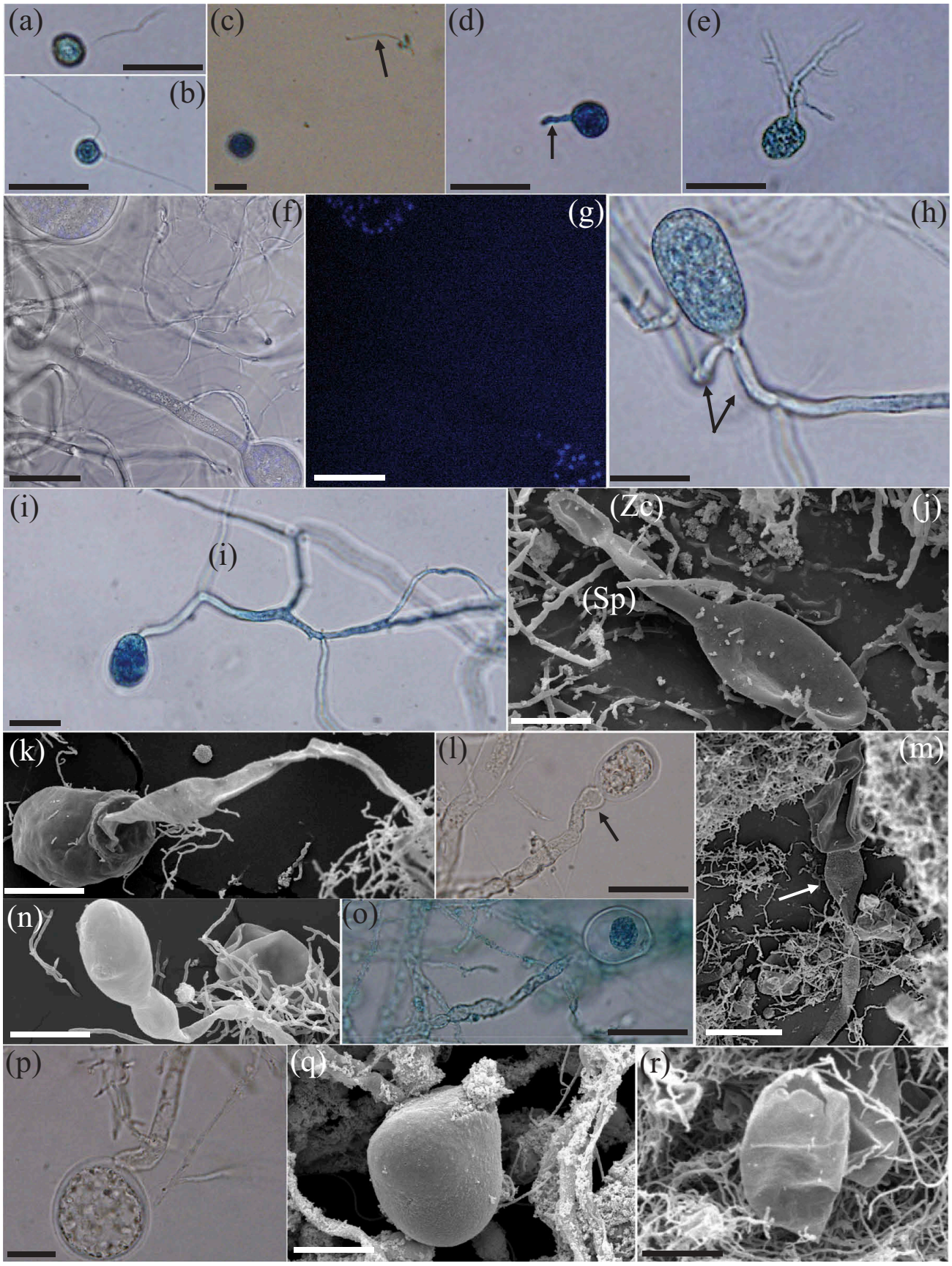
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Figure 4-5. Microscopic features of *Capellomyces elongatus* (clade 3, domesticated goat) strain GFKJa1916 (D-KE). Differential interference contrast (a, d–f, i–l), scanning (g–h), phase-contrast (b), and fluorescence (c) micrographs. a. A monoflagellate zoospore. b–c. Monocentric thalli; nuclei were observed in sporangia, not in rhizoids or sporangiophores. d–g. Endogenous sporangia: d. Globose endogenous sporangium with one main rhizoidal system. e–f. Endogenous sporangia with multiple rhizoidal systems. g. Endogenous sporangium on wheat straw fibers. h–l. Exogenous sporangia: h. Ovoid-shaped sporangium with long sporangiophore. i. Multiple ovoid and globose exogenous sporangia with long sporangiophores. j. Multisporangiate thallus with two sporangia (same shape). k– l. Multisporangiate thallus with two sporangia (different shapes). Bars = 20 μm.

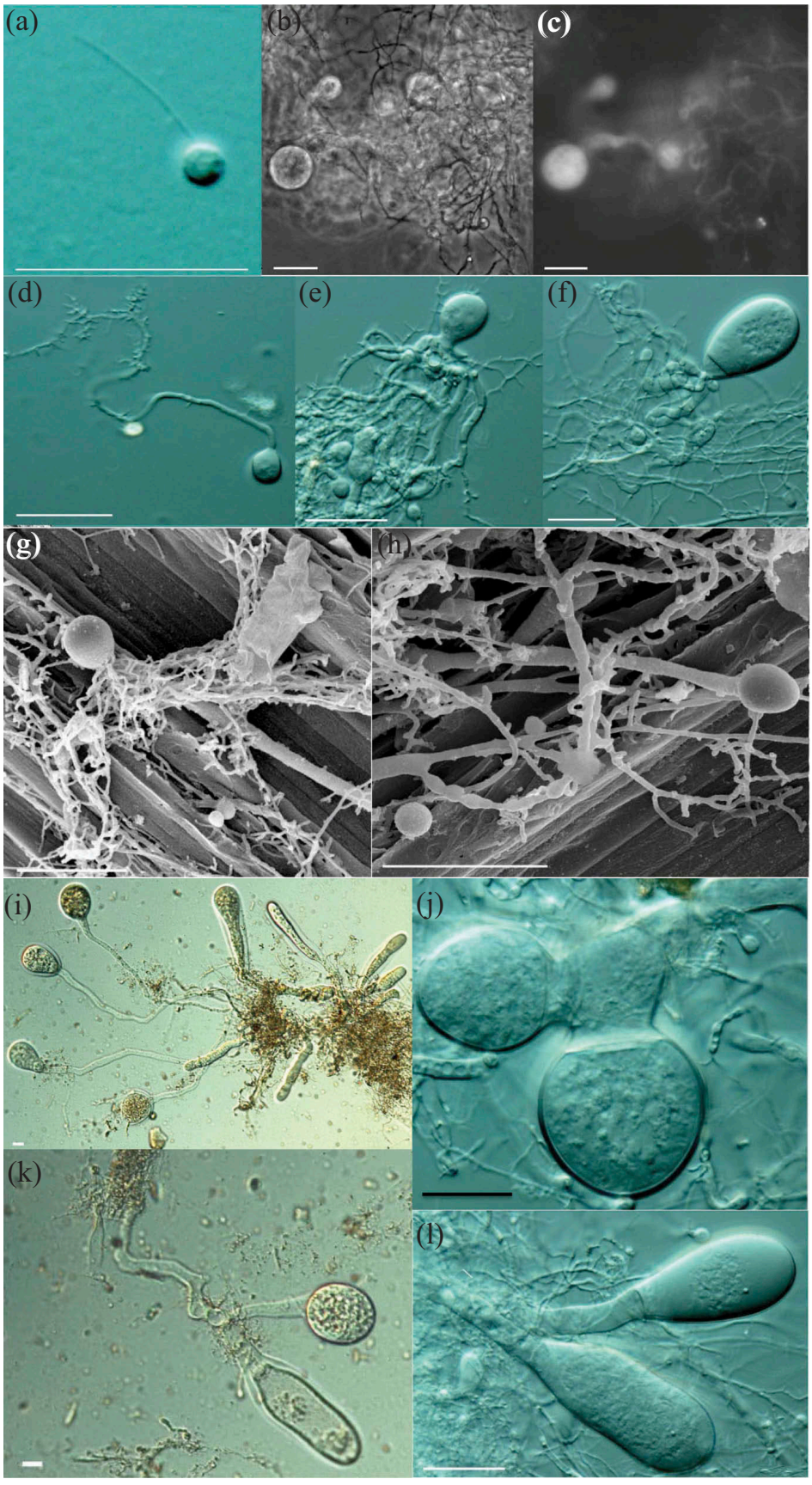
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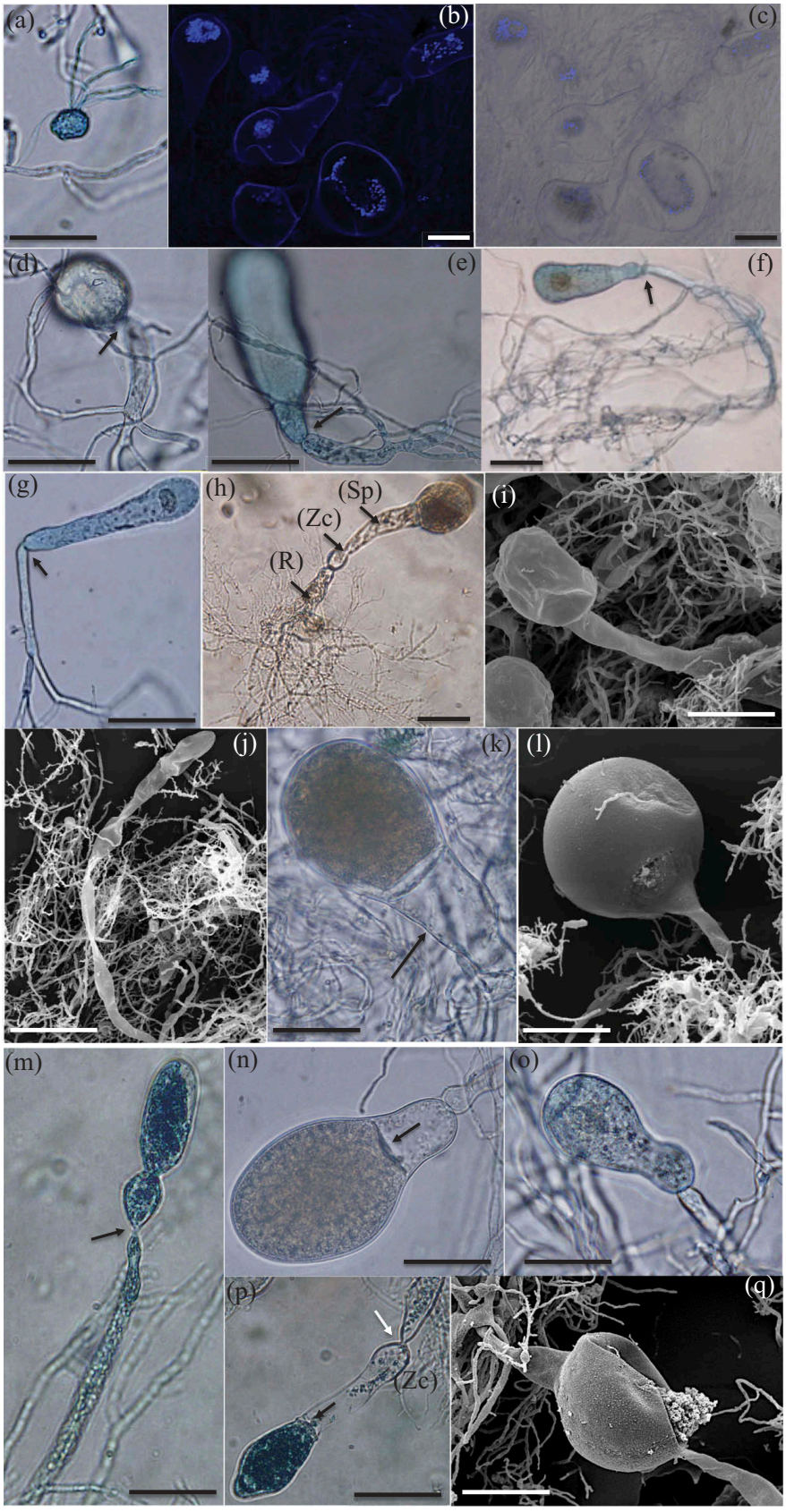
Figure 4-6. Microscopic features of Ghazallomyces constrictus (clade 4, axis deer) strain Axs-31 (W-TX). Light (a–h, f, k, m–p) and scanning electron (i, j, l, q) micrographs. Light microscopy images were examined after staining with lactophenol cotton blue (a, d–h, k, m–p), as well as following staining of nuclei with DAPI (b). c. Overlay image. a. A polyflagellate zoospore. b–c. Monocentric thalli, with nuclei occurring in sporangia, not in rhizoids or sporangiophores. d–g. Endogenous sporangia with tightly constricted necks (arrows): d. Young globose sporangium. e. Young tubular sporangium. f. Mature clavate sporangium. g. Mature ellipsoidal sporangium. h–p. Exogenous sporangia: h. Young sporangium on a short, flattened sporangiophore (Sp); note the persistent empty zoospore cyst (Zc) and the rhizoidal system (R). i. Ovoid sporangium on short sporangiophore. j. Ellipsoidal sporangium on long sporangiophore. k. Ovoid sporangium on an eggcup-shaped sporangiophore (arrow). l. Globose sporangium. m. Constricted ellipsoidal sporangium with tightly constricted neck (arrow) on long sporangiophore. n. Pyriform sporangium; note the fine septum at the base of sporangium (arrow). o. Bowling pin-shaped sporangium. p. Rhomboidal sporangium with constricted neck (white arrow) and fine septum (black arrow); note the persistent empty zoospore cyst (Zc). q. Zoospores are released through apical pore followed by collapse of the sporangial wall. Sp = sporangiophore; Zc = zoospore cyst; R = rhizoid. Bars: a = 20 μm; b–q = 50 μm.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(Zc)
(q)

Figure 4-7. Microscopic features of *Joblinomyces apicalis* (clade 5, domesticated goat and sheep) strain GFH683 (D-HA). Phase-contrast (a, b, c, e), fluorescence (d, f), scanning electron (g–j, m), and differential interference (k–l) micrographs. a. A monoflagellate zoospore. b. A biflagellate spherical zoospore. c–f. Monocentric thalli; nuclei were observed in sporangia, not in rhizoids or sporangiophores; note the empty cup-shaped sporangium after zoospore release (arrow in c). g. Ovoid endogenous sporangium. h–i. Exogenous sporangia: h. Ovoid sporangium with short sporangiophore. i. Subglobose sporangium with long sporangiophore. j–l. Zoospore release: j. Dissolution of the apical portion of sporangial wall (arrow). k–l. Cup-shaped sporangia with wide apical pores and intact sporangial walls (arrows). m. Colonization of rice straw fibers by fungal rhizoids. Bars = 20 μm.

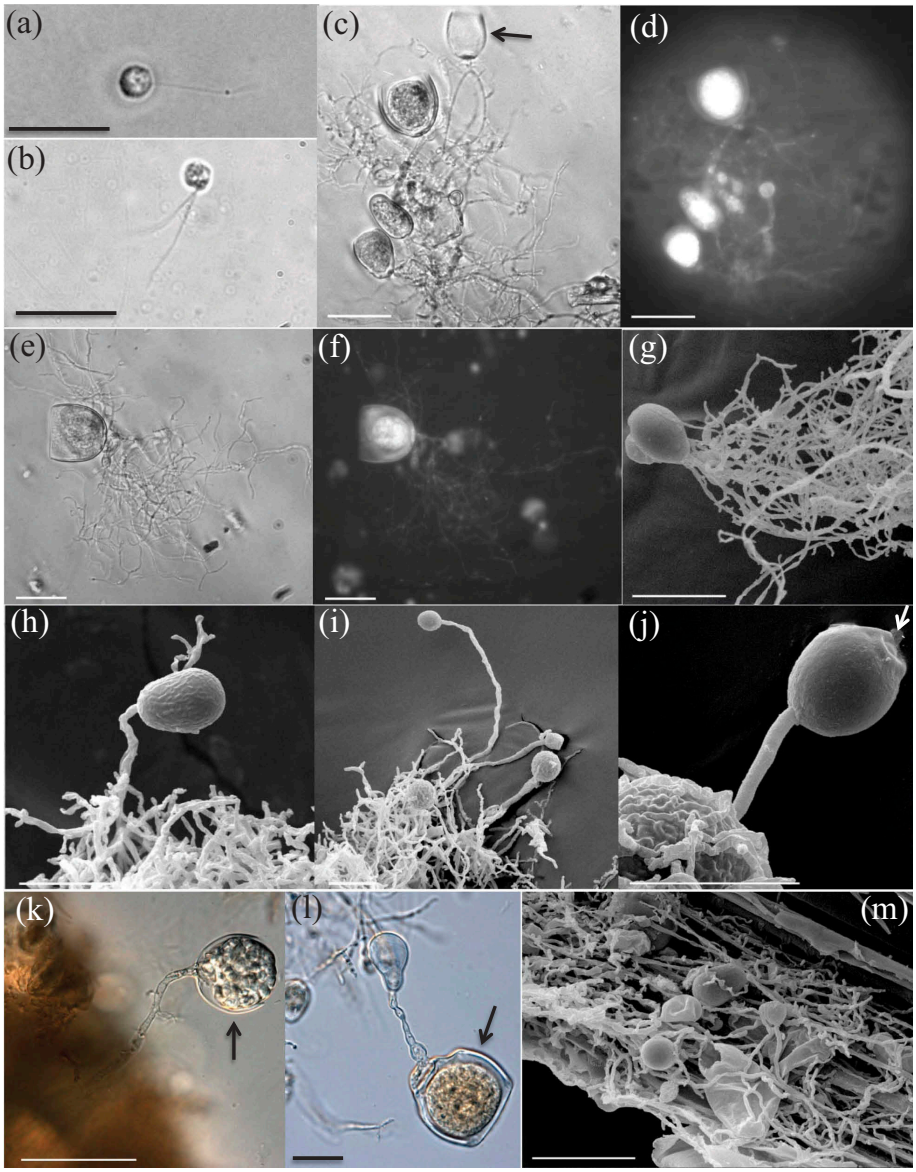
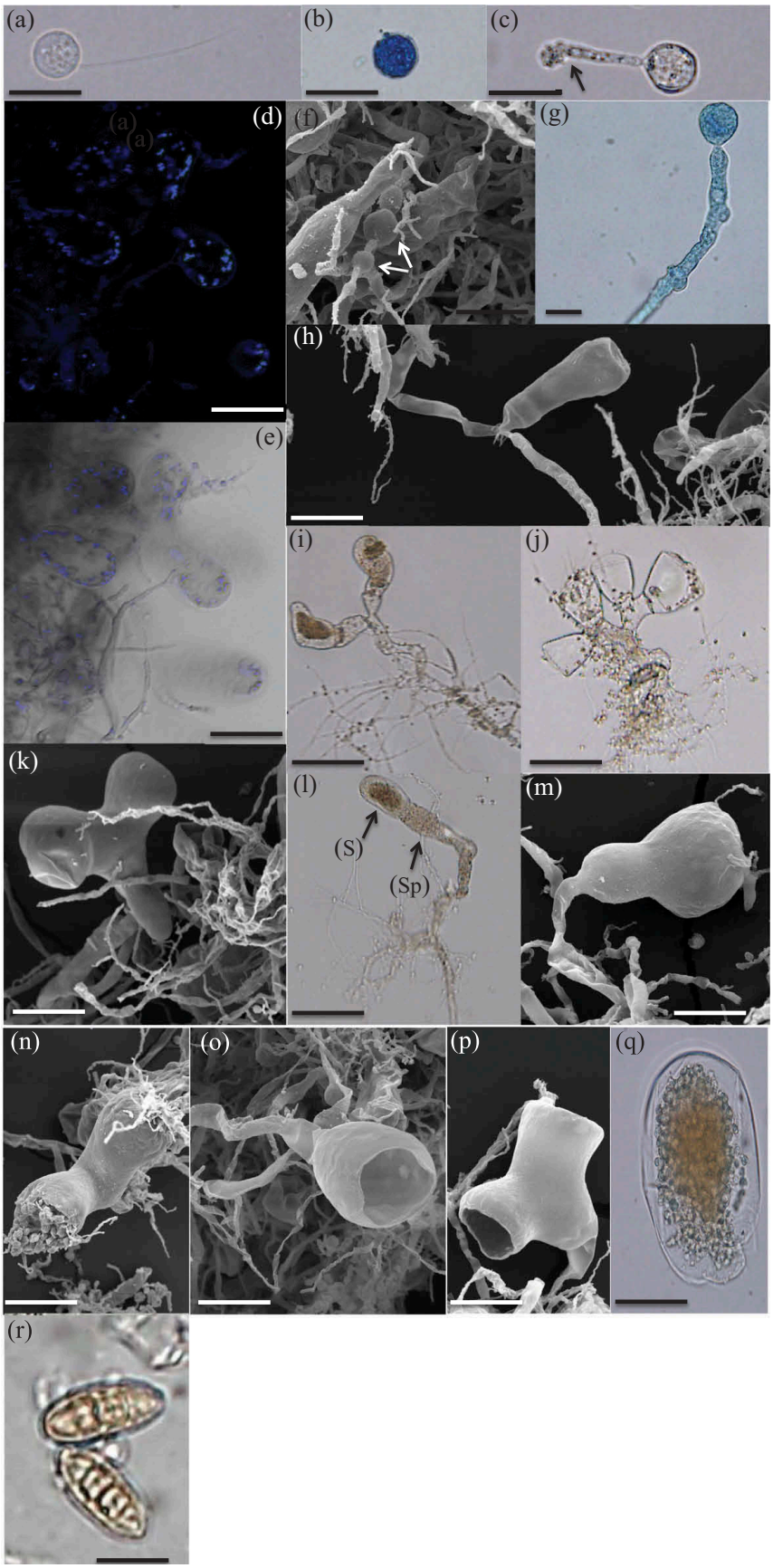


Figure 4-8. Microscopic features of *Khyollomyces ramosus* (clade 6, zebra-horse) strain ZS-33 (Z-OK) (a–q) and distinct resting stage structure from strain HoCal4.A2.2 (r). Light (a–e, g, i–j, l, q) and scanning electron (f, h, k, m, n–p) micrographs. d. DAPI staining for visualizing nuclei using a fluorescence microscope equipped with a Brightline DAPI high-contrast filter set. e. Overlay image. a. A monoflagellate zoospore. b. Zoospore cyst after shedding of the flagellum. c. Germinating zoospore cyst producing a germ tube (arrow). d–e. Monocentric thalli, with nuclei occurring in sporangia, not in rhizoids or sporangiophores. f. Hyphal structures with intercalary swellings in wide hyphae (arrows). g–h. Endogenous sporangial development: g. Young subglobose sporangium with single rhizoidal system. i. Mature ellipsoidal sporangium with two main rhizoidal systems. i–m. Exogenous sporangial development: i. Multisporangiate thallus with two sporangia. j. Multisporangiate thallus with four sporangia. k. Heart-shaped sporangium. l. Ovoid sporangium (S) on a wide flattened sporangiophore (Sp). m. Pyriform sporangium. n. Zoospores are released through apical pore. o– p. Empty sporangia with intact sporangial walls after zoospores discharge. q. Mature sporangia detached from hyphae or sporangiophores. r. Resting stages from strain HoCal4.A2.2. Bars: a–c, f–g = 20 μm; d–e, k, m–q = 50 μm; h–j, l = 100 μm.


(a)
(b)
(c)
(d)
(e)
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(j)
(k)
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(m)
(n)
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(r)

Figure 4-9. Microscopic features of *Tahromyces munnarensis* (clade 7, Nilgiri tahr) strain TDFKJa193 (W-KE). Differential interference contrast (a, f, i–l), phase-contrast (b, d, g, h), and fluorescence (c, e) micrographs. a. Monoflagellate and triflagellate zoospores. b–e. Monocentric thalli; nuclei were observed in sporangia, not in rhizoids or sporangiophores. f–g. Endogenous sporangia: f. Ovoid endogenous sporangium with two rhizoidal systems; note the subsporangial swelling (arrow). g. Endogenous sporangium with branched rhizoids. h–l. Exogenous sporangia: h. Globose sporangium with short swollen sporangiophore (arrow). i–j. Exogenous sporangia with subsporangial swellings and constricted necks (arrows). k. Ovoid sporangium with septum at the sporangial base (arrow). l. Zoospore release through dissolution of a wide apical pore. Bars = 20 µm.

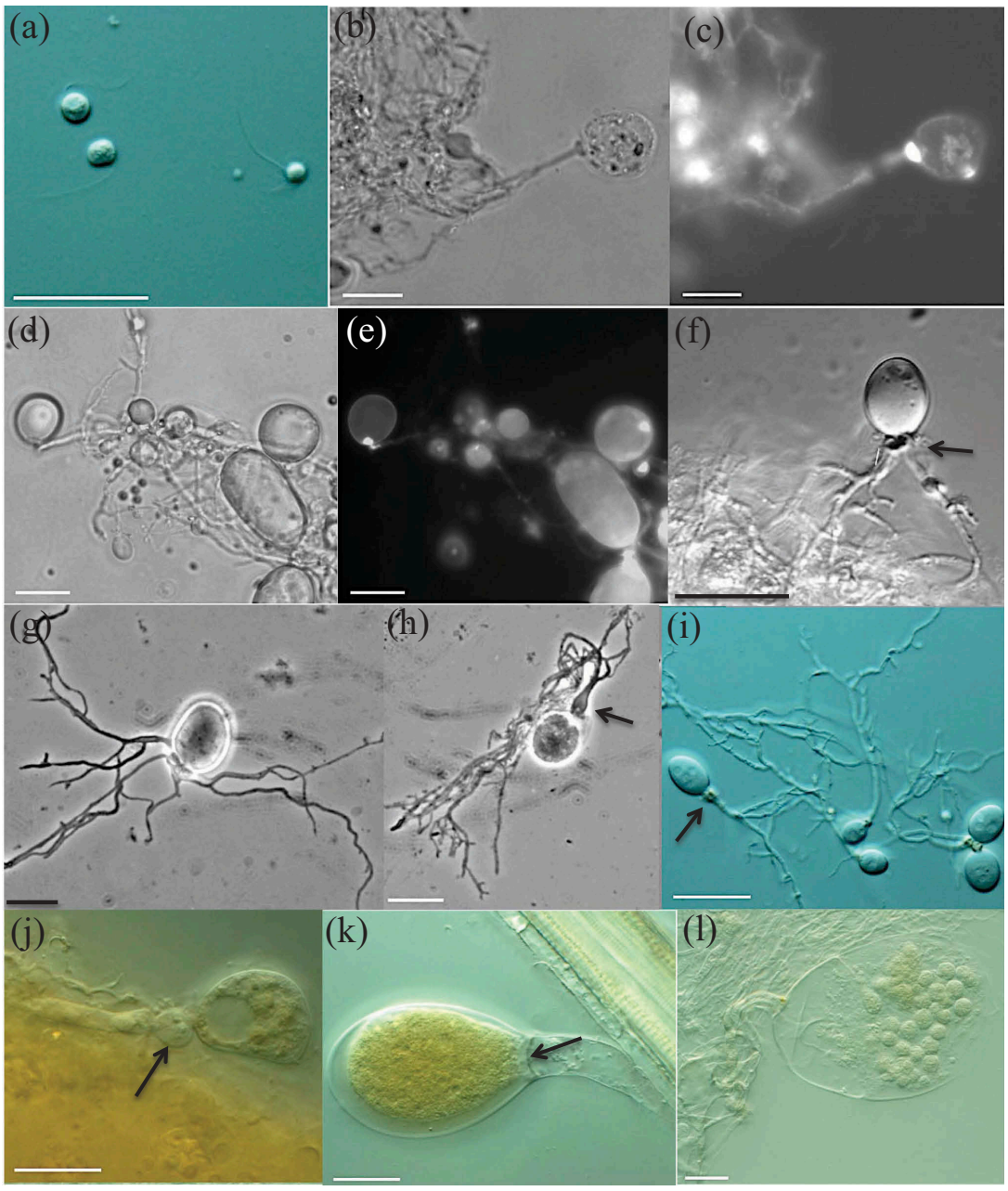

Figure 4-10. Phylogenetic affiliation of the seven newly described genera to other AGF genera based on sequence alignment of (A) the D1–D2 domains of nuc 28S rDNA genes and (B) partial ITS1 sequences. Sequences were aligned in MAFFT (Nakamura et al. 2018), and the alignment was used to construct phylogenetic trees in MEGA7 (Kumar et al. 2016) using a maximum likelihood approach. Bootstrap values from 100 replicates are shown for nodes with more than 70% bootstrap support.

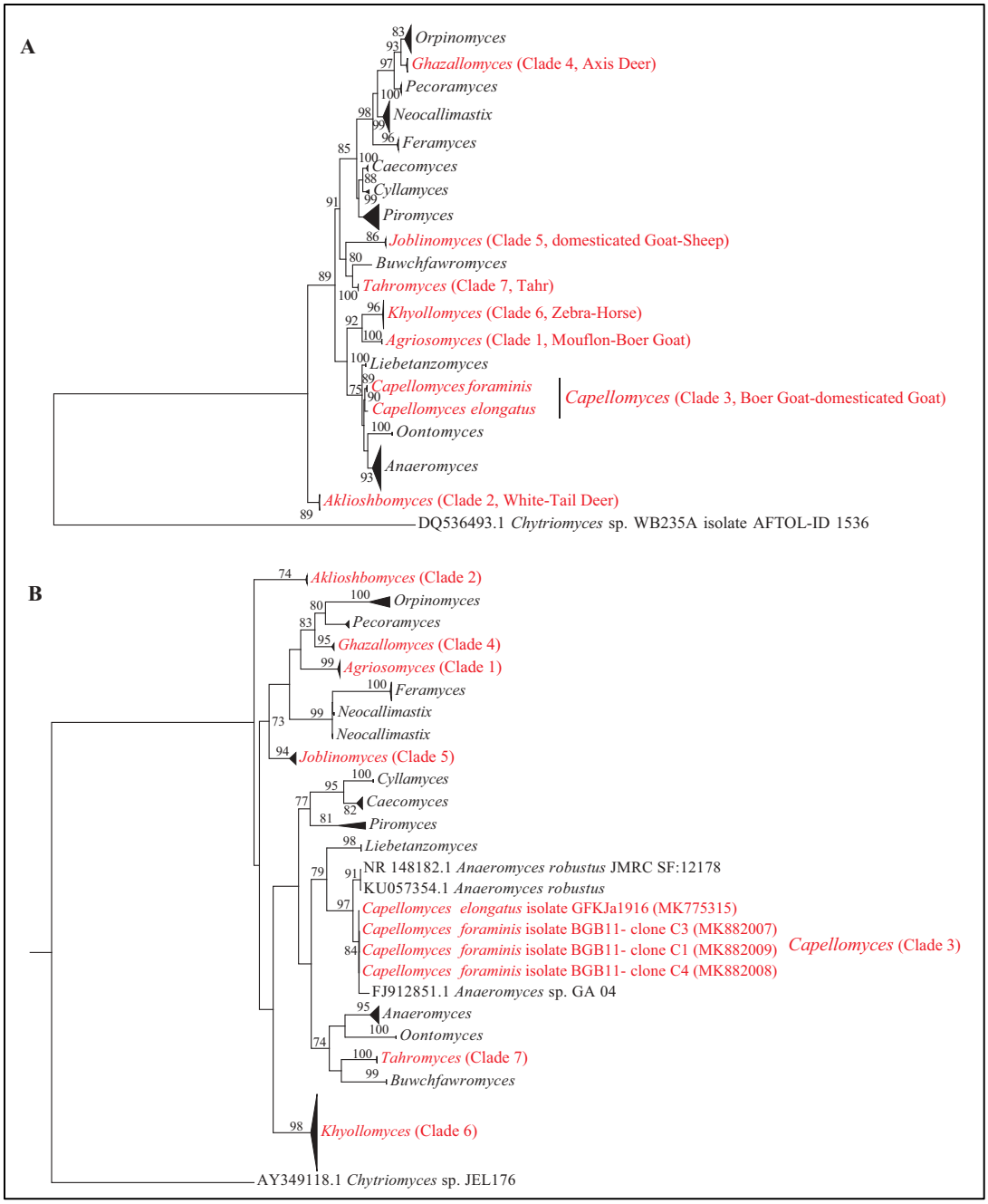

Table 4-1. Summary of Neocallimastigomycota strains reported in this study.

Clade	Genus name	Species name	Source	# of isolates	Type strain	Molecular features					Morphological features			
						LSU sequence inter-lineage variability	% LSU similarity (cultured)	% ITS1 similarity (cultured)	% ITS1 similarity (uncultured)	Accession number	Growth characters	Zoospore	Thallus development	Characteristic morphology
1	***Agriosomyces***	***longus***	Mouflon-Boer goat, Val verde county,TX	3	MS-2	0-1%	91.2% to *Liebetanzomyces polymorphus* strain G1SC (MH468763.1)	83.5% to *Neocallimastix* sp. NYF3 (JQ782544.1)	83.4% wih uncultured clone from Bontebok (GQ76186.1)	LSU: MK881996, ITS1: MK882010, MK882013	On agar: Brown circular colonies (0.2-1 mm diam.); In liquid: Thin fungal biofilm growth (Fig. 4- 1c)	Mono-, bi-flagellated, spherical, 2.7-7.5µm in diameter, flagellum length:16.6-30µm (Fig.4- 4 a-b)	Monocentric/ filamentous (Fig. 4c-d). Sporangia: endogenous (Fig. 4e-f) & exogenous (Fig. 4-4 g-h)	Homogenous globose sporangia, long flagella (5-6 times longer than the zoospore body), swollen sporangiophores & tightly constricted sporangial necks
2	***Aklioshbomyces***	***papillarum***	White-Tailed Deer, Payne county,OK	9	WT-2	0-0.4%	91.3% to *Caecomyces* sp. strain GMLF-60 (MK645804.1)	82% to *Piromyces* sp. strain CN8 (KY368107.1)	82.6% wih uncultured clone from Bontebok (GQ762535.1)	LSU: MK882001, ITS1: MK882038-MK882042	On agar: Beige circular colonies with a brown central core (0.5-2.5 mm diam.); In liquid: Heavy growth of thick biofilms that firmly attached to the tube's glass surface (Fig.4- 1 b).	Mono-, bi-, tri-flagellated, spherical, 4.5-13µm in diameter, flagellum length:12-35µm (Fig.4-3 a-c)	Monocentric/ filamentous (Fig. 3d). Sporangia: endogenous (Fig. 3e-g) & exogenous (Fig.4-3 h-j)	Papillated sporangia and pseudo-intercalary sporangia
3	***Capellomyces***	***foraminis***	Boer goat, Val Verde county, TX	5	BGB-11	0-0.3%	98.6% to *Liebetanzomyces polymorphus* strain G1SC (MH468763.1)	97.3% to *Anaeromyces* sp. GA04 (FJ912851.1) and 93% to *Aaeromyces robustus* (NR_148182.1)	91.6% to uncultured clone from cattle (JN205760.1)	LSU: MK881975, ITS1: MK882007-MK882009	On agar: Small brown circular coloniess with dark central sporangial core (0.1-0.5 mm diam.); In liquid: Thin fungal biofilm growth (Fig. 4-1 d)	Mono-, bi-flagellated, spherical, 4-7µm in diameter, flagellum length:15-25µm (Fig.4-5 a-b)	Monocentric/ filamentous (Fig. 4-5 f-g). Sporangia: endogenous (Fig. 4-5 h-i) & exogenous (Fig. 4-5 j-r)	Zoospores are liberated through a wide apical pore at the top of the sporangia followed by sporangial wall collapse.
		elongatus	Domesticated goat, Munnar, Kerala, India	1	GFKJa1916	NA	97.3% to *Liebetanzomyces polymorphus* strain G1SC (MH468763.1)	96.2% to *Anaeromyces* sp. GA-04 (FJ912851.1) and 92.5% to *Anaeromyces robustus* (NR_148182.1)	90.6% to uncultured clone from cattle (JN205760.1)	LSU: MK775304, ITS1: MK775315	On agar: off-white cottony colonies (2-3 mm) with a compact and fluffy center surrounded by radiating rhizoids; in broth, numerous fungal thalli attach to the glass bottles initially, and later developed into thin mat-like structures (Fig. 4-1 e)	Mono-, bi-or tri-flagellate, globose; size, 4–5 µm diameter, flagellum length, 15-20 µm (Fig 4-6 a)	Monocentric/ filamentous (Fig. 4-6 b-c). Sporangia: endogenous (Fig.4-6d-g) & exogenous (Fig.4-6h-l)	Long & wide sporangiophore in case of exogenous development; multisporangiate thallus with two sporangia of same or different shapes
4	***Ghazallomyces***	***constrictus***	Axis Deer, Sutton county,TX	9	Axs-31	0-0.9%	97.6% to *Orpinomyces* sp. isolate SOI (MF118139.1)	89.7% to *Orpinomyces* sp. C1B (JN943055.1)	90.91% to uncultured clone from Llama (GQ576858.1)	LSU: MK881971, ITS1: MK882043-MK882046	On agar: White circular colonies with a brown central sporangial core (1-4 mm diam.); In liquid: Thick fungal biofilm growth (Fig. 4-1 a)	Polyflagellated, globose, 6-10.5µm in dimeter, 7-14 flagella, flagellum length:16-31µm (Fig. 4-2 a)	Monocentric/ filamentous (Fig. 2b-c). Sporangia: endogenous (Fig. 2d-g) & exogenous (Fig. 4-2 h-p)	Persistent zoospore cyst, Sporangia with tightly constricted necks, narrow ports and fine septum at the base
5	***Joblinomyces***	***apicalis***	Domesticated goat and sheep, Sonipat, Haryana, India	13	GFH683	0-0.4%	86.5% to *Neocallimastix* sp. NYR4 (JQ782549.1)	87.4% to *Orpinomyces* cf. *joyonii* strain D4A (MH045490.1)	87.7% to uncultured clone from horse (MH125224.1)		On agar: small (1-2 mm) colonies with a dense dark central core of abundant sporangial growth, surrounded by long and thin radiating rhizoids; in broth, numerous fungal thalli attach to the glass bottles initially, an later develop into thin mat-like structures (Fig. 1g)	Mono- or bi-flagellate; globose; 5–6 µm diameter; flagellum length, 20-22 µm (Fig. 8a-b)	Monocentric/ filamentous (Fig. 8c-f). Sporangia: endogenous (Fig. 8g) & exogenous (Fig. 8h-i)	Zoospores are discharged after dissolution of a wide apical portion of sporangial wall, resulting in formation of empty cup-shaped sporangia
6	***Khoyollomyces***	***ramosus***	Zebra, Oklahoma City Zoo, OK, and domesticated Horse, Llanbadarn Fawr, Ceredigion	21	ZS-33	0-0.9%	91.8% to *Liebetanzomyces polymorphus* strain G1SC (MH468763.1)	81.3% to *Piromyces* sp. strain CN6 (KY368105.1)	99.6% to uncultured clone Grant's gazelle (GQ782832.1)/ Uncultured clade AL1	LSU: MK881981, ITS1: MK882019	On agar:Yellow to yellowish-brown of irregular shapes; In liquid: Loose fungal thalli with sand-like appearance (Fig. 4-1 f).	Uniflagellated, spherical, 6-17 µm in diameter, flagellum length:18-40µ (Fig.4-7 a)	Monocentric/ filamentous (Fig. 4-7 d-e). Sporangia: endogenous (Fig. 4-7 g-h) & exogenous (Fig. 4-7 i-m)	Multisporangiated thalli & branched sporangiophores
7	***Tahromyces***	***munnarensis***	Nilgiri Tahr, Munnar, Kerala, India	4	TDFKJa193	0-0.1%	94.9% to *Buwchfawromyces eastonii* (NG_058679.1)	87% to *Buwchfawromyces eastonii* (NR_132000.1)	89.4% to uncultured clone from Okapi (GQ600963.1)	LSU: MK775310, ITS1: MK775321	On agar: small (1 mm) white colonies, with a compact and fluffy center, surrounded by dotted circles of fungal thalli; in broth, numerous fungal thalli attach to the glass bottles initially, and later develop into thin mat-like structures (Fig. 4-1 h)	Mono-, bi-or tri-flagellate; globose; 3–4 µm diameter; flagellum length, 12-15 µm (Fig. 4-9 a)	Monocentric/ filamentous (Fig. 4-9 b-e). Sporangia: endogenous (Fig.4-9 f-g) & exogenous (Fig. 4-9 h-l)	Distinct subsporangial swelling with or without constricted neck on a short sporangiophore

Discussion

Here, we report on the isolation and characterization of multiple novel AGF strains from a concerted sampling effort of domesticated, zoo-housed, and wild animals from North America, Europe, and Asia. We propose seven new AGF genera to accommodate these novel strains, hence expanding the AGF genus-level diversity by more than 50% (from 11 to 18). All newly described taxa produced filamentous, monocentric thalli, similar to 7 of the 11 currently described genera. Six of the seven novel genera described here produce exclusively or predominantly monoflagellated zoospores, similar to 8 of the 11 currently described taxa. As such, filamentous taxa with monocentric thalli and exclusively or predominantly monoflagellated zoospore appear to be the most common thallus morphology and zoospore flagellation patterns in the Neocallimastigomycota predominant within currently described AGF genera. It is interesting to note that for decades, microscopy-based identification of AGF strains typically assigned isolates with such morphology to the genus *Piromyces* (Ho et al. 1993). We note broad similarities between the microscopic features of *Aklioshbomyces papillarum* and *P. mae* (papillated sporangia), and between those of *Joblinomyces* and *P. minutus* (zoospores release through a wide apical portion of sporangial wall, resulting in formation of an empty cup-shaped sporangium). Unfortunately, the absence of sequence data and extant cultures of these previously described "*Piromyces*" taxa prevents further investigation into this issue (Ho et al. 1993).

The current isolates were obtained in a multiyear effort to describe novel AGF strains from a wide range of animal hosts in the USA, India, and Wales. The majority of novel taxa described here (5/7 genera, 6/8 species) originated from wild, undomesticated animals (axis deer, white-tailed deer, mouflon, Boer goat, and Nilgiri tahr), underscoring their potential as novel, yet-untapped reservoir of AGF diversity. Such novelty, which has recently been postulated (Hanafy et al. 2018), could be attributed to higher variability in the quality and quantity of ingested plant material and the significant daily and seasonal fluctuations in feeding frequencies.

Culture-independent surveys utilizing ITS1 as a phylogenetic marker (Liggenstoffer et al. 2010; Kittelmann et al. 2012), and subsequent meta-analysis (Kittelmann et al. 2012, 2013), have identified multiple novel, yet-uncultured AGF genus-level lineages. This study has been successful in isolating the first representatives of novel groups AL1 (genus *Khoyollomyces*) from zebra (Z-OK) and horse (D-WA) fecal samples and AL5 (genus *Joblinomyces*) from domesticated goat and sheep (D-HA) fecal samples. In a prior survey of AGF (Liggenstoffer et al. 2010), members of AL1 group were encountered in approximately half of the animal hosts

examined (18/35). AL1-affiliated sequences were more predominant in hindgut fermenters (7/9 hosts) and constituted a relatively high proportion of the AGF community in multiple hosts, e.g., 99.9% in three different zebra individuals, 56.7% and 68.3% in two horses, and 29.6% in a Grant's gazelle). By comparison, they were only encountered in 11/26 foregut fermenters, where they constituted 0.01% to 38% of the AGF community in these animals. Further, a recent seminal spatial analysis that analyzed AGF community in samples directly obtained from various locations along horse digestive tracts (Mura et al. 2019) identified AL1 group as a prominent component of the AGF community in the right ventral (88%) and left dorsal (98%) colons in horses. As such, this novel genus appears to exhibit a preference for hindgut fermenters of the family *Equidae*. The reason for such preferences, and the general preference of some fungal taxa to specific hosts, remains unclear (Callaghan et al. 2015; Dagar et al. 2015).

AL5 group was identified in 14/35 animals examined in Liggenstoffer et al. (2010). However, members of this group, unlike those of AL1, never constituted the majority, or even the plurality, of AGF sequences in any of the examined samples. AL5 appears to constitute a minor component of the AGF community in multiple foregut fermenters. Its presence in hindgut fermenters appears to be exceedingly rare (only in 1/9 hindgut fermenters examined in Liggenstoffer et al. [2010], at 0.06% relative abundance).

Surprisingly, comparative analysis of ITS1 sequences indicated that the mouflon–Boer goat, white-tailed deer, axis deer, Boer goat–domesticated goat, and Nilgiri tahr clades appear to be completely novel and not previously encountered in prior culture-based or culture-independent studies. ITS1 sequences from these five clades did not display mismatches to common ITS1 primers, did not have an atypical length that could hinder its amplification or detection via PCR, and were readily amplified from genomic DNA of pure cultures. As such, we posit that the lack of prior observation of these taxa is biologically relevant and is indicative of their relatively specific host preference and/or predominance in wild, rather than domesticated, herbivores. Indeed, although 30 animals were screened in the current study, three of these seven novel genera were isolated from only a single host (white-tailed deer, axis deer, and Nilgiri tahr), whereas the other four were isolated from only two hosts (mouflon and Boer goat, Boer goat and domesticated goat, domesticated goat and sheep, and zebra–domesticated horse) (TABLE 4-1).

Collectively, the steady identification of novel taxa in culture-based and culture-independent surveys, as well as the sparse overlap between these studies, strongly suggests that the scope of AGF diversity in nature is much broader than currently estimated (Kittelmann et al.

2012; Paul et al. 2018). Compared with the prokaryotic component of the rumen and herbivorous gut, the diversity of the rumen mycobiome remains woefully understudied. To provide a more thorough understanding of the AGF diversity in nature, concerted efforts that systematically assess the AGF diversity and community structure in various spatial (e.g., across various compartments of the herbivorous gut), temporal (e.g., across the life span of an animal), and geographic dimensions in a wide range of domesticated and wild herbivores are needed. Much remains to be understood regarding the diversity and community structure of AGF within various locations of the gastrointestinal tract of an animal host, interspecies stochastic differences between AGF communities in animal subjects, temporal age-related progression of AGF in animal hosts, and the response of the AGF community to various factors, e.g., feeding patterns, antibiotic administration, animal disease, co-housing arrangements, and combinations thereof.

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CHAPTER V

ASSESSING ANAEROBIC GUT FUNGAL DIVERSITY IN HERBIVORES USING D1/D2 LARGE RIBOSOMAL MSUBUNIT SEQUENCING AND MULTI-YEAR ISOLATION

Abstract

The anaerobic gut fungi (AGF, Neocallimastigomycota) reside in the alimentary tracts of herbivores where they play a central role in the breakdown of plant material. Here, we report on the development of the hypervariable domains D1/D2 of the large ribosomal subunit (D1/D2 LSU) as a barcoding marker for the AGF. We generated a reference D1/D2 LSU database for all cultured AGF genera, as well as the majority of candidate genera encountered in prior internal transcribed spacer 1 (ITS1)-based surveys. Subsequently, a D1/D2 LSU- based diversity survey using long read PacBio SMRT sequencing was conducted on faecal samples from 21 wild and domesticated herbivores. Twenty-eight genera and candidate genera were identified, including multiple novel lineages that were predominantly, but not exclusively, identified in wild herbivores. Association between certain AGF genera and animal lifestyles, or animal host family was observed. Finally, to address the current paucity of AGF isolates, concurrent isolation efforts utilizing multiple approaches to maximize recovery yielded 216 isolates belonging to 12 different genera, several of which have no prior cultured representatives. Our results establish the utility of D1/D2 LSU and PacBio sequencing for AGF diversity surveys, the culturability of multiple AGF taxa, and demonstrate that wild herbivores represent a yet-untapped reservoir of AGF diversity.

Introduction

Members of the anaerobic gut fungi (AGF) are strict anaerobes that inhabit the rumen and alimentary tract of a wide range of foregut and hindgut herbivores. The AGF play an important role in the breakdown of ingested plant biomass via enzymatic and physical disruption in the herbivorous gut (Gruninger et al., 2014). AGF represent a distinct basal fungal phylum (Neocallimastigomycota) that evolved 66 (±10) million years ago coinciding, and possibly enabling, mammalian transition from insectivory to herbivory (Wang et al., 2019).

Culture independent amplicon-based diversity surveys have been widely utilized to gauge anaerobic fungal diversity and community structure in herbivores. The ITS1 region within the ribosomal operon has been almost exclusively utilized as the phylogenetic marker of choice in culture-independent sequence-based phylogenetic assessments of AGF diversity (Edwards et al., 2008; Liggenstoffer et al., 2010; Kittelmann et al., 2012; Kittelmann et al., 2013). Such choice is a reflection of its wider popularity as a marker within the kingdom Mycota (Schoch et al., 2012; Nilsson et al., 2018), the high sequence similarity and limited discriminatory power of the 18S rRNA gene between various AGF taxa (Dore and Stahl, 1991), and its relatively shorter length, allowing high throughput pyrosequencing and Illumina-based diversity assessments (Liggenstoffer et al., 2010; Kittelmann et al., 2013). However, concerns for the use of ITS1 in diversity assessment for the Mycota (Kiss, 2012), basal fungi (James et al., 2006) and the Neocallimastigomycota (Edwards et al., 2017) have been voiced. The ITS1 region is polymorphic, exhibiting considerable structural polymorphism (Koetschan et al., 2014), and length (Edwards et al., 2019) variability. Such polymorphism renders automated alignments, reproducible sequence divergence estimates, and classification of sequence data unreliable and highly dependent on alignment strategies and parameters specified. In addition, significant sequence divergence between copies of the ITS1 region within a single strain have been reported (up to 12.9% in (Callaghan et al., 2015), values that exceed cutoffs utilized for species (even genus in some instances) level delineation from sequence data (Liggenstoffer et al., 2010; Kumar et al., 2015; Paul et al., 2018; Mura et al., 2019). Such limitations often necessitate laborious subjective manual

curation and secondary structure incorporation into alignment strategies (Koetschan et al., 2014), although it is well recognized that these efforts only partially alleviate, rather than completely address, such fundamental limitations.

The 28S large ribosomal subunit (LSU) is one of the original genes proposed for fungal barcoding (James et al., 2006). Hypervariable domains 1 and 2 (Guadet et al., 1989) within the LSU molecule (D1/D2 LSU) have previously been utilized for differentiating strains of AGF via molecular typing (Hausner et al., 2000; Fliegerova et al., 2006; Dagar et al., 2011), or sequencing (Wang et al., 2017; Nagler et al., 2018). Compared with ITS1, D1/D2 LSU region exhibits much lower levels of length heterogeneity and intra-strain sequence divergence in fungi (Liu et al., 2012), including the AGF (Dagar et al., 2011). Identification and taxonomic assignment of AGF strains based on D1/D2 LSU have gathered momentum; and D1/D2 LSU-based phylogenetic analysis has been reported in all manuscripts describing novel taxa since 2015 (Callaghan et al., 2015; Dagar et al., 2015; Hanafy et al., 2017; Joshi et al., 2018; Hanafy et al., 2018a; Hanafy et al., 2018b; Hanafy et al., 2020). The potential use of D1/D2 LSU as a marker in culture-independent AGF diversity surveys has been proposed as a logical alternative for ITS1 (Edwards et al., 2017; Edwards et al., 2019). The lack of specific AGF primers and the relatively large size of the region (approximately 750 bp) has long hampered the utilization of short read, high-throughput, Illumina-based amplicon sequencing in such surveys. However, such impediments have recently been alleviated by the development of AGF LSU-specific primers (Dollhofer et al., 2016; Nagler et al., 2018), as well as the standardization and adoption of PacBio long-read sequencing for amplicon-based diversity surveys (Heeger et al., 2018; Tedersoo et al., 2018).

Theoretically, a comprehensive assessment of diversity and community structure of a host-associated lineage necessitates sampling all (or the majority) of hosts reported to harbour such lineage. However, to date, the majority of AGF diversity surveys conducted have targeted a few domesticated herbivores, e.g. cows, sheep and goats (Edwards et al., 2008; Griffith et al., 2009; Kittelmann et al., 2012). ‘Exotic’ animals have been sampled from zoo settings only sporadically, and on an opportunistic basis (Liggenstoffer et al., 2010; Solomon et al., 2016b).

Isolation of AGF taxa enables taxonomic, metabolic, physiological and ultrastructural characterization of indi- vidual taxa. As well, cultures availability enables subsequent—omics, synthetic and system-biology, and biogeography-based investigations (Couger et al., 2015; Seppälä et al., 2016; Haitjema et al., 2017; Calkins et al., 2018; Henske et al., 2018a; Henske et al., 2018b; Murphy et al., 2019), as well as evaluation of evolutionary processes underpinning speciation in the AGF (Youssef et al., 2013; Wang et al., 2019). However, efforts to isolate and maintain AGF strains have lagged behind their aerobic counterparts mainly due to their strict anaerobic nature and the lack of reliable long-term storage procedures. Due to these difficulties, many historic isolates are no longer available and most culture-based studies report on the isolation of a single or few strains using a single substrate/enrichment condition from one or few hosts (Ho et al., 1993; Hanafy et al., 2018b). Indeed, a gap currently exists between the rate of discovery (via amplicon-based diversity surveys) and the rate of isolation of new taxa of AGF, and several yet-uncultured AGF lineages have been identified in culture-independent diversity sur- veys (Paul et al., 2018). Whether yet-uncultured AGF taxa are refractory to isolation, or simply not yet cultured due to inadequate sampling and isolation efforts remains to be seen.

The current study aims to expand our understanding of the diversity of AGF while addressing all three impediments described above. First, we sought to develop D1/D2 LSU as a more robust marker for AGF diversity assessment by building a reference sequence database correlating ITS1 and D1/D2 LSU sequence data from cultured strains and environmental samples. Second, we sought to expand on AGF diversity by examining a wide range of animal hosts, including multiple previously unsampled wild herbivores. Such survey was conducted using D1/D2 LSU fragment, to establish its utility as a new phylogenetic marker for the AGF. Third, we sought to demonstrate the utility of intensive sampling and utilization of various isolation strategies in recovering AGF strains and testing the hypothesis that many yet-uncultured AGF lineages are indeed amenable to cultivation. Collectively, these efforts provide an established framework for future utilization of D1/D2 LSU amplification and PacBio sequencing for AGF community assessment, highlight the value of sampling wild herbivores and establish the culturability of a wide range of AGF taxa.

Materials and Methods

Samples.

Faecal samples were obtained from six domesticated, six zoo-housed and nine wild animals (Table 5-1). The host animals belonged to the families Bovidae (11), Cervidae (6), Equidae (3) and Camelidae (1). The dataset encompassed some replicates from few animal species sometimes with lifestyle variations within a single animal species: Bos taurus (n =2; domesticated cow, and domesticated longhorn cattle), Ovis aries (n = 2; domesticated sheep and wild mouflon ram), Capra aegagrus (n = 3; domesticated goat, wild Boer goat, and zoo- housed dwarf goat) and Ammotragus lervia (n = 2; Aoudad Sheep) (Table 5-1). Samples from domesticated animals were obtained from Oklahoma State University and surrounding farms between September 2016 and May 2018. Samples from the Oklahoma City Zoo were obtained in April 2019. For samples from wild herbivores, we enlisted the help of hunters in four separate hunting expeditions in Sutton, Val Verde, and Coke counties, Texas (April 2017, July 2017 and April 2018), and Payne County, Oklahoma (October 2017). Appropriate hunting licences were obtained and the animals were shot either on private land with the owner's consent or on public land during the hunting season. All samples were stored on ice and promptly (within 20 min for domesticated samples, within 1 h for zoo samples and within 24 h for samples obtained during hunting trips) transferred to the laboratory. Upon arrival, a portion of the sample was immediately used for setting enrichments for isolation efforts, while the rest was stored at −20º C for DNA extraction.

Development of D1/D2 LSU locus as a phylogenetic marker

Amplification of the ITS1-5.8S rRNA-ITS2-D1/D2 LSU from Neocallimastigomycota isolates

Biomass was harvested from 10 ml of 2–4-day old cultures and crushed in liquid nitrogen. DNA was extracted from the ground fungal biomass using DNeasy PowerPlant Pro Kit (Qiagen, Germantown, Maryland) according to the manufacturer's instructions. A PCR reaction targeting the region encompassing ITS1, 5.8S rRNA, ITS2 and D1/D2

region of the LSU rRNA (Fig. 5-1) was conducted using the primers ITS5 (5‘ -GGAAGTAAAAGTCGTAACAAGG-3‘) and NL4 (5‘-TCAACATCCTAAGCGTAGGTA-3‘) (Wang et al., 2017). The PCR protocol consisted of an initial denaturation for 5 min at 95º C followed by 40cycles of denaturation at 95º C for 1 min, annealing at 55º C for 1 min and elongation at 72º C for 2 min, and a final exten-sion of 72º C for 20 min. PCR amplicons were purified using PureLink® PCR cleanup kit (Life Technologies, Carlsbad, California), followed by cloning into a TOPO-TA cloning vector according to the manufacturer's instructions (Life Technologies). Clones (n = 1–12 per isolate) were Sanger sequenced at the Oklahoma State University DNA sequencing core facility. Amplification of the ITS1-5.8S-ITS2-D1/D2 LSU from environmental samples

Faecal material from different animals (0.25–0.5 g) was crushed in liquid nitrogen and total DNA was extracted from the ground sample using DNeasy PowerPlant Pro Kit (Qiagen) according to the manufacturer's instructions. Extracted DNA was then used as a template for ITS1-5.8S- ITS2-D1/D2 LSU PCR amplification. To select for the AGF community and avoid amplifying other fungal phyla, we paired a universal fungal forward primer (ITS5: 5‘ -GGAAGTAAAAGTCGTAACAAGG-3‘) with an AGF-specific reverse primer (GG-NL4: 5‘ -TCAACATCCTAA GCGTAGGTA-3‘) (Nagler et al., 2018). Primers were barcoded to allow PacBio sequencing and multiplexing (Table S2). Amplicons were purified using PureLink PCR cleanup kit (Life Technologies), quantified using Qubit® (Life Technologies), pooled, and sequenced at Washington State University core facility using one cell of the SMRT Pacific Biosciences (PacBio) RSII system.

Environmental PacBio-generated sequences quality control

We performed a two-tier quality control protocol on the generated sequences. First, the raw reads were processed using the official PacBio pipeline (RS_Subreads.1) (http://files.pacb.com/software/smrtanalysis/2.2.0/doc/smrtportal/help/!SSL!/Webhelp/CS_Prot_RS_Subreads. htm). Raw reads were filtered based on the minimum read length, and minimum read quality using default settings. The passing reads were then processed

with the PacBio RS_ReadsOfInsert protocol (http://files.pacb.com/software/smrtanalysis/2.2.0/doc/smrtportal/help/!SSL!/Webhelp/CS_Prot_RS_ReadsOfInsert.htm) for generating single-molecule consensus reads from the insert template. Consensus reads had a minimum of five full passes, 99.95% predicted accuracy and 1000 bp insert length. The resulting consensus reads had a mean number of passes of 20, mean read length of insert of 1429 bp and mean polymerase read quality of 0.99.

Subsequently, sequence quality control procedures were conducted in Mothur (Schloss et al., 2009) to assess the quality of the generated consensus reads utilizing stringent protocols previously suggested for assessing bacterial, archaeal and fungal diversity for similar sized amplicons (Schlaeppi et al., 2016; Schloss et al., 2016; Tedersoo et al., 2018; Bahram et al., 2019). Reads were filtered in Mothur using trim.seqs to remove reads longer than 2000 bp, reads with average quality score below 25, reads with ambiguous bases, reads not containing the correct barcode sequence, reads with more than 2 bp difference in the primer sequence and reads with homopolymer stretches longer than 12 bp. Reads with the primer sequence in the middle were identified by performing a standalone Blastn-short using the primer sequence as the query, and were subsequently removed using the remove.seqs command in Mothur.

A mock community (constituted of equal concentration of PCR products of five different strains (Aklioshbomyces papillarum strain WT2, Feramyces austinii isolate DS10, Liebetanzomyces sp. isolate Cel1A, Piromyces sp. isolate A1 and Piromyces sp. isolate Jen1) from our culture collection and for which we have obtained at least five Sanger clone sequences) was also sequenced. To establish whether the above approaches for overall read and sequence-based quality control are adequate, we compared PacBio-generated mock sequences to the corresponding Sanger-generated clone sequences. The median percentage similarity between PacBio-generated sequences affiliated with a certain strain and the Sanger-generated clone sequences obtained for that strain (99.05 ± 0.6 to 99.64 ± 0.47) were not significantly different from the median percentage similarities between different clones of the same strain (98.91 ± 0.6 to 99.72 ± 0.47) (Student t-test p-value >0.1) attesting to the adequacy of the above quality control measures in removing low-quality sequences.

A D1/D2 LSU reference database for cultured and yet- uncultured AGF taxa

A reference D1/D2-LSU sequence database for all Neocallimastigomycota cultured genera present in our culture collection was created via amplification, cloning and sequencing of the ITS1-5.8S-ITS2-D1/D2 LSU all- owing for a direct correlation and cross-referencing of the ITS1 and the D1/D2 LSU regions. To obtain D1/D2 LSU sequences representing yet-uncultured candidate genera previously defined by ITS1 sequence data (Liggenstoffer et al., 2010; Herrera et al., 2011; Kittelmann et al., 2012; Koetschan et al., 2014; Paul et al., 2018), the ITS1 region from the PacBio-generated ITS1-5.8S-ITS2-D1/D2 LSU environmental amplicons was extracted in Mothur using the pcr.seqs command with the sequence of the MNGM2 reverse primer and the flag rdiffs = 2 to allow for two differences in primer sequence. The trimmed sequences corresponding to the ITS1 region were compared, using blastn, to a manually curated Neocallimastigomycota ITS1 database encompassing all known cultured genera, as well as yet-uncultured taxa previously identified in culture-independent studies (Liggenstoffer et al., 2010; Herrera et al., 2011; Kittelmann et al., 2012; Koetschan et al., 2014; Paul et al., 2018) (Fig. 5-1). Sequences were classified as their first hit taxonomy if the percentage similarity to the first hit was >96% and the two sequences were aligned over >70% of the query sequence length. A taxonomy file was then created that contained the name of each sequence in the PacBio-generated environmental dataset and its corresponding taxonomy and was used for assigning taxonomy to the D1/D2 LSU sequence data.

Comparison of D1/D2-LSU versus ITS1 as phylogenetic markers

We used the dataset of full length PacBio-generated sequences described above, in addition to 116 Sanger- generated clone sequences from this study and previous studies (Nicholson et al., 2005; Wang et al., 2017; Hanafy et al., 2018a; Hanafy et al., 2018b), as well as genomic rRNA loci from two Neocallimastigomycota genomes (*Pecoramyces ruminantium* strain C1A and Neocallimastix californiae strain G1) (Youssef et al., 2013; Haitjema et al., 2017) in which the entire rrn operon sequence data are available to compare the ITS1 and D1/D2-LSU regions with respect to heterogeneity

in length and intra-genus sequence divergence. For every sequence, the ITS1, and the D1/D2-LSU regions were extracted in Mothur using the pcr.seqs command (with the reverse primer MNGM2, and the forward primer NL1, for the ITS1, and the D1/D2-LSU regions respectively) and allowing for two differences in the primer sequence. The trimmed sequences (both ITS1 and D1/D2-LSU) were then sorted into files based on their taxonomy such that for each genus/taxon two fasta files were created, an ITS1 and a D1/D2-LSU. These fasta files were then used to compare length heterogeneity and intra-genus sequence divergence as follows. Sequences lengths in each fasta file were obtained using the summary.seqs command in Mothur. Intra-genus sequence divergence values were obtained by first creating a multiple sequence alignment using the MAFFT aligner (Katoh and Standley, 2013) with the –auto flag and the default parameters for gap extension and gap opening penalties, followed by generating a sequence divergence distance matrix using the dist.seqs command in Mothur. Box plots for the distribution of length and sequence divergence were created in R (R Core Team, 2020).

AGF diversity assessment using D1/D2 LSU locus

Phylogenetic placement

The majority of the D1/D2-LSU sequences extracted from environmental sequences were assigned to an AGF genus as described above. D1/D2-LSU sequences that could not be confidently assigned to an AGF genus were sequentially inserted into a reference LSU tree (with representatives from all cultured AGF genera as well as representatives of uncultured AGF genera identified in this study) to assess novelty. Briefly, sequences were aligned using the MAFFT aligner (Katoh and Standley, 2013) with the auto flag and the default parameters for gap extension and gap opening penalties, and the alignment was used to construct maximum likelihood phylogenetic trees in FastTree (Price et al., 2009, 2010) using the gtr model. Furthermore, the associated ITS1 sequences (obtained from the same amplicon) were similarly inserted into a reference ITS1 tree for confirmation. Sequences were assigned to a novel candidate genus when both loci (LSU and ITS1) cluster as novel, independent genus-level clades with high (>70%) bootstrap support in both trees.

Genus and species level delineation

Genus level assignments were conducted via a combination of similarity search and phylogenetic placement as described above. We chose not to depend on sequence divergence cutoffs for OTU delineation at the genus level since some genera exhibit high sequence similarity between their D1/D2-LSU sequences (e.g. *Liebetanzomyces*, *Capellomyces* and *Anaeromyces* D1/D2-LSU sequence divergence ranges between 1.8% and 2.5%), while other genera are highly divergent (e.g. *Piromyces* intra-genus sequence divergence cutoff of the D1/D2-LSU region ranges between 0% and 5.7%), and as such 'a one size fits all' approach should not be applied. On the other hand, a similar approach for OTUs delineation at the species equivalent level is problematic due to uncertainties in circumscribing species boundaries, and inadequate numbers of species representatives in many genera. Therefore, for OTU delineation at the species equivalent level, we reverted to using a sequence divergence cutoff. Historically, cutoffs of 3% (Mura et al., 2019) to 5% (Liggenstoffer et al., 2010) were used for ITS1-based species equivalent delineation. However, D1/D2-LSU sequence data are more conserved when compared with ITS1 data in the Neocallimastigomycota (Callaghan et al., 2015; Dagar et al., 2015; Hanafy et al., 2017; Joshi et al., 2018; Hanafy et al., 2018a; Hanafy et al., 2018b; Hanafy et al., 2020), as well as other fungi (Guadet et al., 1989). To obtain an appropriate species equivalent cutoff, we used the 116 Sanger-generated clone sequences from this study and previous studies (Nicholson et al., 2005; Wang et al., 2017; Hanafy et al., 2018a; Hanafy et al., 2018b), as well as genomic rRNA loci from two Neocallimastigomycota genomes where the entire ribosomal operon sequence is available (*Pecoramyces ruminantium* strain C1A, and *Neocallimastix californiae* strain G1) (Youssef et al., 2013; Haitjema et al., 2017). The ITS1 and D1/D2-LSU regions were extracted and sorted to separate fasta files. Sequences in each file were then aligned using MAFFT (Katoh and Standley, 2013) with the auto flag and the default parameters for gap extension and gap opening penalties, and the alignment was used to create a distance matrix for every possible pairwise comparison using dist.seqs command in Mothur. The obtained pairwise distances for the ITS1, and the D1/D2-LSU regions were then correlated to obtain values of D1/D2-LSU sequence divergence cutoffs corresponding to the 3%–5% range in ITS1. This was equivalent to 2.0%–2.2%, and hence, for this study, a cutoff of 2% was used for OTU

generation at the species equivalent level using the D1/D2-LSU region.

Diversity and community structure assessments

Genus and species equivalent OTUs generated as described above were used to calculate alpha diversity indices (Chao and Ace richness estimates, Shannon diver- sity index and Simpson evenness index) for the different samples studied using the summary.seqs command in Mothur with the subsample option to normalize for sample size by randomly subsampling a number of sequences from each sample equivalent to the number of sequences in the smallest sample. A shared OTUs file created in Mothur using the make.shared command was used to cal- culate Bray–Curtis beta diversity indices between different samples (using the summary.shared command in Mothur). The shared OTUs file was also used as a starting point for ranking the samples based on their diversity using both an information-related diversity ordering method (Renyi generalized entropy) and an expected number of species- related diversity ordering method (Hulbert family of diversity indices) (Table 5-2). For community structure visualization, Bray–Curtis indices at the genus level were also used to perform non-metric multidimensional scaling using the metaMDS function in the Vegan package in R (Oksanen et al., 2019). Also, the percentage abundance of different genera across the samples studied was used in principal components analysis using the prcomp function in the labdsv package in R (Roberts, 2019). Ordination plots were generated from the two analyses (NMDS and PCA) using the ordiplot function.

Statistical analysis

Correlations of the diversity estimates to animal host factors including the animal lifestyle (domesticated, zoo- housed and wild) and the animal host families (Bovidae, Cervidea, Equidae and Camelidae) were calculated using χ^2-contingency tables followed by measuring the degree of association using Cramer's V statistics as detailed before (Liggenstoffer et al., 2010). In addition, to identify factors impacting AGF diversity, Student's t-tests were used to identify significant differences in the above alpha diversity estimates based on animal lifestyle (zoo- housed, domesticated and wild) and host phylogeny (families Bovidae, Equidae and Cervidae). To test the effect of the above host

factors on the AGF community structure, Student's t-tests were used to identify significant associations between specific AGF taxa percentage abundances and animal lifestyle (zoo-housed, domesticated and wild) or host family (families Bovidae, Equidae and Cervidae). In addition, analysis of similarity was performed using the Bray–Curtis indices and the anosim function in the Vegan package in R (Oksanen et al., 2019) to test the significance of the above factors on the samples clustering in the ordination plots.

Isolation efforts

Faecal samples (either freshly obtained or stored at −20º C in sterile, airtight plastic tubes) were used for isolation. Care was taken to avoid sample repeated freezing and thawing. Samples were first enriched by incubation for 24 h at 39ºC in rumen-fluid-cellobiose (RFC) medium supplemented with antibiotics (50 µg/ml kanamycin, 50 µg/ml penicillin, 20µg/ml streptomycin and 50µg/ mL chloramphenicol) (Calkins et al., 2016; Hanafy et al., 2017; Hanafy et al., 2018a; Hanafy et al., 2018b; Hanafy et al., 2020). Subsequently, enrichments were serially diluted by adding approximately 1 ml of enriched samples to 9 ml of RF medium supplemented with 1% cellulose or a mixture of 0.5% switchgrass and 0.5% cellobiose. Since fungal hyphae and zoospores are usually attached to the coarse particulates in the enrichment, serial dilutions were conducted using Pasteur pipettes rather than syringes and needles, as the narrow bore of the needle prevented the faecal clumps from being transferred. Serial dilutions up to a 10^{-5} dilution were incubated at 39º C for 24–48 h. Dilutions showing visible signs of growth (clumping or floating plant materials, a change in the colour of cellulose and production of gas bubbles) were then used for the preparation of roll tubes (Hungate, 1969; Bryant, 1972) on RFC agar medium. At the same time, and as a backup strategy in case the roll tubes failed to produce visible colonies, the dilution tubes themselves were subcultured and transferred to fresh medium with the same carbon source. Single colonies on roll tubes were then picked into liquid RFC medium, and at least three rounds of tube rolling and colony picking were conducted to ensure purity of the obtained colonies. To maximize the chances of obtaining isolates belonging to different genera, special attention was given, not only to picking colonies of different shapes and sizes but also to picking several colonies of the same shape, as representatives of different genera could

produce colonies with very similar macroscopic features. Isolates were maintained by biweekly subculturing into RFC medium. For long-term storage, cultures were stored on agar medium according to the procedure described by Calkins et al. (2016).

Results

The overall workflow of this study is shown in Figure 5-1 and described in details in the methods section. Briefly, A 1.4–1.5-kbp amplicon products encompassing the ITS1-5.8S-ITS2-D1/D2 LSU region was amplified and sequenced from AGF pure cultures and environmental samples to correlate the D1/D2 LSU region to the corresponding ITS1 region, and to provide a reference D1/D2 database for future utilization in high-throughput diversity surveys of AGF. Intra-genus length variability, intra-genus sequence divergence, within strain length variability and within strain sequence divergence were subsequently compared for the two markers, to demonstrate the utility and value of utilizing D1/D2 LSU as a phylogenetic marker. Subsequently, the D1/D2 region was extracted from all amplicons and utilized to assess the diversity and community structure of AGF in a wide range of wild and domesticated herbivores to provide insights into the AGF community in novel hosts, and demonstrate the feasibility and practicality of using D1/D2 as a phylogenetic marker in diversity surveys.

A reference D1/D2-LSU dataset for the Neocallimastigomycota

A 1.4–1.5-kbp amplicon product encompassing the ITS1-5.8S-ITS2-D1/D2 LSU region was amplified and sequenced from AGF pure cultures and environmental samples to correlate the D1/D2 LSU region to the corresponding ITS1 region, and to provide a reference D1/D2 database for future utilization in high-throughput diversity surveys. Using this approach, representative D1/D2 LSU of all the previously cultured AGF genera *Agriosomyces, Aklioshbomyces, Anaeromyces, Buwchfawromyces, Caecomyces, Capellomyces, Cyllamyces, Ghazallomyces, Joblinomyces, Feramyces, Khyollomyces, Liebetanzomyces, Neocallimastix, Orpinomyces, Pecoramyces, Piromyces* and *Tahromyces* were obtained (Table 5-1). Representatives of the genus *Oontomyces* were not generated in this study, but reference LSU and ITS1 sequences were obtained from prior publication (Dagar et al., 2015). In addition, representative sequences of D1/D2 LSU of candidate genera AL3, AL4, AL8, MN3, MN4, SK3 and SK4, previously identified in ITS1 culture-independent datasets, were also obtained (Table 5-1). Finally, representa- tives of six completely novel AGF candidate genera (RH1-RH6) were also

identified (Table 5-1) and confirmed as novel independent clades in ITS1 and D1/D2 LSU-based phylogenetic analysis (Fig. 5-5). It should be noted that multiple previously reported yet-uncultured (candidate) genera have recently been successfully isolated, e.g. AL1 (*Khyollomyces*), AL5 (*Joblinomyces*), AL6 (*Feramyces*), AL7 (*Piromyces finnis*), MN1 (*Cyllamyces*), SP4 (*Liebetanzomyces*) and SK2 (*Buwchfawromyces*). In addition, some previously proposed candidate genera clustered as members of already existing genera in our analysis, e.g. SP8 with *Cyllamyces*, and SP6 with Neocallimastix (Table 5-1). As such, we estimate that only representatives of candidate genera BlackRhino, SP1, SP2 (Paul et al., 2018), and the relatively rare AL2, DA1, DT1, JH1/SP5 (ITS1 sequence representatives of these two candidate genera are 99.6% similar and so they should be considered as one candidate genus), KF1, MN2, SK1, SP3 and SP7 (Tuckwell et al., 2005; Fliegerová et al., 2010; Liggenstoffer et al., 2010; Nicholson et al., 2010; Herrera et al., 2011; Kittelmann et al., 2012; Koetschan et al., 2014; Paul et al., 2018) were not encountered in this study, and hence no reference LSU sequence data for these candidate genera are currently available (Table 5-1).

D1/D2 LSU versus ITS1 as a taxonomic marker

Intra-genus length variability. The ITS1 and D1/D2 LSU regions were extracted from the 116 Sanger-generated clone sequences from this study and previous studies (Nicholson et al., 2005; Wang et al., 2017; Hanafy et al., 2018a; Hanafy et al., 2018b) (accession numbers in Table 5-1), the rRNA loci from two Neocallimastigomycota genomes (*Pecoramyces ruminantium* strain C1A and *Neocallimastix californiae* strain G1) (Youssef et al., 2013; Haitjema et al., 2017) in which the entire rrn operon sequence data are available, and from the PacBio- generated environmental amplicons in this study. The ITS1 region displayed a high level of length heterogeneity, ranging in size between 141 and 250 bp (median 191 bp, Fig. 5-2 A), with 75% of sequences ranging between 182 and 208 bp in length. Some genera had shorter than median ITS1 region length, e.g. *Cyllamyces* (range 141–173 bp), *Buwchfawromyces* (range 155–169 bp), and candidate genus AL3 (range 145–148 bp), while others exhibited a longer than median ITS1 region length, e.g. Liebetanzomyces (range 198–225 bp), and candidate genus RH5 (range 191–224 bp) (Fig. 5-2 A). A third group of genera displayed a wide range of length

heterogeneity, e.g. *Neocallimastix* (range 160–244 bp), *Caecomyces* (range 192–250) and *Piromyces* (range 173–225 bp). Few genera and candidate genera displayed a fairly narrow range of ITS1 length, e.g. AL3 (141–148 bp), but this is potentially a reflection of the paucity of sequences belonging to these genera obtained in this study (Fig. 5-2 A).

As expected, a much lower level of length heterogeneity was identified in the D1/D2 LSU (Fig. 5-2 B), ranging in size between 740 and 767 bp (median 760 bp), and where 75% of the sequences ranged between 757 and 761 bp, with all genera consistently displaying a much narrower D1/D2-LSU length heterogeneity, ranging between 11 bp in RH4 and 26 bp in the genera *Neocallimastix* and *Aklioshbomyces*.

Intra-genus sequence divergence. The ITS1 region displayed intra-genus sequence divergence ranging from 0.4% to 21% (median 3.2%), with 75% of the pairwise divergence values ranging between 1.7% and 6%. Gen- era displaying the highest level of divergence were Cae- comyces (1%–18.9%, median 8.3%), Cyllamyces (0.6%– 19.6%, median 5.5%) and Neocallimastix (0.4%–19.3%, median 5.5%) (Fig. 5-2 C). On the other hand, intra-genus sequence divergence of the D1/D2 LSU ranged between 0.1% and 9.2% (median 1.4%), with 75% of the pairwise divergence values ranging between 0.8% and 2.1%. Genera displaying highest level of divergence were Feramyces (0.1%–7.8%), Joblinomyces (0.1%–8.7%, Caecomyces (0.1%–9%) and Piromyces (0.1%–9.2%) (Fig. 5-2 D).

Within strain length variability. Within strain length heterogenicity examined in 19 strains with two or more sequenced clones ranged between 0 and 5 bp (Fig. 5-3 A) for ITS1 region and 0–1 bp for the LSU region (Fig. 5-3 B).

Within strain sequence divergence. Examining the 19 strains with more than two sequenced clones, the full ITS1 region showed intra-strain sequence divergence ranging from 0.1% to 10.01% (Fig. 5-3 C). Similar, and even higher levels of intra-strain ITS1 variability was previously reported, e.g. up to 12.9% in *Buwchfawromyces* eastonii strain GE09 (Callaghan et al., 2015). On the other hand, within strain D1/D2 LSU rRNA region showed a much lower sequence divergence, ranging from 0.13% to 1.84% (Fig. 5-3 d).

Culture-independent assessment of Neocallimastigomycota diversity in wild and domesticated herbivores using D1/D2 LSU as a phylogenetic marker

Phylogenetic diversity and novelty. A total of 17 697 high-quality long-read amplicons were obtained. Phylogenetic analysis using the D1/D2 LSU amplicons assigned all sequences into 28 different genera/candidate genera (Figs 5-4 A and 5-5A) and 298 species level OTUs$_{0.02}$. AGF genera identified in this study included members of the previously described genera *Anaeromyces*, *Buwchfawromyces*, *Caecomyces*, *Cyllamyces*, *Liebetanzomyces*, *Neocallimastix*, *Orpinomyces*, *Pecoramyces* and *Piromyces*. In addition, sequences representing multiple novel genera were also identified (Figs 5-4 A and 5-5 A), some of which have been subsequently isolated, named and characterized in separate publications, e.g. Feramyces (Hanafy et al., 2018a), *Aklioshbomyces*, *Agriosomyces*, *Ghazallomyces* and *Khyollomyces* (Hanafy et al., 2020). Finally, six novel candidate genera were identified and designated RH1–RH6 (Figs 5-4 A and 5-5 A). All of these six novel genera were encountered in extremely low abundance in a few samples (Fig. 5-4 A), with the notable exception of RH5, which was present in high relative abundance in multiple animals, e.g. domesticated sheep (96.22%), blackbuck deer (52.41%), axis deer (20.71%) and an aoudad sheep sample (11.75%).

Diversity estimates and distribution patterns. The number of AGF genera encountered per sample varied widely from 5 (in Pere David's deer, and Longhorn cattle) to 16 (in one Aoudad sheep sample) (Table 5-2; Figs 5-4 A and 5-5 A). However, in each of these samples a distribution pattern was observed in which a few genera represent the absolute majority of the sequences obtained. Excluding genera present in less than 1% abundance would lower the number of genera encountered per animal to 1 (in white-tail deer and dwarf goat) -10 (domesticated goat). Usually, 1–5 taxa were present in >10% abundance per animal (Fig. 5-4 B).

Using arbitrary cutoffs for ubiquity (presence in at least 50% of the animals studied) and abundance (above 1%), we identify five different distribution patterns for AGF genera encountered in this study (Fig. 5-5 B); (i) Ubiquitous mostly abundant genera: These are the genera identified in at least 50% of the animals studied and where

their relative abundances exceed 1% in at least 50% of their hosts: This group includes *Piromyces, Feramyces, Khyollomyces,* RH5, *Neocallimastix*, *Cyllamyces* and *Caecomyces*. (ii) Ubiquitous mostly rare genera: These are the genera identified in at least 50% of the animals studied and where their relative abundances were lower than 1% in at least 50% of their hosts. This group includes *Orpinomyces* and *Pecoramyces*. (iii) Less ubiquitous but mostly abundant genera: These are the genera identified in <50% of the animals studied but where their relative abundances exceed 1% in at least 50% of their hosts. This group includes *Ghazallomyces*, RH4, MN4, *Joblinomyces*, SK4, *Buwchfawromyces*, AL3, RH1 and RH3. (iv) Less ubiquitous mostly rare genera: These are the genera iden- tified in <50% of the animals and where their relative abundances were lower than 1% in at least 50% of their hosts. This group includes *Liebetanzomyces*, *Anaeromyces*, AL8, *Aklioshbomyces*, RH2 and *Agriosomyces*. (v) Less ubiquitous consistently rare genera: These are the genera identified in <50% of the animals and where their relative abundances never exceeded 1% in any of their hosts. This group includes RH6, AL4, MN3 and SK3.

Multiple diversity estimates (number of observed genera, Chao and Ace richness estimates, Shannon diversity index, Simpson evenness, as well as diversity rankings) were computed for each sample (Table 5-2). The highest genus-level richness was observed in aoudad sheep, dwarf goat, oryx, domesticated cow, domesticated goat, miniature donkey, zebra and blackbuck deer samples, while the highest genus-level diversity (based on diversity ranking and Shannon index) was observed in domesticated goat, alpaca, axis deer, blackbuck deer, mouflon ram, miniature donkey, oryx and domesticated horse. On the other hand, the lowest genus-level richness was observed in longhorn cattle, Pere David's deer, Boer goat, domesticated horse, domesticated sheep and alpaca, while the lowest genus-level diversity was observed in Fallow deer, zebra, domesticated sheep, dwarf goat and white-tail deer.

When correlated to animal host phylogeny or animal lifestyle (24 possible combinations), all diversity estimates showed low correlation coefficients (Cramer's V statistic <0.49) at both the genus and the species equivalent levels. Student's t-tests were used to examine the significance of the difference in diversity estimates at the genus and

species equivalent levels between animal host families (families Bovidae, Cervidae and Equidae) as well as animal lifestyle (zoo-housed, wild, and domesticated). Only three of these showed a significant difference (Student's t-test p-value <0.05): Family Bovidae had a significantly higher observed number of genera and significantly higher Chao estimate at the genus level, and zoo- housed animals had significantly lower Shannon diversity at the species equivalent level.

Community structure. We used a combination of ordination methods and Student's t-tests to identify associations between AGF genera and host factors. Non-metric multi-dimensional scaling based on the genus-level Bray–Curtis indices (Fig. 5-6 A and B) identified a few patterns. The genra *Aklioshbomyces, Ghazallomyces, Joblinomyces, Feramyces, Buwchfawromyces* and *Pecoramyces* seem to be more prevalent in some wild animals (e.g. black buck deer, mouflon, oryx, axis deer and white-tailed deer; filled squares in Fig. 5-6 A), while some zoo-housed animals (e.g. elk, dwarf goat and miniature donkey; grey squares in Fig. 5-6 A) clustered together based on the abundance of *Neocallimastix, Caecomyces* and *Liebetanzomyces*. A few domesticated animals (e.g. domesticated goat, long horn, alpaca and domesticated cow; open squares in Fig. 5-6 A) clustered together based on the abundance of *Cyllamyces*, AL8, MN3, MN4, RH1, RH3, RH4 and RH6. Animal host family had a slightly less apparent effect on AGF community structure (Fig. 5-6 B) with the exception of the importance of *Aklioshbomyces* and *Ghazallomyces* in family Cervidae, and AL3 and *Khyollomyces* in family Equidae. Indeed, analysis of similarity (ANOSIM) identified that the animal lifestyle had a more significant effect on community structure when compared with animal host family (p-values of 0.003, and 0.096 for animal lifestyle, and host family respectively).

To test the significance of these observed patterns, Student's t-tests were used to identify significant associations between specific AGF taxa and host phylogeny (families Bovidae, Equidae and Cervidae), or animal lifestyle (zoo- housed, domesticated and wild). From all possible associations (168 total; 28 genera × 3 host families and three life styles), significant differences were observed only in the following cases. The AGF genera AL3, *Khyollomyce*s and *Piromyces* were significantly more abundant in family Equidae (p-value = 0.014, 0.018 and 0.034 respectively), while the genera

Aklioshbomyces, *Ghazallomyces* and *Joblinomyces* were significantly more abundant in family Cervidae (p-value = 0.074, 0.072 and 0.075 respectively). On the other hand, the animal lifestyle had slightly more significant effect on AGF community structure as follows: The genus Neocallimastix was significantly more abundant in zoo-housed animals (p-value = 0.007), the genera *Aklioshbomyces*, *Buwchfawromyces* and *Pecoramyces* were significantly more abundant in wild animals (p- value = 0.047, 0.028 and 0.014 respectively), and the genera *Cyllamyces*, AL8, RH1, RH4 and RH6 were significantly more abundant in domesticated animals (p- value = 0.001, 0.001, 0.011, 0.018 and 0.054 respectively). Finally, for individual animals species with enough replication in our study, the genera *Cyllamyces*, AL8 and RH1 were significantly more abundant in Bos taurus (p- values = $1.86E^{-}11$, 3E-5 and $2.27E^{-}9$ respectively), the genera Caecomyces and RH5 were significantly more abundant in Ovis aries (p-values = 0.006, and 0.004 respectively), and the genera Feramyces and SK4 were significantly more abundant in Ammotragus lervia (p- values = 0.002, and 0.0006 respectively). Furthermore, some genera were only encountered in one animal, dem- onstrating a probable strong AGF genus–host preference. These genera include *Ghazallomyces* only encountered in axis deer, AL4 only encountered in domesticated sheep, MN3 only encountered in domesticated cow and MN4 only encountered in domesticated goat.

In addition to Student's t-tests, ANOSIM was used to test the effect of animal lifestyle and animal host family on the community structure as a whole. Results showed that the animal lifestyle had a more significant effect on community structure when compared with animal host family (p-values of 0.003, and 0.096 for animal lifestyle, and host family respectively).

Neocallimastigomycota isolation

A total of 216 AGF isolates were obtained from 21 animals (Table 5-3). Success in isolation and maintenance of that large number of isolates was enabled by the implementation of various techniques for isolation, and the development of a reliable storage procedure (Calkins et al., 2016). Isolates obtained belonged to 12 different genera (Table 5-3), six of which were exclusively isolated in this study, and characterized in

separate publications (*Akhlioshbomyces, Ghazallomyces, Capellomyces, Agriosomyces, Khoyollomyces* (AL1) and *Feramyces* (AL6) (Hanafy et al., 2018a; Hanafy et al., 2020). In general, 1–3 AGF genera were isolated per sample. Isolation efforts captured anywhere between 6.3% (1 of 16 genera) and 27.3% (3 of 11 genera) of AGF genera identified in a single sample using culture-independent D1/D2 LSU gene-based analysis. However, these values are highly affected by the fact that sequencing efforts are capable of identification of AGF genera present in extremely low levels of relative abundance. Indeed, excluding rare taxa (those present at <1% abundance), the culturability goes up to 10% (1 of 10 genera)-100% (2 of 2 genera).

We sought to determine how community structure and isolation efforts correlate, and whether obtaining isolates belonging to a specific genus could be predicted from the community structure of the sample. We observed a strong Pearson correlation (r = 0.79) between the abundance of a certain genus in a sample and the frequency of its isolation. On the other hand, the success of isolation of the most domi- nant member of the community was negatively affected by the sample evenness (Pearson correlation coefficient = −0.87). Indeed, our ability to isolate the novel genera *Aklioshbmyces, Ghazallomycota* and *Khyollomyces* could be attributed to their presence in high relative abundance in samples from which they were recovered (Table 5-3), as opposed to their rarity/absence in other samples (Figs 5-4 and 5-5). Within ubiquitous genera, we observed that the abundance-success of isolation correlation described above is stronger for monocentric taxa (Pearson correlation coefficients = 0.83, 0.96, 0.92 and 1 for *Pecoramyces, Feramyces, Neocallimastix* and *Agriosomyces* respectively), while such relationship was much weaker in polycentric taxa (Pearson correlation coefficients = 0.31, and 0.58 for *Orpinomyces*, and *Anaeromyces* respectively). However, the polycentric nature of these genera (ability to propagate even in the absence of zoospore production, and the larger colony size on roll tubes) enabled their isolation even when they constituted a minor fraction of the total community.

Table 5-1. Animals sampled in this study, numbers of sequences obtained (N), number of observed OTUs, and various diversity indices both at the species equivalent (0.02) and at the genus levels.

	Host description			Observed number of OTUs		Chao		Ace		Simpson evenness		Shannon		Diversity ranking[b]		Coverage[c]	
Sample	Family	Lifestyle	*N*[a]	Sp. Eq.	Genus	Sp. Eq.	Genus	Sp. Eq.	Genus	Sp. Eq.	Genus	Sp. Eq.	Genus	Sp. Eq.	Genus	Sp. Eq.	Genus
Alpaca	Camelidae	Domestic	240	19	9	26.2	9.5	30.5	10.8	0.27	0.50	1.99	1.64	17.3	18.8	0.94	0.99
American bison	Bovidae	Zoo	183	17	11	22.6	11.3	41.6	12.2	0.13	0.14	1.45	0.90	9.8	7.5	0.93	0.99
American elk	Cervidae	Zoo	99	11	9	16	11	28.2	15.3	0.17	0.21	1.12	1.01	6.3	10.5	0.92	0.96
Aoudad sheep (1)	Bovidae	Wild	3381	80	17	111	23	159.4	27	0.03	0.15	1.54	1.17	13.5	7	0.99	1
Aoudad sheep (2)	Bovidae	Wild	1779	55	13	57	13	59.9	13.4	0.05	0.13	1.78	0.91	11.2	11.8	0.99	1
Axis deer	Cervidae	Wild	367	18	9	18.6	9.5	19.6	11.6	0.36	0.49	2.17	1.61	19	19.5	0.99	0.99
Blackbuck deer	Bovidae	Wild	145	21	13	34.8	16	67.7	17	0.18	0.24	1.93	1.59	16	16.7	0.91	0.97
Boer goat	Bovidae	Wild	2503	41	9	49.1	9	58.8	9	0.05	0.21	1.12	0.88	6.5	8.3	0.99	1
Domestic cow	Bovidae	Domestic	727	45	13	46.7	16	48.5	15.4	0.06	0.14	1.95	1.03	13.5	10.2	0.98	1
Domestic goat	Bovidae	Domestic	162	23	15	39.5	16	70.7	17.2	0.40	0.43	2.52	2.10	20.3	21	0.87	1
Domestic horse	Equidae	Domestic	498	15	8	15.5	8.3	17.7	10	0.11	0.35	0.94	1.18	5.3	13.7	0.99	1
Domestic sheep	Bovidae	Domestic	1349	33	9	33.7	9	34.5	9.5	0.04	0.12	0.72	0.25	2.5	3	1	1
Dwarf goat	Bovidae	Zoo	519	15	8	20.3	18	36.7	17.5	0.08	0.13	0.49	0.14	1	2	0.98	0.99
Fallow deer	Cervidae	Wild	1368	43	12	46.7	12.3	48.7	13.2	0.08	0.13	1.67	0.78	14.5	5.3	0.99	1
Longhorn cattle	Bovidae	Domestic	62	9	5	16.5	5	47.2	5.8	0.28	0.41	1.37	0.96	11	10	0.82	0.98
Miniature donkey	Equidae	Zoo	56	12	8	30	11	191.6	18	0.23	0.46	1.50	1.46	11	17.2	0.80	0.93
Mouflon ram	Bovidae	Wild	297	17	11	35	11.3	111	14.3	0.23	0.31	1.80	1.55	16	17.2	0.95	0.99
Oryx	Bovidae	Wild	780	34	15	36.2	16	41.9	17.7	0.26	0.16	2.52	1.35	20.7	13.8	0.98	0.98
Pere David's deer	Cervidae	Zoo	169	7	6	10	6.5	17	7.8	0.32	0.36	0.96	0.88	6.7	10.5	0.96	0.99
White-tail deer	Cervidae	Wild	946	23	6	23	7.5	23.4	14.7	0.06	0.17	0.75	0.07	2.8	1	1	1
Zebra	Equidae	Zoo	2067	55	11	73	13	85.4	15	0.03	0.15	1.10	0.76	6	6	0.99	1

[a]N refers to the number of sequences obtained for each of the sampled.

[b]Diversity ranking is the average rank obtained using both an information-related diversity ordering method (Renyi generalized entropy), and an expected number of species-related diversity ordering method (Hulbert family of diversity indices). Samples are ranked from the least diverse (rank 1) to the most diverse (rank 21). [c]Coverage refers to the Good's coverage index.

Table 5-2. Representatives full length sequences spanning the region 'ITS1-5.8S rRNA-ITS2-D1/D2 LSU'.

Name	GenBank Accession number[a]	Number of ITS1-5.8S-ITS2-D1/D2 LSU sequences		Position (number refers to the position within the accession where the region starts)				Allternate names	References
		# of isolates (# of clone sequences)	# of SMRT-generated environmental sequences	ITS1	5.8S	ITS2	LSU		
Cultured genera									
Agriosomyces		1 (2 clones)	222						
Agriosomyces longus strain MS2, clone B	MT085709			1–226	227–406	407–587	588–1372		This study
Agriosomyces longus strain MS2, clone C	MT085708			1–219	220–401	402–582	583–1367		This study
Aklioshbomyces		1 (5 clones)	1009						
Aklioshbomyces papillarum strain WT2, clone 7	MT085737			1–182	183–357	358–540	541–1326		This study
Aklioshbomyces papillarum strain WT2, clone 8	MT085738			1–182	183–357	358–540	541–1326		This study
Aklioshbomyces papillarum strain WT2, clone 9	MT085739			1–182	183–360	361–538	539–1323		This study
Aklioshbomyces papillarum strain WT2, clone 10	MT085740			1–182	183–357	358–540	541–1325		This study
Aklioshbomyces papillarum strain WT2, clone 12	MT085741			1–184	185–359	360–538	539–1324		This study
Anaeromyces		7 (15 clones)	76						
Anaeromyces contortus isolate C3G Clone 10	MG605705.1			1–222	223–401	402–572	573–1356		29
Anaeromyces contortus isolate C3J Clone 2	MG605699.1			1–226	227–405	406–576	577–1362		29
Anaeromyces contortus isolate G3A Clone 1	MG605688.1			1–221	222–400	401–572	573–1358		29
Anaeromyces contortus isolate G3A clone 2	MG605684.1			1–219	220–397	398–569	570–1353		29
Anaeromyces contortus isolate G3A Clone 3	MG605681.1			1–221	222–399	400–571	572–1355		29
Anaeromyces contortus isolate G3A Clone 5	MG605697.1			1–223	224–402	403–573	574–1357		29
Anaeromyces contortus isolate G3C Clone 4	MG605685.1			1–217	218–395	396–567	568–1353		29
Anaeromyces contortus isolate G3C Clone 5	MG605679.1			1–221	222–399	400–572	573–1358		29
Anaeromyces contortus isolate G3C Clone 6	MG605683.1			1–221	222–399	400–571	572–1355		29
Anaeromyces contortus isolate G3G Clone 10	MG605690.1			1–220	221–398	399–571	572–1357		29
Anaeromyces contortus isolate G3G Clone 8	MG605686.1			1–216	217–394	395–566	567–1350		29
Anaeromyces contortus isolate G3G Clone 9	MG605691.1			1–221	222–399	400–572	573–1358		29

Table 5-2, continued

Anaeromyces contortus isolate Na Clone 5	MG605704.1			1–223	224–402	403–573	574–1357		29
Anaeromyces contortus isolate Na Clone 6	MG605701.1			1–226	227–406	407–578	579–1362		29
Anaeromyces contortus isolate X4 Clone 2	MG605706.1			1–223	224–402	403–573	574–1357		29
Buwchfawromyces		0	55					SK2	
Buwchfawromyces eastonii	AoudOld_160513			1–168	169–326	326–536	537–1235		This study
Caecomyces		3 (3 clones)	879						
Caecomyces sp. isolate DS1 Clone C3	MT085702			1–205	206–381	382–583	584–1366		This study
Caecomyces sp. isolate CYF	JQ782554.1			65–280	281–456	457–654	655–1379		23
Caecomyces sp. isolate CYR	JQ782555.1			65–274	275–450	451–646	647–1371		23
Capellomyces		2 (5 clones)	0						
Capellomyces foraminis isolate BGB11 Clone C1	MT085700			1–220	221–400	401–577	578–1360		This study
Capellomyces foraminis isolate BGB11 Clone C2	MT085697			1–220	221–401	402–578	579–1362		This study
Capellomyces foraminis isolate BGB11 Clone C3	MT085698			1–220	221–401	402–579	580–1363		This study
Capellomyces foraminis isolate BGB11 Clone C4	MT085699			1–220	221–401	402–578	579–1363		This study
Capellomyces elongatus	MT085701			1–250	251–432	433–609	610–1393		This study
Cyllamyces		1 (clones 5)	704					MN1, SP8	
Cyllamyces sp. isolate TSB2 Clone B10	MT085707			1–170	171–347	348–537	538–1320		This study
Cyllamyces sp. isolate TSB2 Clone B11	MT085705			1–170	171–347	348–538	539–1321		This study
Cyllamyces sp. isolate TSB2 Clone B12	MT085703			1–170	171–347	348–537	538–1320		This study
Cyllamyces sp. isolate TSB2 Clone B8	MT085704			1–168	169–344	345–536	537–1319		This study
Cyllamyces sp. isolate TSB2 Clone B9	MT085706			1–168	169–345	346–535	536–1318		This study
Ghazallomyces		1 (4 clones)	102						
Ghazallomyces constrictus isolate AXS31 Clone B1	MT085693			1–189	190–370	371–564	565–1348		This study
Ghazallomyces constrictus isolate AXS31 Clone B2	MT085695			1–186	187–364	365–556	557–1339		This study
Ghazallomyces constrictus isolate AXS31 Clone B3	MT085694			1–186	187–364	365–556	557–1339		This study
Ghazallomyces constrictus isolate AXS31 Clone B5	MT085696			1–189	190–367	368–552	553–1335		This study
Joblinomyces		2 (10 clones)	1076					AL5	
Joblinomyces apicalis isolate GFH681 Clone1	MT085665			1–213	214–388	389–561	562–1344		This study

Table 5-2, continued

Joblinomyces apicalis isolate GFH681 Clone2	MT085666			1–213	214–390	391–564	565–1347		This study
Joblinomyces apicalis isolate GFH681 Clone4	MT085667			1–215	216–393	394–568	569–1351		This study
Joblinomyces apicalis isolate GFH681 Clone5	MT085668			1–215	216–393	394–568	569–1351		This study
Joblinomyces apicalis isolate GFH681 Clone6	MT085669			1–213	214–389	390–563	564–1346		This study
Joblinomyces apicalis isolate GFH683 Clone1	MT085670			1–213	214–389	390–563	564–1346		This study
Joblinomyces apicalis isolate GFH683 Clone2	MT085671			1–213	214–389	390–563	564–1346		This study
Joblinomyces apicalis isolate GFH683 Clone3	MT085672			1–212	213–388	389–562	563–1345		This study
Joblinomyces apicalis isolate GFH683 Clone4	MT085673			1–213	214–389	390–563	564–1346		This study
Joblinomyces apicalis isolate GFH683 Clone5	MT085674			1–213	214–389	390–563	564–1346		This study
Feramyces		4 (11 clones)	2373					AL6	
Feramyces austinii isolate DS10 Clone 11	MG584196.1			1–192	193–368	369–570	571–1353		28
Feramyces austinii isolate DS10 Clone 12	MG584194.1			1–192	193–368	369–570	571–1353		28
Feramyces austinii isolate DS10 Clone 7	MG584192.1			1–192	193–368	369–571	572–1354		28
Feramyces austinii isolate DS10 Clone 8	MG584200.1			1–192	193–368	369–570	571–1352		28
Feramyces austinii isolate DS10 Clone 9	MG584197.1			1–192	193–368	369–570	571–1353		28
Feramyces austinii isolate F3A Clone 3	MG584193.1			1–192	193–368	369–570	571–1353		28
Feramyces austinii isolate F3B Clone 10	MG584190.1			1–192	193–368	369–570	571–1352		28
Feramyces austinii isolate R4A Clone 1	MG584191.1			1–192	193–368	369–570	571–1353		28
Feramyces austinii isolate R4A Clone 2	MG584199.1			1–192	193–368	369–570	571–1353		28
Feramyces austinii isolate R4A Clone 3	MG584198.1			1–192	193–368	369–570	571–1353		28
Feramyces austinii isolate R4A Clone 5	MG584195.1			1–192	193–368	369–570	571–1353		28
Khyollomyces		1 (1 clone)	2553					AL1	

Table 5-2, continued

Khyollomyces ramosus isolate ZS33 Clone 8	MT085710			1–193	194–369	370–543	544–1327		This study
Liebetanzomyces		1 (7 clones)	31					SP4	
Liebetanzomyces sp. isolate Cel1A Clone 2	MT085726			1–225	226–403	404–577	578–1361		This study
Liebetanzomyces sp. isolate Cel1A Clone 3	MT085727			1–225	226–403	404–577	578–1361		This study
Liebetanzomyces sp. isolate Cel1A Clone 4	MT085728			1–225	226–403	404–577	578–1361		This study
Liebetanzomyces sp. isolate Cel1A Clone 6	MT085729			1–225	226–403	404–577	578–1362		This study
Liebetanzomyces sp. isolate Cel1A Clone 7	MT085730			1–223	224–401	402–576	577–1362		This study
Liebetanzomyces sp. isolate Cel1A Clone 8	MT085731			1–225	226–403	404–577	578–1361		This study
Liebetanzomyces sp. isolate Cel1A Clone 9	MT085732			1–223	224–401	402–575	576–1359		This study
Neocallimastix		14 (14 clones)	794					SP6	
Neocallimastix californiae strain G1[b]	MCOG01000947.1			12 566–12 742	12 743–12 917	12 918–13 113	13 114–13 895		36
Neocallimastix californiae strain G1[b]	MCOG01000947.1			2806–2982	2983–3157	3158–3354	3355–4136		36
Neocallimastix californiae strain G1[b]	MCOG01001752.1			2193–2369	2018–2192	1821–2017	1820–1036		36
Neocallimastix cf. *cameroonii* isolate G3	MT085722			1–178	179–356	357–554	555–1338		This study
Neocallimastix sp isolate Hef5 Clone 1	MT085723			1–229	230–408	409–588	589–1371		This study
Neocallimastix sp isolate Hef6 Clone 6	MT085724			1–240	241–419	420–602	603–1385		This study
Neocallimastix sp isolate Hef7 Clone 3	MT085725			1–229	230–407	408–585	586–1368		This study
Neocallimastix cf. *frontalis* isolate NYF1	JQ782542.1			67–308	309–486	487–665	666–1390		23
Neocallimastix cf. *frontalis* isolate NYF2	JQ782543.1			67–307	308–485	486–672	673–1397		23
Neocallimastix cf. *frontalis* isolate NYF3	JQ782544.1			67–296	297–474	475–654	655–1379		23
Neocallimastix cf. *frontalis* isolate NYF4	JQ782545.1			67–310	311–488	489–676	677–1401		23
Neocallimastix cf. *frontalis* isolate NYR1	JQ782546.1			67–307	308–486	487–669	670–1394		23
Neocallimastix cf. *frontalis* isolate NYR2	JQ782547.1			67–317	318–496	497–676	677–1401		23
Neocallimastix cf. *frontalis* isolate NYR3	JQ782548.1			67–317	318–495	496–678	679–1403		23
Neocallimastix cf. *frontalis* isolate NYR4	JQ782549.1			67–295	296–473	474–654	655–1380		23
Neocallimastix cf. *frontalis* isolate NYR5	JQ782550.1			67–309	310–488	489–667	668–1392		23

Table 5-2, continued

Oontomyces		0	0						
Oontomyces anksri strain SSD-CIB1	JX017310.1			60–291	292–467	468–642	643–695		26
Oontomyces anksri strain SSD-CIB1	JX017314.1						1–772		26
Orpinomyces		23 clones	349						
Orpinomyces sp. OUS1	AJ864475.1			842–1056	1057–1241	1242–1418	1419–2201		49
Orpinomyces cf. *joyonii* isolate D3A Clone 3	MT085735			1–183	184–360	361–539	540–1322		This study
Orpinomyces cf. *joyonii* isolate D3A Clone F11	MT085736			1–182	183–357	358–537	538–1320		This study
Orpinomyces cf. *joyonii* isolate D3A Clone G09	MT085733			1–184	185–359	360–538	539–1321		This study
Orpinomyces cf. *joyonii* isolate D3A Clone H09	MT085734			1–187	188–362	363–540	541–1323		This study
Orpinomyces sp. OYF	JQ782551.1			67–267	268–447	448–630	631–1356		23
Orpinomyces sp. OYR2	JQ782553.1			65–253	254–431	432–610	611–1335		23
Pecoramyces		2 (4 clones)	248						
Pecoramyces ruminantium isolate C1A[b]	ASRE01020932.1			909–1095	1096–1271	1272–1452	1453–2235		43
Pecoramyces ruminantium isolate C1A[b]	ASRE01007038.1			790–976	977–1152	1153–1333	1334–2116		43
Pecoramyces ruminantium isolate C1A[b]	ASRE01022884.1			2760–2946	2584–2759	2403–2583	1620–2402		43
Pecoramyces ruminantium isolate S4B	MT085711			1–184	185–360	361–542	543–1325		This study
Piromyces		5 (26 clones)	3818					AL7, UC1	
Piromyces finnis strain finn	MCFH01000027.1			1034–1105	1106–1285	1286–1470	1471–2253		36
Piromyces finnis strain finn	MCFH01000027.1			9568–9639	9640–9819	9820–10,004	10,005–10,787		36
Piromyces finnis strain finn	MCFH01000027.1			18 102–18 173	18 174–18 353	18 354–18 538	18 539–19 321		36
Piromyces sp. isolate A1 Clone A1	MT085682			1–199	200–375	376–548	549–1333		This study
Piromyces sp. isolate A1 Clone A12	MT085684			1–198	199–374	375–547	548–1330		This study
Piromyces sp. isolate A1 Clone A2	MT085679			1–199	200–375	376–548	549–1331		This study
Piromyces sp. isolate A1 Clone A3	MT085683			1–199	200–375	376–548	549–1331		This study
Piromyces sp. isolate A1 Clone A4	MT085685			1–199	200–375	376–548	549–1331		This study

Table 5-2, continued

Piromyces sp. isolate A1 Clone A5	MT085688		1–199	200–375	376–548	549–1335	This study
Piromyces sp. isolate A1 Clone A6	MT085687		1–199	200–375	376–548	549–1331	This study
Piromyces sp. isolate A1 Clone A7	MT085686		1–199	200–375	376–548	549–1334	This study
Piromyces sp. isolate A1 Clone A8	MT085681		1–199	200–375	376–548	549–1331	This study
Piromyces sp. isolate A1 Clone A9	MT085680		1–199	200–375	376–548	549–1331	This study
Piromyces sp. isolate Cel1B Clone 1	MT085717		1–198	199–375	376–548	549–1331	This study
Piromyces sp. isolate Cel1B Clone 10	MT085721		1–198	199–374	375–547	548–1332	This study
Piromyces sp. isolate Cel1B Clone 2	MT085718		1–198	199–375	376–548	549–1331	This study
Piromyces sp. isolate Cel1B Clone 3	MT085719		1–198	199–375	376–549	550–1334	This study
Piromyces sp. isolate Cel1B Clone 6	MT085720		1–198	199–374	375–547	548–1332	This study
Piromyces sp. isolate DB3 Clone B2	MT085690		1–226	227–404	405–592	593–1350	This study
Piromyces sp. isolate DB3 Clone B3	MT085691		1–227	228–405	406–592	593–1359	This study
Piromyces sp. isolate DB3 Clone B4	MT085689		1–225	226–403	404–588	589–1351	This study
Piromyces sp. isolate Jen1 Clone 1	MT085712		1–201	202–378	379–552	553–1335	This study
Piromyces sp. isolate Jen1 Clone 2	MT085713		1–201	202–378	379–552	553–1335	This study
Piromyces sp. isolate Jen1 Clone 3	MT085714		1–201	202–377	378–550	551–1333	This study
Piromyces sp. isolate Jen1 Clone 4	MT085715		1–201	202–377	378–550	551–1333	This study
Piromyces sp. isolate Jen1 Clone 5	MT085716		1–201	202–378	379–552	553–1335	This study
Tahromyces		4 (4 clones)					
Tahromyces munnarensis isolate TDFKJa1924	MT085677		1–178	179–358	359–537	538–1316	This study
Tahromyces munnarensis isolate TDFKJa1926	MT085676		1–178	179–358	359–537	538–1307	This study
Tahromyces munnarensis isolate TDFKJa1927	MT085678		1–178	179–358	359–537	538–1320	This study
Tahromyces munnarensis isolate TDFKJa193	MT085675		1–178	179–358	359–537	538–1313	This study
Uncultured lineages							
Identified in this study (accession number is the sequence name in Supplementary Datasets 1–3)							
AL3	DwGoat_61688	86	57–200	201–352	353–604	605–1358	This study
AL4	Sheep_129918	1	67–268	269–425	426–630	631–1394	This study
AL8	Cow_130070	151	88–273	274–432	433–653	654–1424	This study

Table 5-2, continued

MN3	Cow_90808	3	76–280	281–438	439–640	641–1423	This study
MN4	OSUGoat_119881	3	69–276	277–434	435–666	667–1444	This study
SK3	Aoud18_104764	3	67–279	280–436	437–676	677–1444	This study
SK4	Aoud18_141177	1387	71–264	265–421	422–644	645–1421	This study
Not identified in this study (GenBank accession number of a representative ITS1 sequence)							
AL2	GQ826457						3
BlackRhino	JF423850						5
DA1	JX184822						13
DT1	GQ850291						48
JH1/SP5	GU911240						17, 46
KF1	GQ850345						45
MN2	AM690075						47
SK1	JF423570						5
SP1	GQ678747						17
SP2	GQ698377						17
SP3	GQ657498						17
SP7	GU910219.1						17
Novel lineages (accession number is the sequence name in Supplementary Datasets 1–3)							
RH1	Cow_130696	13	66–222	223–378	379–593	594–1369	This study
RH2	Oryx_79099	74	67–270	271–425	426–660	661–1440	This study
RH3	AmBis_130671	3	71–246	247–404	405–613	614–1374	This study
RH4	Cow_156860	13	68–238	239–393	394–597	598–1379	This study
RH5	Sheep_119174	1681	69–280	281–438	439–646	647–1426	This study
RH6	Cow_144271	2	78–289	290–446	447–653	654–1433	This study

GenBank accession numbers are shown for all clone sequences obtained from representative AGF isolates in our culture collection. For yet-uncultured AGF taxa, accession numbers SMRT generated sequence name in Datasets 1–3. Start and end positions of ITS1, 5.8S rRNA gene, ITS2 and the D1/D2 region of the LSU are shown. [a]GenBank Accession numbers are provided for Sanger sequenced clones from all fungal isolates. PacBio-generated datasets are present in GenBank in the Bioproject accession number PRJNA609702, Biosample accession numbers SAMN14258225, and Targeted Locus Study project accession KDVX00000000. FASTA files of ITS1-5.8S-ITS2-D1/D2 LSU region, as well as bioinformatically extracted ITS1 region and D1/D2 LSU regions are provided as supplementary documents (Datasets 1–3). [b]Sequences extracted from a genomic assembly.

Table 5-3. Number and sources of isolates obtained in thus study.

AGF genus	Source	Number of isolates	% Abundance in PacBio culture-independent assessment
Agriosomyces	Mouflon	4	3.28
	Boer goat	1	8.5
Aklioshbomyces	White-tail deer	9	98.95
Anaeromyces	Domesticated cow	4	0.68
	domesticated goat	12	2.44
	American bison	4	1.63
	Alpaca	4	17.01
Caecomyces	Fallow deer	1	0.44
Capellamyces	Boer goat	5	ND
Feramyces	Aoudad sheep (1)	5	55.31
	Fallow deer	1	4.46
Ghazallomyces	Axis deer	11	27.79
Khyollomyces	Zebra	16	74.5
Neocallimastix	Dwarf goat	7	97.88
	Fallow deer	10	1.24
	Pere David's deer	10	54.44
	American elk	12	72
Orpinomyces	Domesticated cow	8	1.09
	Longhorn	3	3.03
	American bison	6	80.43
	Alpaca	6	33.2
Pecoramyces	Domesticated sheep	10	0.37
	Mouflon	3	12.79
	Oryx	11	13.78
	Aoudad sheep (2)	13	0.39
Piromyces	Domesticated cow	5	1.64
	domesticated sheep	3	1.6
	Mouflon ram	2	23.61
	Axis deer	1	28.88
	Blackbuck deer	7	6.21
	Domesticated horse	6	80.12
	Miniature donkey	16	69.64

Figure 5-1. Workflow diagram describing the methods employed in this study.

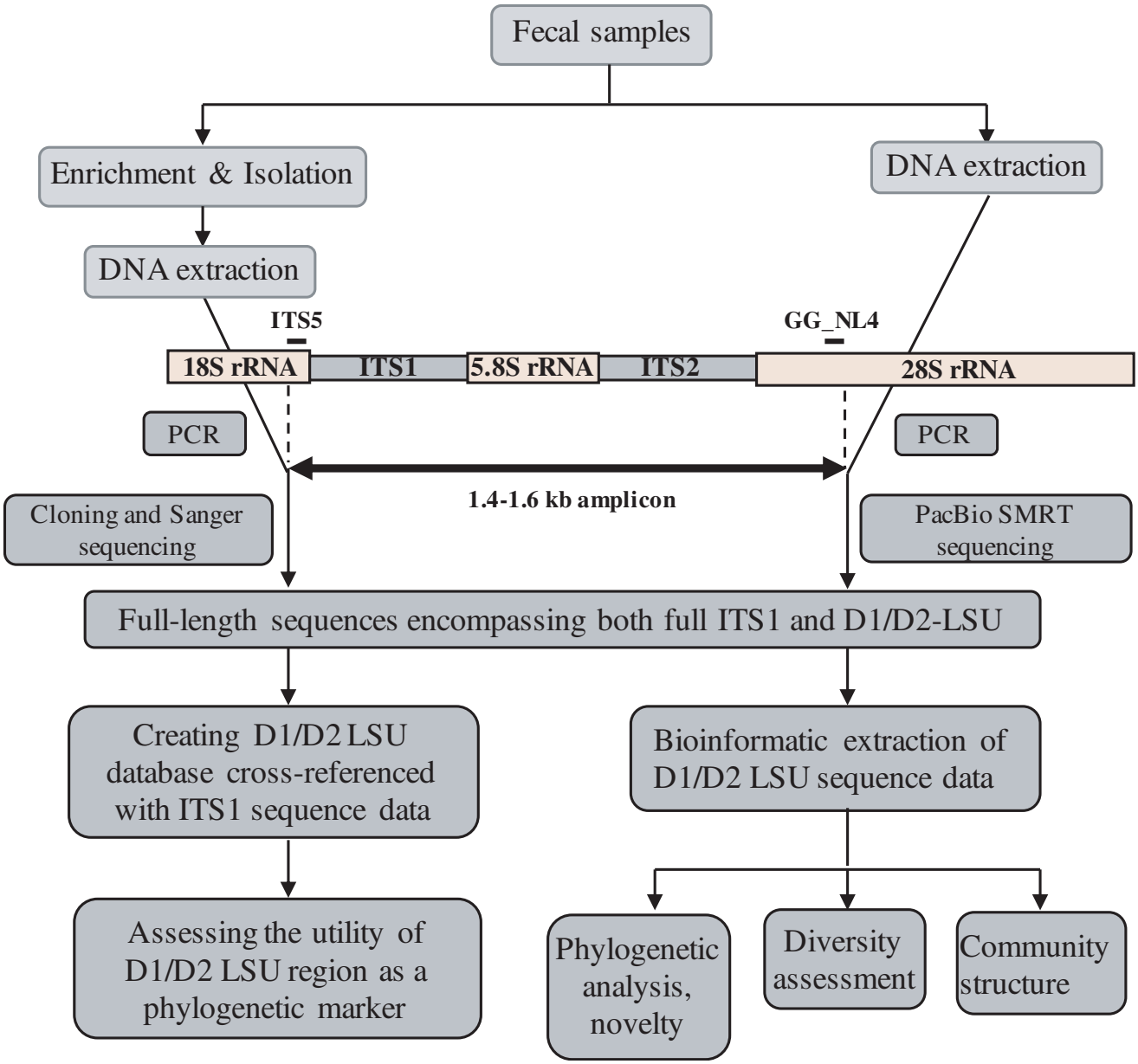


Figure 5-2. Box and whisker plots showing the variability in intra-genus length (A, B) and sequence divergence cutoff (C, D) for the ITS1 (A, C) and D1/D2 LSU (B, D) regions. A cartoon of the rRNA locus is shown on top. Genera and candidate genera with at least five sequences encompassing the full region 'ITS1-5.8S-ITS2-D1/D2 LSU' were used to construct this plot as detailed in the methods section. The candidate genera AL4, MN3, MN4, RH3, RH6 and SK3 had only a few sequence representatives

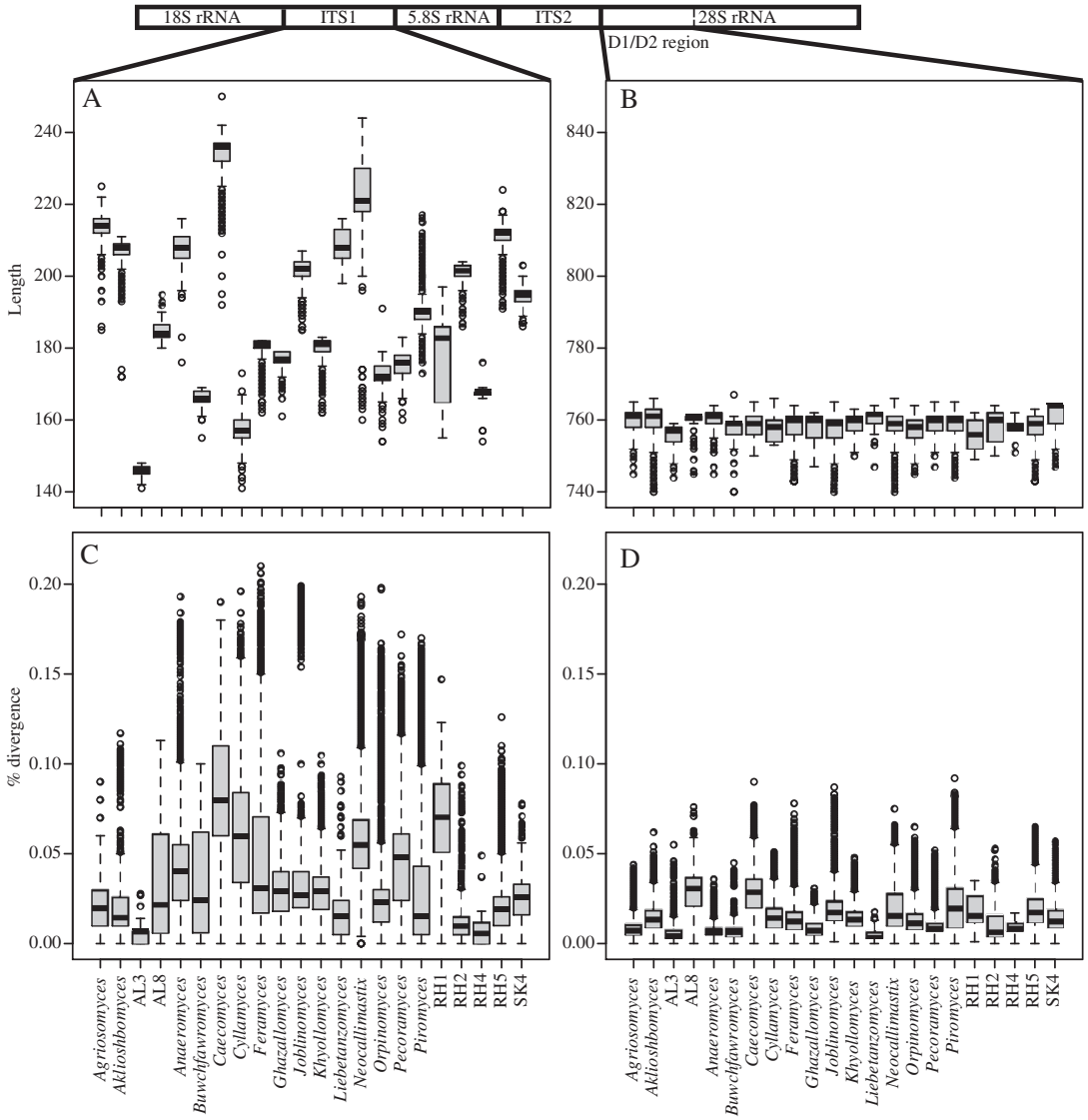


Figure 5-3. Box and whisker plots showing the variability in intra-strain length (A, B) and sequence divergence cutoff (C, D) for the ITS1 (A, C) and D1/D2 LSU (B, D) regions.

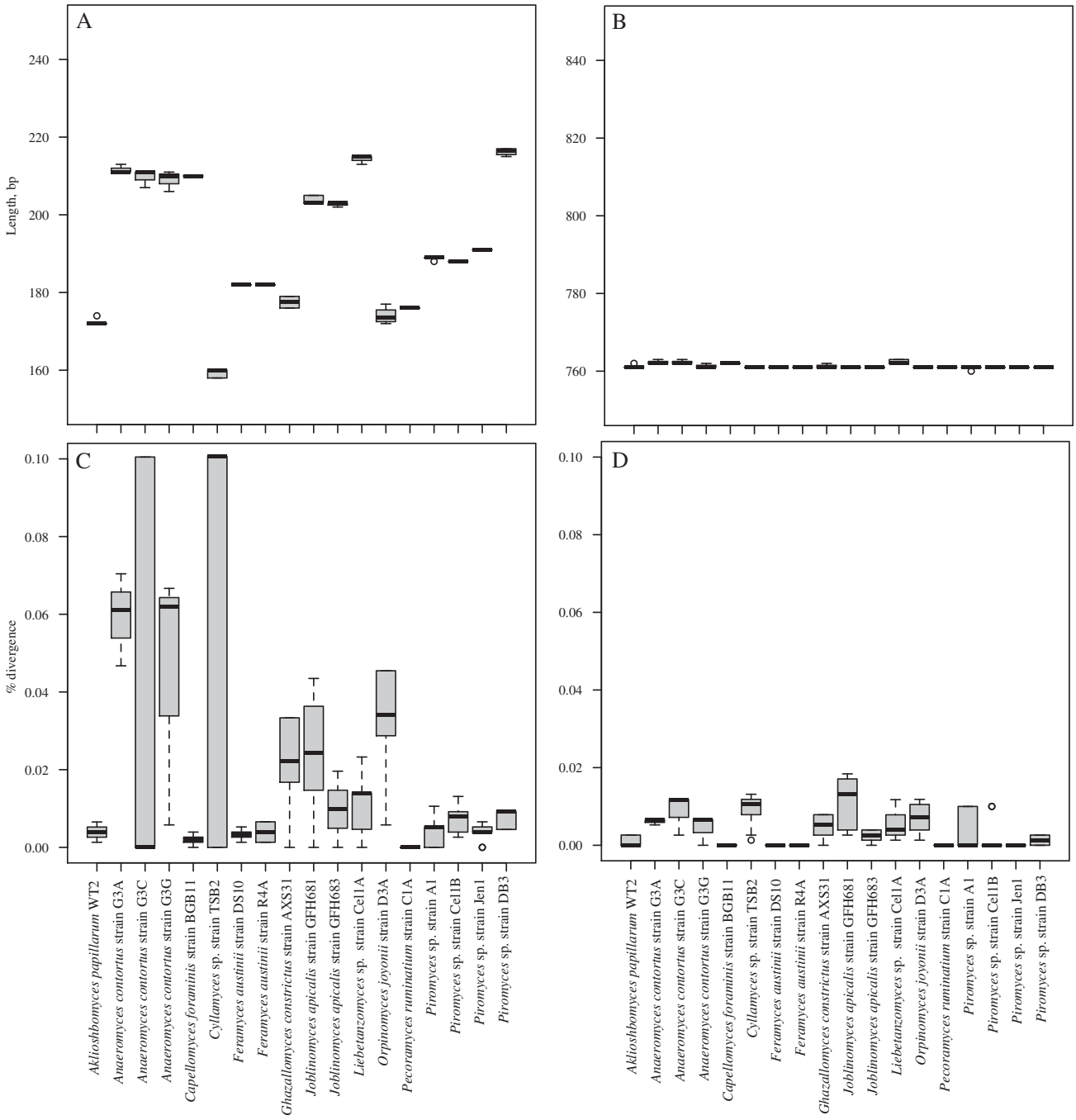
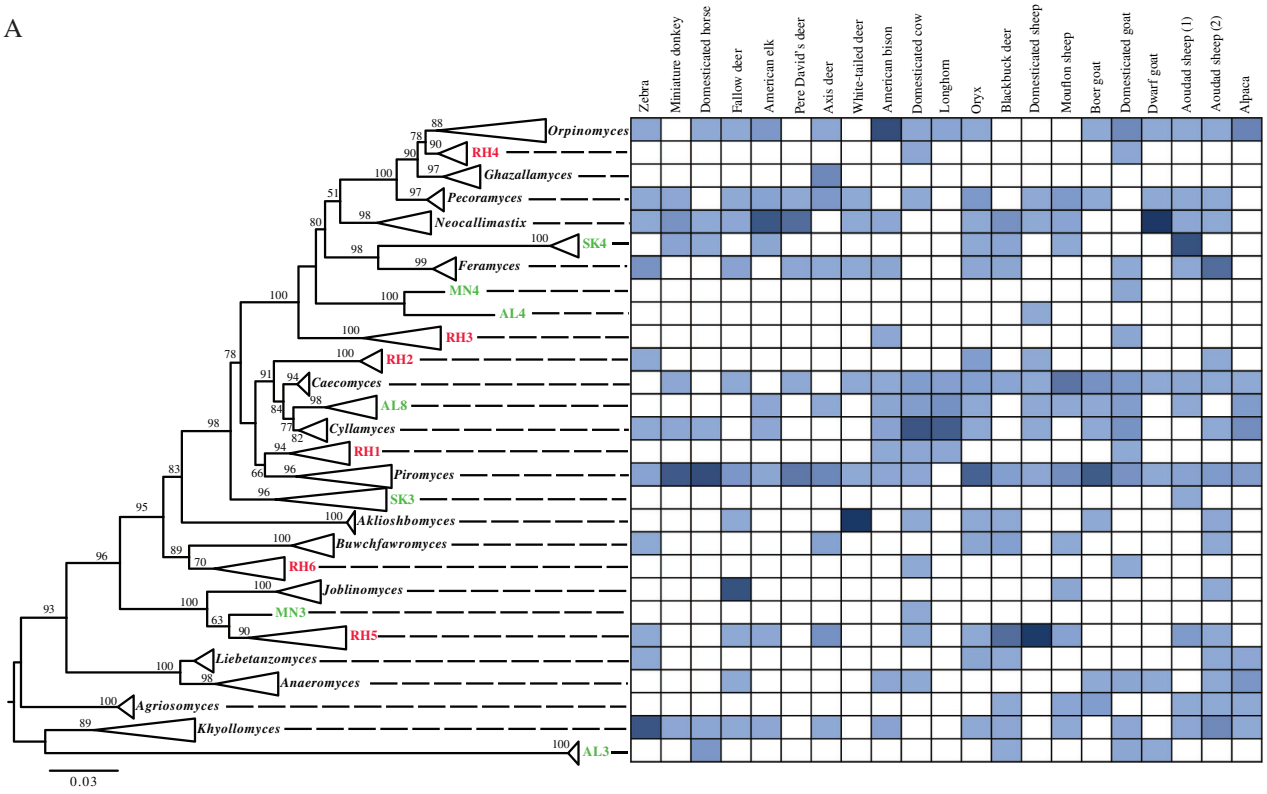


Fig 5-4. AGF genera distribution across the animal studied. (A) Percentage abundance of AGF genera in the animals studied. The tree is intended to show the relationship between the animals and is not drawn to scale. Host phylogeny (family), lifestyle and gut type are shown for each animal. The X-axis shows the percentage abundance of AGF genera.

Figure 5-5. (A) Phylogenetic tree constructed using the D1/D2 LSU sequences of representatives of each of the 28-genera/candidate genera identified in this study. Sequences were aligned using the MAFFT aligner with the –auto flag and the default parameters for gap extension and gap opening penalties and maximum likelihood tree was constructed in FastTree (Price et al., 2009, 2010). Bootstrap values are based on 100 replicates and are shown for branches with >50% bootstrap support. Genera with cultured representatives are shown in black, uncultured candidate genera identified in previous ITS1-based studies are shown in green, while the six novel genera identified in the current study are shown in red. The distribution of each of these genera/candidate genera in the animals studied is shown as a heatmap on the right. (B) AGF genera distribution patterns. The total number of animals harbouring each of the genera identified in this study is shown on the Y-axis, with the different coloured stacked bars reflecting the number of animals where the genus was the most abundant member, occurred with high (>5%) abundance, occurred with medium (1%–5%) abundance, or occurred with low (<1%) abundance. AGF genera are classified into one of the five distribution patterns shown on top of the graph using arbitrary cutoffs for ubiquity (presence in at least 50% of the animals studied, shown as the dotted line across the bottom bar chart), as well as the fraction of animals where the genus abundance was above 1% (shown as the top bar chart).


A
Zebra
Miniature donkey
Domesticated horse
Fallow deer
American elk
Pere David's deer
Axis deer
White-tailed deer
American bison
Domesticated cow
Longhorn
Oryx
Blackbuck deer
Domesticated sheep
Mouflon sheep
Boer goat
Domesticated goat
Dwarf goat
Aoudad sheep (1)
Aoudad sheep (2)
Alpaca
Orpinomyces
RH4
Ghazallamyces
Pecoramyces
Neocallimastix
SK4
Feramyces
MN4
AL4
RH3
RH2
Caecomyces
AL8
Cyllamyces
RH1
Piromyces
SK3
Aklioshbomyces
Buwchfawromyces
RH6
Joblinomyces
MN3
RH5
Liebetanzomyces
Anaeromyces
Agriosomyces
Khyollomyces
AL3
0.03

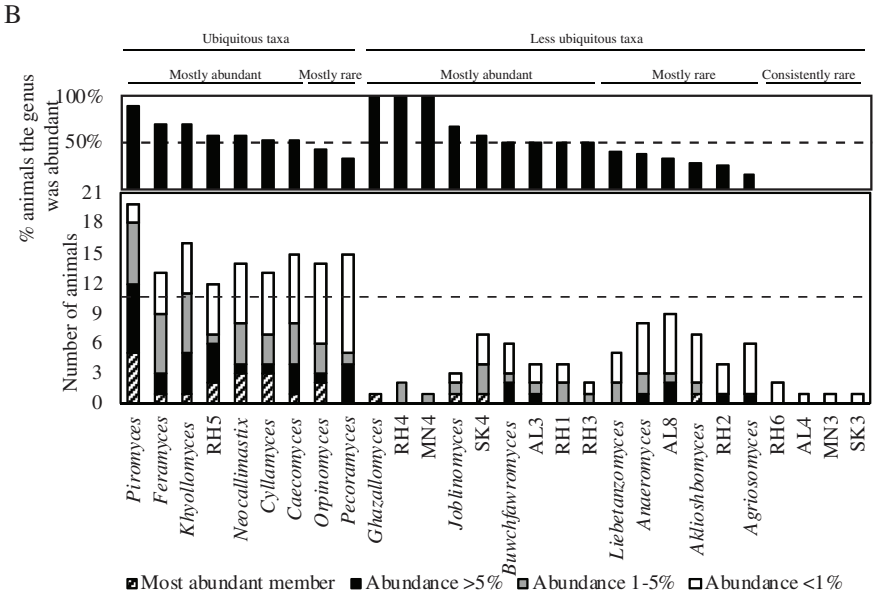
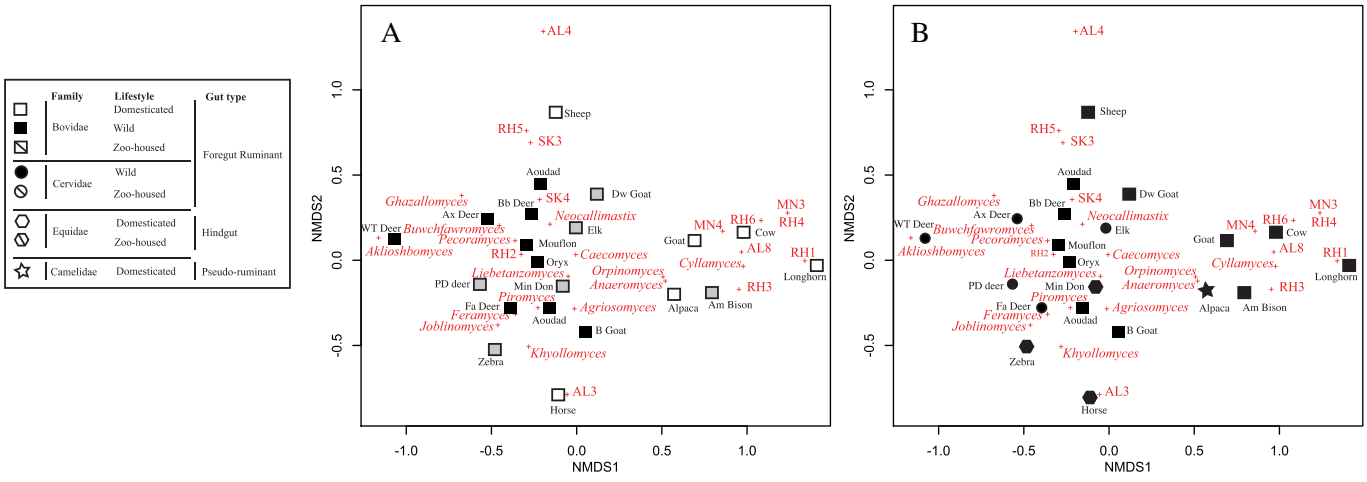

B
Ubiquitous taxa
Less ubiquitous taxa
Mostly abundant
Mostly rare
Mostly abundant
Mostly rare
Consistently rare
% animals the genus was abundant
100%
50%
Number of animals
21
18
15
12
9
6
3
0
Piromyces
Feramyces
Khyollomyces
RH5
Neocallimastix
Cyllamyces
Caecomyces
Orpinomyces
Pecoramyces
Ghazallomyces
RH4
MN4
Joblinomyces
SK4
Buwchfawromyces
AL3
RH1
RH3
Liebetanzomyces
Anaeromyces
AL8
Aklioshbomyces
RH2
Agriosomyces
RH6
AL4
MN3
SK3
Most abundant member
Abundance >5%
Abundance 1-5%
Abundance <1%

Figure 5-6. Non-metric multidimensional scaling based on pairwise Bray–Curtis dissimilarity indices at the genus level. Samples are shown as symbols and displayed in black text while AGF genera are shown as '+' and displayed in red text. (A) Symbols reflect lifestyle with domesticated animals shown as white squares, zoo-housed animals shown as grey squares and wild animals shown as black squares. (B) Symbols reflect animal host phylogeny with family Bovidae shown as squares, family Cervidae shown as circles, family Equidae shown as hexagons and family Camelidae shown as a star. Abbreviations: Am Bison, American bison; Axe deer, Axis deer; B goat, Boer goat; Bb deer, Blackbuck deer; Dw Goat, Dwarf goat; Fa deer, Fallow deer; Min Don, Miniature donkey; PD deer, Pere David's deer; WT deer, White-tail deer.



Discussion

LSU as a phylogenetic marker for AGF diversity surveys

In this study, we highlight and quantify the advantages associated with the utilization of D1/D2 LSU as a phylomarker for the AGF when compared with the currently utilized ITS1 region (Figs 5-2 and 5-3). To address the lack of reference LSU sequence data, we undertook a multi-year isolation effort to provide a comprehensive D1/D2-LSU database from a wide range of AGF taxa, a necessary approach given the lack of LSU sequence data from multiple historic taxa, unavailability of AGF in culture collections and difficulties in maintenance of this fastidious group of organisms. To correlate D1/D2 LSU data to currently available ITS1 datasets and overcome amplicon length constrains, we utilized a single molecular real time (SMRT)-PacBio sequencing approach to obtain sequences comprising the region spanning from the start of the ITS1 region to the end of the D1/D2-LSU region in the rRNA locus (1400–1500 bp). In the process, we not only increased the representation of D1/D2-LSU sequences from all cultured taxa but also identified D1/D2-LSU sequences of yet-uncultured taxa previously defined only by their ITS1 sequences (e.g. AL3, AL4, AL8, MN3, MN4, SK3 and SK4), as well as defined six completely novel AGF candidate genera (RH1-RH6). Collectively, this dataset (17 697 sequences of environmental D1/D2 LSU annotated by their taxonomy, plus 116 Sanger-generated clone sequences and genomic rRNA loci sequences (Table 5-2 with accession numbers) could pave the way for future diversity surveys solely based on D1/D2 LSU amplification and analysis. We anticipate that future AGF diversity studies employing PacBio sequencing of the D1/D2-LSU region (rather than the full ITS1-5.8S-ITS2-D1/D2 LSU region) would be further enabled by the shorter amplicon length (~700 as opposed to ~1300–1400 bp), as well as recent (e.g. Sequel II) and future anticipated improvements in SMRT sequencing technology. Use of D1/D2 LSU as a phylogenetic marker could certainly be employed for assessing diversity of various fungal lineages outside the Neocallimastigomycota, although comparing its utility as a phylogenetic marker to the ITS1 region, similar to that conducted in this study (Figs 5-2 and 5-3), is warranted (Schoch *et al*., 2012).

Discovery and characterization of novel AGF lineages

D1/D2 LSU-based diversity assessment of 21 faecal samples identified multiple novel AGF candidate genera (Figs 5-4 and 5-5), five of which were subsequently isolated and described in separate publications (*Feramyces* (Hanafy *etal*., 2018a), *Aklioshbomyces*, *Agriosomyces*, *Ghazallomyces* and *Khyollomyces* (Ha nafy *et al*., 2020). These results clearly demonstrate that the scope of AGF diversity is much broader than implied from prior studies. This conclusion is in apparent disagreement with the recent work of Paul *et al*. (2018), where the authors utilized a rarefaction-based approach on publicly available ITS1 AGF sequence

data to suggest that AGF sampling efforts have reached saturation. However, we argue that using a rarefaction curve approach on publicly available datasets only elucidates coverage within samples already in the database, and not the broader AGF diversity in nature. Many prior studies have used relatively low throughput sequencing technologies, and repeatedly sampled a few domesticated animals, and such pattern would result in encountering highly similar populations between different studies. We attribute the discovery and characterization of a wide range of novel AGF taxa within our dataset to sampling previously unsampled animal hosts, and the use of high-throughput sequencing that enabled access to rare members of the AGF community. Multiple novel AGF genera were isolated from animals previously unsampled for AGF diversity, e.g. *Aklioshbomyces* from white-tailed deer where it represented 98.5% of the community, *Ghazallomyces* from axis deer where it represented 27.8% of the community and *Feramyces* from an aoudad sheep sample where it represented 55.3% of the community. It is notable that many of these novel taxa were only encountered in wild herbivores. Whether this novelty is a reflection of differences in lifestyle, with wild herbivores experiencing a more diverse diet and infrequent feeding intervals and domesticated herbivores experiencing a monotonous feeding schedule and less diverse diets selecting for specific taxa, or a reflection of simply lack of prior sampling of wild animals due to logistic difficulties remains to be seen. This clearly demonstrates that novel AGF taxa remain to be discovered by sampling hitherto unsampled/poorly sampled animal hosts.

Furthermore, a significant fraction of novel AGF candidate genera identified were present in extremely low relative abundance. The discovery and characterization of the rare members of AGF community could significantly expand the scope of AGF diversity in nature. The dynamics, rationale for occurrence, mechanisms of maintenance, putative role in ecosystems and evolutionary history of rare members of the community are currently unclear. It has been suggested that a fraction of the rare biosphere could act as a seed bank of functional redundancy that aids in ecosystem response to periodic (e.g. occurring as part of growth of the animal host, or due to seasonal changes in feed types) or occasional (i.e. due to unexpected disturbances) changes in the gut *in situ* conditions, where environmental stressors could promote the growth of specific members of the rare biosphere that are more metabolically and physiologically adapted to the new condition, a process that could potentially contribute to the overall ecosystem functional flexibility (Marchant *et al*., 2002; Elshahed *et al*., 2008; Lennon and Jones, 2011; Crump *et al*., 2012; Coveley *et al*., 2015). Regardless, such pattern highlights the value of deeper sampling (to capture rare biosphere), as well as more extensive time-series, rather than snapshot, sampling to capture patterns of promotion and demotion of members of the AGF community within the lifespan of an animal.

The value of AGF isolation efforts

The strict anaerobic nature of AGF necessitates the implementation of special techniques for their isolation and maintenance (Hungate, 1969; Bryant, 1972). Furthermore, while several storage methods based on cryopreservation have been proposed (Solomon *et al*., 2016a), the decrease in temperature to the ultra-low values and the incidental O_2 exposure during revival of the cryopreserved strains were shown before to be detrimental for some isolates. The lack of reliable long-term storage procedures often necessitates frequent subculturing of strains (every 3–4 days), which often leads to either the production of sporangia that do not differentiate to zoospores or the outright failure to produce sporangia (Ho and Barr, 1995).

Through a multi-year effort, we obtained 216 isolates representing 12 AGF genera. Obtaining this diverse collection was enabled by employing a variety of isolation procedures, e.g. enrichment on multiple carbon sources, paying special attention to picking colonies of different shapes and sizes, and picking several colonies of the same shape, as representatives of different genera are known to produce colonies with very similar macroscopic features. More importantly, isolation of novel AGF taxa was also enabled by utilizing a wide range of faecal samples from animals with varying host lifestyle, gut type and phylogeny as an inoculum. The success of isolation of a certain genus was, in general, attributed to its abundance in the sample (Pearson correlation coefficient = 0.79), especially for monocentric genera (e.g. *Pecoramyces*, *Feramyces*, *Neocallimastix* and *Agriosomyces*), and was negatively correlated to the sample evenness (Pearson correlation coefficient = −0.87). It remains to be seen if this is true and reproducible for all samples and across laboratories. More efforts are certainly needed to develop targeted isolation strategies for specific taxa that we failed to obtain in pure cultures despite our best effort and despite their abundance in their respective sample (e.g. SK4 in one of the aoudad sheep samples, and RH5 in the domesticated sheep and the axis deer samples).

In conclusion, our results establish the utility of D1/D2 LSU and PacBio sequencing for AGF diversity surveys, and the culturability of a wide range of AGF taxa, and demonstrate that wild herbivores represent a yet-untapped reservoir of AGF diversity.

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CHAPTER VI

AESTIPASCUOMYCES DUPLICILIBERANS GEN. NOV, SP. NOV., THE FIRST CULTURED REPRESENTATIVE OF THE UNCULTURED SK4 CLADE FROM THE WILLD AUODAD SHEEP

Abstract

We report the isolation of representatives of the previously uncultured Neocallimastigomycota SK4 lineage from a wild Aoudad sheep (*Ammotragus lervia*) rumen sample using a sequence guided isolation strategy. Morphologically, the isolates formed medium-sized (2–5 mm), white filamentous colonies with a white center of sporangia on agar roll tubes and a heavy biofilm in liquid media. Microscopic analysis revealed monocentric thalli with both endogenous and exogenous sporangial development patterns, and spherical polyflagellated zoospores with 7–20 flagella. Zoospore release occurred through an apical pore as well as by sporangial wall rupturing, a duality that is unique amongst described AGF strains. The type strain, isolate R4, was capable of growing on a wide range of mono-, oligo-, and polysaccharides substrates as the sole carbon and energy source, including glucose, fructose, mannose, xylose, glucuronic acid, cellulose, xylan, starch, inulin, and raffinose. Phylogenetic assessment based on the D1-D2 region of the large rRNA subunit (D1-D2 LSU) and Internal transcribed spacer-1 (ITS-1) regions showed low sequence identity (92.4% and 86.7%) to their closest cultured relatives. D1-D2 LSU phylogenetic trees grouped the isolates as a new monophyletic clade within the *Orpinomyces- Neocallimastix-Pecoramyces-Feramyces-Ghazallamyces* supragenus group. D1-D2 LSU and ITS-1 sequences from the obtained isolates were either identical or displayed extremely high sequence similarity to sequences recovered from the same Aoudad sheep sample on which isolation were conducted, as well as several sequences recovered from domestic sheep and few other ruminants. We propose accommodating these novel isolates in a new genus, *Aestipascuomyces* (derived from the Latin word for "summer pasture"), and a new species, *A. dupliciliberans*. The type strain is *Aestipascuomyces dupliciliberans* strain R4.

Introduction

The herbivorous gut harbors a wide range of bacterial, archaeal, protozoan, and fungal communities that collectively mediate the transformation of plant biomass into fermentable sugars and short-chain fatty acids (SCFA) [1]. Within such complex assemblages, members of
the anaerobic gut fungi (AGF, phylum Neocallimstigomycota) remain the most enigmatic [2, 3].

During the last few decades, an increased understanding of the AGF diversity, ecology, and metabolic capabilities has been accumulating, and it is now broadly agreed that AGF play an integral role in the anaerobic degradation of recalcitrant lignocellulosic material [4, 5], through hyphal penetration of plant material and production of a wide array of polysaccharide-degrading enzymes [1, 2, 6].

To date, eighteen different cultured AGF genera have been described, the majority of which were isolated in the last few years [7-19]. Generally, AGF genera are distinguished morphologically based on several structural features such as thallus development pattern [7, 16], zoospore flagellation, and thallus morphology. Monocentric thallus development pattern (i.e. zoospore cyst germination is not accompanied by nucleus migration into the germ tube, resulting in anucleated rhizoidal system) is the most abundant among AGF genera, as opposed to polycentric thallus development (i.e. nucleus enters the germ tube, developing a nucleated rhizomycelium) that is thus far only observed in three genera, e.g. Anaeromyces [18], Orpinomyces [7], and Cyllamyces [19]. Additionally, the majority of AGF genera produce monoflagellated zoospores (with 1-4 flagella), with only four genera thus far (e.g. Orpinomyces [7], Neocallimastix [16], Feramyces [14], and Ghazallomyces [15]) known to produce polyflagellated zoospores (7-30 flagella). Finally, all currently described genera exhibit filamentous thalli, except for the phylogenetically-related genera Cyllamyces [19] and Caecomyces [8], both known to display 60 bulbous thalli morphology.

Culture-independent diversity surveys have clearly demonstrated that AGF diversity is much broader than previously inferred from culture-based approaches. Such studies have identified several novel yet-uncultured lineages, mostly through the use of the ITS-1 and D1/D2 LSU regions as phylogenetic markers [20-23,24]. Despite multiple

recent efforts to isolate and characterize novel AGF lineages [11-15], many candidate genera remain uncultured. A recent study that combined amplicon based diversity survey with isolation efforts has suggested that the success of isolation of AGF taxa is positively correlated to its relative abundance in a sample and negatively correlated to the sample evenness [24]. Based on this observation, we adopted a sequence-guided isolation strategy, where samples harboring relatively high proportions of yet-uncultured genera are prioritized for AGF isolation efforts. Here, we employed this strategy to isolate members of the previously uncultured lineage SK4 from a sample where it constituted the majority of AGF sequences in a culture-independent survey [24].

In addition to expanding the number of cultured AGF genera, our work shows the value of a sequence-guided culturing approach and argues that a large number of the yet-uncultured AGF genera are indeed culturable, given the right sampling and isolation conditions.

Materials and Methods

Samples. Fresh fecal and rumen contents were collected in several sterile 50-ml falcon tubes from a wild Aoudad sheep (Ammoragus lervia) during a hunting trip in Sutton County, Texas in April 2018. Tubes were filled completely to ensure the absence of oxygen. Samples were stored on ice and transferred to the laboratory within 24 hours, where they were either directly utilized for DNA extraction or stored at -20^{0}C.

Isolation. A sample exhibiting a relatively high abundance (76.6%) for members of the yet-uncultured SK4 lineage [24] was chosen for targeted enrichment and isolation. Isolation efforts were conducted on fecal, as well as rumen samples from the same animal. Rumen samples used in the isolation process were stored unopened at -20ºC. Fecal samples were opened once in an anaerobic chamber (Coy laboratories, Grass Lake, Michigan, USA) to obtain 0.5 gram for use in culture-independent diversity survey efforts, then stored at -20ºC. Isolation efforts were conducted 46 months post sample collection and DNA extraction. The isolation procedure was conducted by enriching the samples in autoclaved rumen fluid-cellobiose (RFC) medium [25] for 24h at 39ºC. Enriched tubes were serially diluted into anaerobic rumen fluid medium (RF) supplemented with either 1% w/v cellulose or a (1:1) mixture of cellobiose and switchgrass (1% w/v), and an antibiotics mixture of 50 µg/mL kanamycin, 50 µg/mL penicillin, 20 µg/mL, streptomycin, and 50 µg/mL chloramphenicol. Following enrichment, serial dilutions up to 10-5 were performed, and the dilution tubes were incubated for 3 days at 39ºC. Dilutions showing visible signs of growth (change in the color of cellulose, clumping and floating of the switch grass, and production of gas bubbles) were used to prepare roll tubes [26] using RFC medium with 2% agar. Roll tubes were incubated for 2-3 days at 39ºC, after which single colonies were transferred into RFC medium. Roll tube preparation and colony picking were repeated at least 3 times to ensure the purity of the obtained isolates. Obtained isolated are being maintained via bi-weekly sub-culturing into RFC media. Cultures are stored on agar medium for long-term storage as previously described in [25].

Morphological characterization. Both light and scanning electron microscopies were utilized to observe various microscopic features at different growth stages. For light microscopy, fungal biomass was collected from an actively growing 2-3d old culture

grown in RFC medium. Fungal biomass was stained with lactophenol cotton blue for examination of various thallus features including: hyphae, sporangia, zoospores, and other specific microscopic structures as previously described in [13-15]. For nuclear localization, samples were stained with DNA-binding dye 4, 6 diamidino-2-phenylindole (DAPI, final concentration of 10 μg/ml), followed by incubation in the dark for 10 min at room temperature. All light microscopy examinations were conducted using an Olympus BX51 microscope (Olympus, Center Valley, Pennsylvania) equipped with a Brightline DAPI high contrast filter set for DAPI fluorescence and a DP71 digital camera (Olympus). Sample preparation and fixation for scanning electron microscopy was conducted as previously described in [13]. The prepared samples were then examined on a FEI Quanta 600 scanning electron microscope (FEI Technologies Inc., Hillsboro, Oregon, United States).

Substrate utilization. To assess the substrate utilization capabilities of the type strain R4, twenty-four different substrates were used to replace the cellobiose in RFC medium at a final concentration of 0.5% w/v (Table 6-1) [13, 14]. The ability of strain R4 to utilize a specific substrate was considered positive if it exhibited viable growth on the tested substrate after four successive transfer events [13, 14, 18]. All results were compared to substrate-free medium.

Phylogenetic analysis and ecological distribution. DNA was extracted 124 from 10 ml of 2-3 d old cellobiose-grown cultures of five strains using DNeasy PowerPlant Pro Kit (Qiagen Corp., Germantown, MD) according to the manufacturer's instructions. The extracted DNA was used as a template to amplify the region encompassing ITS-1, 5.8S rRNA, ITS-2, and the D1/D2 domains of 28S nuc-rDNA gene using the modified the primers ITS5F (5'- GGAAGTAAAAGTCGTAACAAGG-3') -NL4R (5′ GGTCCGTGTTTCAAGACGG-3′) [14,15] using the following PCR protocol: Initial denaturation at 94ºC for 5 min followed by 39 cycles of denaturation at 94º C for 1 min, annealing at 55º C for 1 min, and elongation at 72º C for 2 min. A final elongation step at 72º C for 10 min was added. PCR amplicons were cloned into TOPO-TA cloning vector (Life Technologies®, Carlsbad, CA) following the manufacturer's instructions, and 22 clones from 5 strains were Sanger-sequenced at the Oklahoma State University DNA sequencing core facility. For every clone sequence obtained, the ITS1, and the D1/D2-

LSU regions were extracted in Mothur using the pcr.seqs command (with the reverse primer MNGM2, and the forward primer NL1, for the ITS1, and the D1/D2-LSU regions, respectively). The trimmed sequences were aligned to anaerobic fungal reference ITS-1 and D1/D2-LSU sequences using MAFFT [27] and the alignments were manually curated in Mega7 [28]. The refined alignments were used to construct maximum likelihood trees to assess the phylogenetic position of the obtained sequences using Mega7. *Chytriomyces* sp. WB235A, isolate AFTOL-ID 1536 was used as the outgroup (DQ536498 for ITS1, DQ536493 for 28S). Bootstrap values were calculated on the basis of 100 replicates.

To assess the ecological distribution of this novel lineage, we queried the trimmed ITS-1 sequences against a manually curated Neocallimastigomycota ITS1 database encompassing all known cultured genera, as well as yet-uncultured taxa previously identified in culture independent studies [20, 21, 23, 24, 29, 30] using blastn. Hits with significant sequence similarity (>87%) were evaluated by insertion into ITS-1 phylogenetic trees. We also queried the D1/D2 dataset generated in our prior effort [24], and hits with >93% sequence similarity were further evaluated by insertion into D1/D2-LSU phylogenetic trees.

Data and culture accession. Clone sequences are deposited in GenBank under accession numbers MW019479- MW019500.

Results

Isolation. Ten fecal isolates (FS1-FS10) and five rumen isolates (R1-R5) were obtained from a fecal and rumen samples of a single Aoudad sheep individual. The fecal isolates (n=10; designated FS1-FS10) were obtained from enrichment on cellobiose/switchgrass mixture, while the rumen isolates (n=5; designated R1-R5) were obtained from enrichment on cellulose.Phylogenetic and morphological analyses identified 9 of the fecal isolates (FS1-FS9) as members of the genus *Pecoramyces*, and the remaining fecal isolate (FS10) as belonging to the genus *Piromyces*. The rumen isolates (R-R5), on the other hand, were not phylogenetically affiliated with any of the previously cultured genera (see below) and so were deemed representing a novel genus. Preliminary morphological and microscopic characterization as well as phylogenetic analysis showed no distinguishable differences between the five isolates. One isolate (strain R4) was chosen as the type strain and used for subsequent analysis. Below, we present detailed characterization of the putative novel genus morphology and phylogenetic affiliation.

Colony morphology and liquid growth pattern. On solid media, strain R4 formed circular, white filamentous colonies with a white center of sporangia (Figure 6-1a). Colony size ranged from 2-5 mm. In liquid media, strain R4 produced a heavy fungal loose biofilm-like growth that doesn't attach to the tube's glass surface (Figure 6-1b).

Microscopic features.

Zoospores. Strain R4 produced globose zoospores with an average diameter of 9.3 ± 2.1 μm (standard deviation for 60 zoospores, range: 5–14 μm) (Figure 6-2a). All zoospores were polyflagellated, with 7-20 flagella and an average flagellum length of 28.1 ± 4.8 μm (average ± standard deviation from 60 zoospores, range: 19–36 μm).

Thalli and sporangia. Zoospores germination in strain R4 resulted in monocentric thalli with highly branched anucleated rhizoids (Figure 6-2b-e). Strain R4 displayed both endogenous and exogenous thallus development. Endogenous thalli were developed as a result of enlargement of zoospore cysts into sporangia with one (Figure 6-2f), or two adjacent (Figure 6-2g) rhizoidal systems. Endogenous sporangia displayed different shapes and sizes including ovoid (20–70 μm L x 15–45 μm W) (Figure 6-2f), rhomboid (30–70 μm L x 40–85 μm W) (Figure 6-2g), and elongated (25–90 μm L x 15–40 μm W) (Figure 6-2h). No intercalary or pseudo-intercalary sporangia (sporangia present between

two main rhizoidal systems) were observed. Exogenous sporangia were mainly developed at the end of unbranched sporangiophores that ranged in length between 10–300 μm (Figure 6-2i-j). Wide flattened sporangiophores (Figure 6-2i) and sporangiophores ending with sub-sporangial swellings (Figure 6-2k) were also frequently encountered. Mature exogenous sporangia ranged in size between (40–90 μm L x 15–35 μm W), and exhibited different morphologies including obpyriform (Figure 6-2i), ellipsoid (Figure 6-2j), globose (Figure 6-2k), ovoid (Figure 6-2m), and constricted ellipsoid (Figure 6-2 n). Sporangial necks (point between sporangia and rhizoids) were either tightly constricted (Figures 6-2f, 6-2k, and 6-2q) or broad (Figure 6-2j, 6-2l, 6-2n, and 6-2o). The neck opening, port, was either narrow (Figure 6-2k), or wide (Figure 6-2l).

Zoospore release. Zoospore release in strain R4 was achieved through two mechanisms, either from an apical pore (Figure 6-2o) as previously observed in *Feramyces* [14],) or through rapturing of the sporangial wall (Figure 6-2p) as previously observed in *Neocallimastix* [16]. To our knowledge, the simultaneous utilization of both mechanisms by a single strain has not been previously reported in other AGF taxa. Sporangial walls either stayed intact (Figure 6-2o) or completely disintegrated after zoospore discharge (Figure 6-2q).

Substrate utilization. Strain R4 utilized a wide range of substrates as the sole carbon and energy source (Table 6-1). These included monosaccharides, e.g., glucose, fructose, mannose, xylose, and glucuronic acid, but not arabinose, galactose, or ribose. Strain R4 was able to metabolize and vigorously grow on all disaccharides tested including cellobiose, lactose, maltose, sucrose, and trehalose. Among the polymers tested, strain R4 was able to grow on cellulose, xylan, starch,inulin, and raffinose, but not alginate, chitin, pectin, poly- galacturonate, peptone, or tryptone.

Phylogenetic analysis and ecological distribution. Phylogenetic analysis using the D1/D2 domains of 28S nuc-rDNA gene (D1/D2 LSU) reproducibly grouped all six isolates in a single, monophyletic cluster within the *Orpinomyces-Neocallimastix-Pecoramyces-Feramyces-Ghazallamyces* supragenic clade (Figure 4a). The obtained isolates showed very low D1/D2 LSU inter-sequence length heterogeneity (749–751 bp long, average 750 bp), low within strain divergence between copies (0–1.74%), as well as

low inter-sequence divergence between strains (0–1.6%). The closest cultured representative to the obtained isolates was *Feramyces austinii* (MG584193; 92.4% similarity). When compared to amplicon sequences, D1/D2 LSU sequences of the isolates showed highest similarity (93-100%) to amplicon sequences assigned to the uncultured lineage SK4 [24], originating from fecal material of the same aoudad sheep individual whose rumen sample was used for isolation (n=1338 sequences), as well as sequences recovered from Blackbuck deer (n=4), elk (n=2), domesticated horse (n=1), miniature donkey (n=2), mouflon ram (n=2), and oryx (n=2).

On the other hand, the obtained isolates showed a slightly higher ITS-1 length heterogeneity (196–200 bp; average 197.5 bp), within strain divergence between copies (0–4.38%), as well as inter-sequence divergence between strains (0–5.84%). ITS-1 phylogeny (Figure 4b) placed the obtained isolates close to the genus *Feramyces*. Blastn against our custom ITS-1 database identified 1327 sequences with ≥87% sequence similarity. All hits were affiliated with the SK4 clade (originally identified in domesticated sheep and red deer samples in NZ [21, 22]). The majority of hits were from the same wild Aoudad sheep samples from which the US isolates were obtained (n=1311), domesticated sheep (n=5) previously reported in NZ [21], as well as oryx, blackbuck deer, horse, miniature donkey, mouflon, and elk (n=11). Analysis of all available SK4-affiliated sequences obtained from prior studies [21, 22, 24] and the current study indicates a clade ITS-1 sequence divergence range of 0–13.2%, with two well-defined subclades. Interestingly, divergent ITS-1 sequences originating from one isolate routinely clustered within both clades (Figure 4b), precluding equating subclades to two distinct species and highlighting the difficulty associated with species-level OTU assignment using ITS-1 data in the Neocallimastigomycota.

Notably, it seems that members of the SK4 clade exhibit higher abundance when animals graze on summer pasture. For example, in New Zealand's domesticated sheep, SK4 was only identified as part of the AGF community when the animals were grazing on summer, but not winter, pasture [21, 22]. In addition, while the exact feed of other animal hosts harboring the SK4 lineage (e.g. aoudad sheep, oryx, blackbuck deer, horse, miniature donkey, mouflon, and American elk) is not available [24], all the above samples were collected during summer months (between April and October), suggesting

a potential relationship between the enrichment of SK4 in the AGF community and the season feed type.

Taxonomy

Aestipascuomyces Radwa Hanafy, Mostafa Elshahed, &. Noha Youssef, gen. nov.
MycoBank ID: MB837524
Typification: Aestipascuomyces dupliciliberans Radwa Hanafy, Mostafa Elshahed, &. Noha Youssef (holotype).
Etymology: Aestipascuo = derived from Aesta, Latin for summer, and Pascui, Latin for pasture; *myces* = the Greek name for fungus.
Obligate anaerobic fungus that produces globose polyflagellated zoospores (7-20 flagella). Zoospores germinate into determinate monocentric thalli with highly branched anucleated rhizoids that lack constriction and intercalary swellings. The clade is defined by the sequences MW019479- MW019500 (ITS-1, 5.8S rDNA, ITS2, D1-D2 28S rDNA). The most genetically similar genera are *Feramyces*, which is characterized by its poly-flagellated zoospores (7-16) and monocentric thalli that usually produce a single terminal sporangium, and in some occasions produce pseudo-intercalary and sessile sporangia, and *Neocallimastix*, which is characterized by the production of polyflagellate zoospores (7-30), monocentric thalli, and empty zoospore cysts that remain at the base of sporangiophores.
Aestipascuomyces dupliciliberans Radwa Hanafy, Mostafa Elshahed, &. Noha Youssef, sp. nov.
Mycobank ID: MB837526
Typification: The holotype is figure 6-2b in this manuscript derived from the following: U.S.A. TEXAS: Sutton county, 30.591 N and 100.138 W ~300 m above sea level, 3d old culture of strain R4, which is isolated from the frozen rumen content of a female aoudad sheep (*Ammotragus lervia*), collected in April 2018 by Mr. Jim Austin. Ex-type strain R4 is stored on solid agar media at 39ºC at Oklahoma State University, Department of Microbiology and Molecular Genetics. GenBank accession numbers MW019494-MW019497 (for ITS-1, 5.8S rDNA, ITS2, D1-D2 28S rDNA).

Etymology: *duplici*= Latin for dual, *liberans*= Latin for liberation or release. The species epithet highlights the dual zoospore release mechanisms.

An obligate anaerobic fungus that produce globose (5–14 μm diam) zoospores with 7-20 flagella (19–36 μm long). Zoospores germinate into determinate monocentric thallus with highly branched anucleated rhizoids that lack constriction and intercalary swellings. Both endogenous and exogenous thalli developments are observed. Mature endogenous sporangia were mainly rhomboid (30–70 μm L x 40–85 μm W), and elongated (25–90 μm L x 15–40 μm W). Mature exogenous sporangia ranged in size between (40–90 μm L x 15–35 μm W), and displayed a variety of shapes including obpyriform, ellipsoid, globose, constricted ellipsoid, and ovoid. Sporangiophores varied in length between (10–300 μm). Wide flattened sporangiophores and sporangiophores ending with sub-sporangial swellings were occasionally encountered. Zoospores are released either through an apical pore or through the lysis of the sporangial wall. Sporangial walls remain intact or are completely collapsed after zoospore release. Produces white filamentous colonies with a white center of sporangia (2-5 mm diam.) on agar roll tubes, and heavy fungal biofilm-like growth that doesn't attach to the tube's glass surface in liquid media. The clade is defined by the sequences MW019494- MW019497 (for ITS-1, 5.8S rDNA, ITS2, D1-D2 28S rDNA).

Additional specimens examined: Radwa Hanafy strains R1 (MW019480, MW019481, MW019483, MW019484, MW019485, MW019486), R2 (MW019482, MW019487), R3 (MW019488, MW019490, MW019491, MW019492, MW019494), and R5 (MW019498, MW019489, MW019499, MW019479, MW019500) (GenBank accession number of clones in parenthesis), isolated from the same frozen rumen content of a female aoudad sheep (*Ammotragus lervia*) from which the type strain was isolated, in April 2018.

Figure 6-1. Macroscopic features of *Aestipascuomyces dupliciliberans* type strain R4. (a) Heavy fungal biofilm-like growth in liquid medium. (b) Circular, white filamentous colonies with a white center of sporangia on cellobiose agar roll tube.

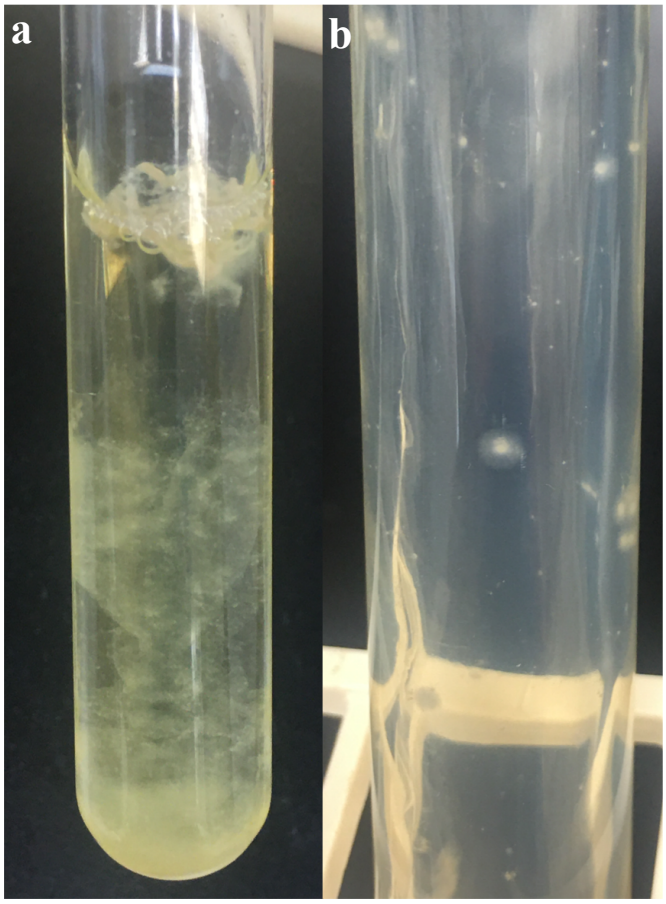
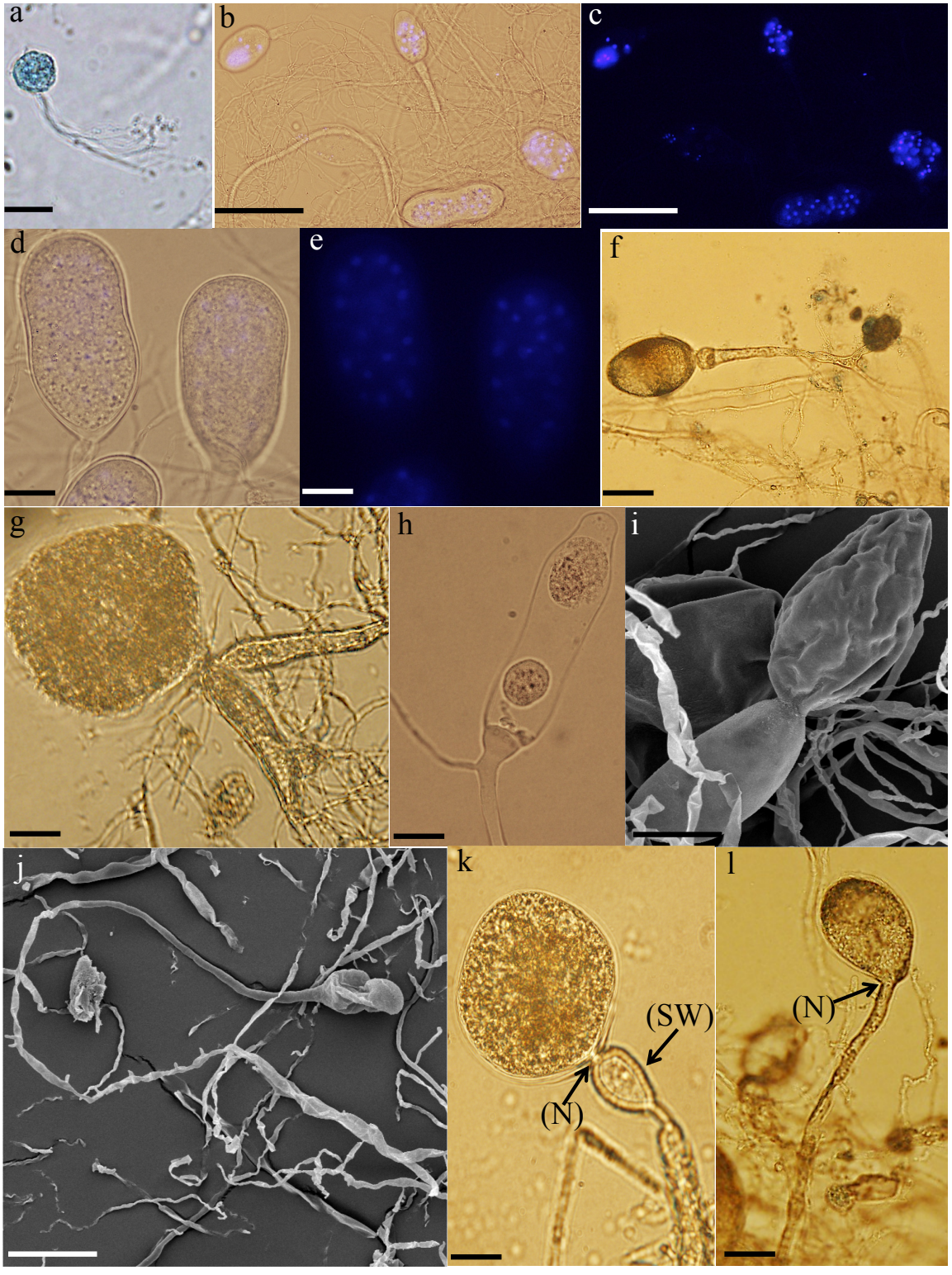


Figure 6-2. Microscopic features of *Aestipascuomyces dupliciliberans* type strain R4. Light (a-h, k-n and p-q), fluorescence (c and e), and scanning electron (i, j and o) micrographs are shown. (b-c), and (d-e) each depict the same field with c, and e showing the fluorescence field and b and d showing the overlay of fluorescence and phase contract micrographs. (a) A spherical polyflagellated zoospore. (b-e) Monocentric thalli; nuclei were observed in sporangia, not in rhizoids or sporangiophore. (f-h) Endogenous sporangia: (f) Ovoid sporangium with single rhizoidal system, (g) rhomboid sporangium with two adjacent rhizoidal systems, (h) elongated sporangium. (i-n) Exogenous sporangia: (i) obpyriform sporangium on a flattened sporangiophore, (j) ellipsoidal sporangium on a long sporangiophore, (k) globose sporangium with sub-sporangial swelling and tightly constricted neck, (l) Ovoid sporangium with broad neck and wide port, (m) mature ovoid sporangium full of zoospores, (n) constricted ellipsoidal sporangium. (o-q) Zoospore release mechanisms: (o) An empty sporangium with intact wall after zoospore release through an apical pore (arrow), (p) zoospore release through rupturing the sporangial wall, (q) collapse and disintegration of the sporangial wall after zoospore release. (SW), sub-sporangial swelling; (N), neck. Bar =20 μm (a, f-h, k-n, p-q). Bar =50 μm (b-e, i, and o). Bar =100 μm (j).


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

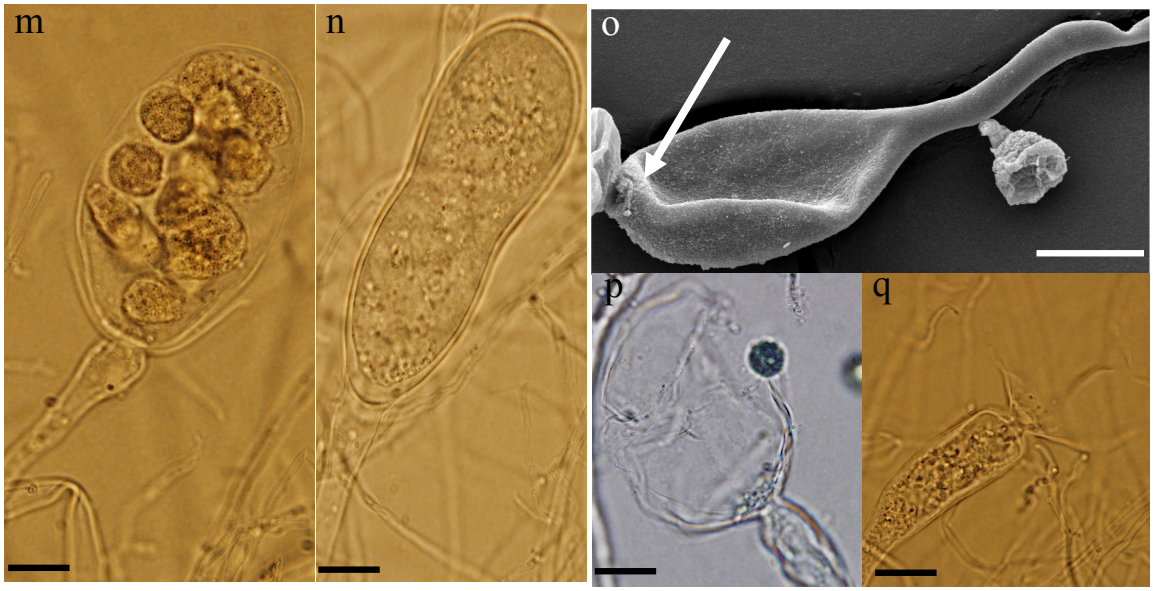

m
n
o
p
q

Figure 6-3. Phylogenetic affiliation of the *Aestipascuomyces* genus to other AGF genera based on the sequences of the D1–D2 domains of nuc 28S rDNA gene (a), and partial ITS-1 sequences (b) Sequences were aligned in MAFFT [27] and the alignment was used to construct phylogenetic trees in MEGA7 [28] using a maximum likelihood approach. Bootstrap values from 100 replicates are shown for nodes with more than 70% bootstrap support.

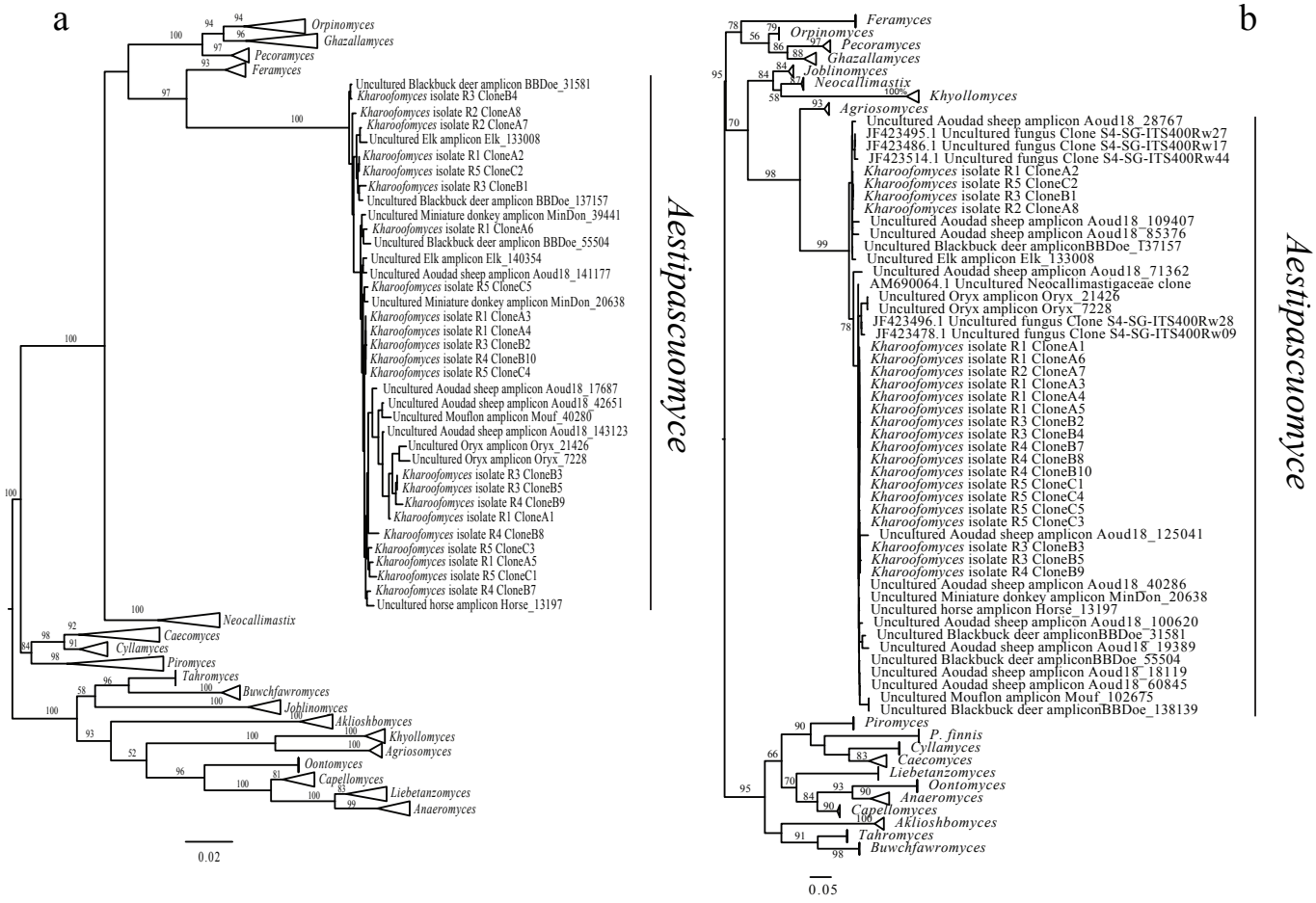


Table 6-1. Substrate utilization patterns by *Aestipascuomyces dupliciliberans* strain R4.

Substrate		Growth*
Monosaccharides	Glucose	+
	Xylose	+
	Mannose	+
	Fructose	+
	Glucuronic acid	+
	Arabinose	-
	Ribose	-
	Galactose	-
Disaccharides	Cellobiose	+
	Succrose	+
	Maltose	+
	Trehalose	+
	Lactose	+
Polysaccharides	Cellulose	+
	Xylan	+
	Starch	+
	Inuline	+
	Raffinose	+
	Poly-galcturonate	-
	Chitin	-
	Alginate	-
	Pectin	-
Peptides	Peptone	-
	Tryptone	-

*Positive results are reported after four subcultures on the substrate.

Discussion

Here, we report on the isolation and characterization of the first cultured representatives of the previously uncultured Neocallimastigomycota lineage SK4 from the rumen contents of a wild Aoudad sheep. Phylogenetic analysis using the D1/D2-LSU region showed that the five isolates obtained formed a single, monophyletic cluster within the *Orpinomyces-Neocallimastix-Pecoramyces-Feramyces-Ghazallamyces* supragenus clade [24, 31]. All members in this clade are characterized by the production of polyflagellated zoospore, with the notable and peculiar exception of the genus *Pecoramyces*, which produces monoflagellated zoospores. This suggests an acquisition pattern of zoospore polyflagellation at ~ 46.3 Mya (the most current estimate of this clade emergence per [31]), followed by a recent loss and reverting to zoospor monoflagellation for the relatively recently evolved genus *Pecoramyces* (current estimates of emergence at 19.1 Mya, [31]). Similarly, all members of this supragenus clade form monocentric thalli with the exception of *Orpinomyces* genus that is known to develop polycentric thalli, also suggesting that the development of polycentric thalli is a recent independent event that happened multiple times in the Neocallimastigomycota tree (for example with the emergence of *Orpinomyces*, *Anaeromyces*, and *Cyllamyces*). The closest cultured representatives of the SK4 clade are the genera *Feramyces* and *Neocallimastix*. While the three genera share similar morphological and growth patterns (e.g. polyflagellated zoospores, and monocentric thalli development), they exhibit several distinct macroscopic and microscopic features. For example, members of the SK4 genus produce zoospores with 7-20 flagella, as opposed to 7-16 for *Feramyces* [14] and 7-30 for *Neocallimastix* [16]. Additionally, SK4 members produce terminal sporangia, while the *Feramyces* genus members produce terminal, pseudo-intercalary and sessile sporangia [14]. Also, and perhaps most notably, members of the SK4 genus show two zoospore release mechanisms; either through an apical pore or via rupturing of the sporangial wall.

On the other hand, *Neocallimastix* members are known to release zoospores through complete rupturing and lysis of the sporangial wall (Figure 25 in [16]) while Feramyces members release zoospores through apical pores (Figure 2x in [14]). To our knowledge, the dual zoospore release mechanism has not been encountered before in any of the cultured AGF genera members and hence is highly characteristic of the SK4 genus.

Within the microbial world, a large fraction remains uncultured. This is more commonly encountered within the bacterial and archaeal domains, although a similar pattern has been suggested with Fungi [32-35]. Within the anaerobic fungal phylum Neocallimastigomycota, multiple putative novel genera were identified in culture-independent studies [20, 21, 29]. Failure to obtain these taxa in pure culture could be attributed to several reasons. First, some AGF taxa are extremely fastidious and might require special nutritional and culturing requirements, and hence would evade isolation using routinely utilized isolation and enrichment protocols [1, 36]. Second, some AGF taxa might exhibit a very limited ecological distribution pattern and could be confined to few phylogenetically-related animal hosts. Indeed, many novel genera recently isolated appear to be of limited distribution, being observed only in very few samples from which they have been successfully isolated (e.g. *Aklioshbomyces* from white-tailed deer, *Ghazallomyces* from Axis deer, *Khyollomyces* (AL1) in the Equidae [15]. We argue that, in addition to mere presence, the relative abundance of the target lineage in the sample could be an important determinant for isolation success in the AGF. Our recent efforts [24 suggest that while some AGF genera are generalists, present in low abundance in a large number of samples and are often readily recovered from these samples, e.g. *Orpinomyces*, and *Anaeromyces*; others show a clear correlation between the success of their isolation and their relative abundance within a sample, especially in samples where one or a few lineages make 272 up the majority (>90%) of the AGF community.

Therefore, this study clearly demonstrates the value of the sequence-guided isolation strategy we employed here, whereby samples are initially prescreened using culture-independent approaches followed by targeting promising samples exhibiting a high proportion of novel/wanted genera for isolation efforts using a wide range of substrates, sample types, and growth conditions. Evidently, this approach will unfortunately involve storing the samples at - 20^0 C for a certain amount of time to allow for sequencing and data analysis to be conducted. Nevertheless, while some AGF taxa might not survive prolonged freezing, we have been successful in recovering isolates from samples stored frozen, especially when tubes were unopened, or at least where repeated freezing and thawing cycles were avoided, and where tubes were filled to the top with little to no room for air [14].

Based on morphological, physiological, microscopic, and phylogenetic characteristics, we propose accommodating these new isolates into a new genus, for which the name *Aestipascuomyces* (from aesta, latin for summer, and pascui, latin for pasture, to indicate the apparent enrichment of the clade during animal feeding on summer pasture) is proposed. The type species is *Aestipascuomyces dupliciliberans* (to indicate the two zoospore release mechanisms exhibited by members of the clade), and the type strain is *Aestipascuomyces dupliciliberans* strain R4.

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CHAPTER VII

CONCLUSIONS

In this dissertation, I employed a combination of culture-based and culture-independent approaches to expand our understanding of the AGF diversity. Assessing the diversity of the AGF has previously been hindered by the inherent deficiencies associated with the lack of dedicated isolation efforts for culturing these strict fastidious anaerobes, the use of the Internal Transcribed Spacer-1 (ITS-1) region as a phylogenetic marker, and restriction of prior sampling efforts to a few domesticated animal hosts.

To address the paucity of AGF isolates, I have conducted a multi-year isolation effort utilizing a variety of isolation procedures, e.g. enrichment on multiple carbon sources, sequence-guided isolation strategy, and sampling a wide range of herbivorous animals with varying lifestyle, gut type, and host phylogeny. Such concerted effort has resulted in the isolation and characterization of ten novel AGF genera, including the first cultured representatives of the hitherto uncultured AGF lineages AL1, AL5, AL6, AL7, and SK4, and proved that yet-uncultured AGF genera are not refractive to isolation given the right sampling and isolation conditions.

Additionally, my culture-independent study resulted in proposing the utilization of D1/D2 of the large ribosomal subunit as a more robust phylomarker for AGF diversity assessment to replace the problematic ITS1 region, generating the first comprehensive reference D1/D2 LSU database encompassing all cultured AGF genera, and the majority of candidate genera, providing interesting clues on the role of host phylogeny and life style in shaping the AGF diversity, and confirming the notion that wild herbivores represent an untapped reservoir for AGF diversity.

Moving forward, more comprehensive culture-independent future studies targeting domesticated and wild hosts known to harbor AGF, as well as multiple novel hosts (e.g. avian, reptilian, and marsupial herbivores, mammalian foliovores, and specific marine invertebrates) that possess an alimentary tract architecture and digestive strategies conducive to AGF establishment and colonization, are timely. Such studies will expand on my efforts in this dissertation and should result in a global-level assessment of the herbivorous mycobiome. Also, an updated taxonomic framework for culture-independent assessment of AGF taxa using the novel phylomarker (D1/D2 region in the LSU) is direly needed and could be attained with the availability of enough datasets. Isolation efforts also need to continue with the aim of obtaining

cultured representatives of several more uncultured lineages, as well as generating novel AGF taxa monograms and standards for taxa description and naming. Finally, long-term storage efforts are needed to facilitate maintenance and exchange of AGF cultures between interested labs.

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