*Article*

# *Aestipascuomyces dupliciliberatus* gen. nov, sp. nov., the first cultured representative of the uncultured SK4 clade from Aoudad Sheep and Alpaca

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**Abstract:** We report on the isolation of the previously uncultured Neocallimastigomycota SK4 lineage by two independent research groups from a wild aoudad sheep rumen sample (Texas, USA) and an alpaca fecal sample (Baden-Württemberg, Germany). Isolates from both locations showed near identical morphological and microscopic features, forming medium-sized 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, 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. Isolates were capable of growing on a wide range of mono-, oligo-, and polysaccharides substrates. Phylogenetic assessment based on the D1-D2 large rRNA subunit (D1-D2 LSU) and internal transcribed spacer-1 (ITS-1) regions demonstrated high sequence identity (minimum identity of 99.07% and 96.96%, respectively) between all isolates; but low sequence identity (92.4% and 86.7%, respectively) 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 was conducted, as well as several sequences recovered from domestic sheep and few other herbivores. Interestingly, members of the SK4 clade seem to be encountered in animals grazing on summer pasture. We hence propose accommodating these novel isolates in a new genus, *Aestipascuomyces* (derived from the Latin word for "summer pasture"), and a new species, *A. dupliciliberatus*. The type strain is *Aestipascuomyces dupliciliberatus* strain R4.

**Keywords:** Anaerobic gut fungi; sequence-guided isolation; Neocallimastigomycota SK4 lineage

## 1. 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 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 [20-24]. Despite multiple recent efforts to isolate and characterize novel AGF lineages [11-15], many candidate genera remain uncultured. A recent study combining amplicon-based diversity survey with isolation efforts suggested that the success of isolation of an AGF taxon is positively correlated to its relative abundance in a sample and negatively correlated to the sample evenness [24]. Further multiple culture-based [11, 15], and culture-independent [20, 24] studies have provided evidence that poorly sampled animal hosts harbor a wide range of hitherto uncharacterized AGF taxa. Based on these observations, we adopted two strategies to isolate novel AGF taxa: A targeted sequence-guided isolation strategy, where samples harboring relatively high proportions of yet-uncultured genera are prioritized for AGF isolation efforts, and a sampling strategy targeting animals from which no prior isolation efforts have been reported. Intriguingly these efforts, driven by two different hypotheses, and sampling different animals (a wild aoudad sheep and a zoo-housed alpaca) from two different geographical locations (Texas, USA, and Baden-Württemberg, Germany) have yielded almost identical strains of a hitherto uncultured AGF lineage (SK4, originally identified in samples from New Zealand). This study demonstrates the global distribution of AGF lineages across multiple continents, suggests that some yet-uncultured AGF genera are not refractive to isolation given the right sampling and isolation conditions, and highlights the value of implementing a sequence-guided culturing approach as well as directing isolation efforts to poorly sampled animals.

## 2. 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, USA in April 2018. Fecal samples were collected from an Alpaca (*Vicugna pacos*) at the Karlsruhe Zoo, Germany in August 2019. Tubes were filled completely to ensure the absence of oxygen. Aoudad sheep 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. Alpaca fecal samples were stored at room temperature until the next day where they were used for isolation.

**Isolation.** The aoudad sheep sample exhibited a relatively high abundance (76.6%) of the yet-uncultured SK4 lineage in a prior study [24], and hence was chosen for targeted enrichment and isolation. Isolation efforts were conducted on fecal, as well as rumen samples. 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 22 months post sample collection and DNA extraction. Samples were enriched 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 0.1% w/v cellulose or a (1:1) mixture of cellobiose and switchgrass (0.1% 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].

Isolation efforts from the Alpaca sample were conducted by suspending 1g of fecal material in a 1:1 mixture of anoxic salt solutions A [g/l: KH2PO4 (3.0), (NH4)2SO4 (6.0), NaCl (6.0), MgSO4 .7H20 (0.6) and CaCl2. 2H2O (0.6)] and B [g/l: K2HPO4 (3.0)], followed by inoculation in serum bottles with Enriched Rumen Fluid medium (ERF) adapted from the basal medium described by [12] and supplemented with dissolved xylan solution (0.2%), cellobiose (0.2%), and wheat straw milled to a particle size of 1 mm (0.5%). Inoculated serum bottles were then incubated for 7 days at 39 °C in the dark. Fungal growth was monitored by light microscopy and serum bottles with signs of anaerobic fungal growth were then used to inoculate roll tubes followed by incubation for 4 days at 39 °C in the dark. Single colonies were transferred into fresh medium. Roll tube preparation and colony picking were repeated at least 3 times to ensure the purity of the obtained isolates.

**Morphological characterization.** For aoudad sheep isolates, 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 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, Center Valley, Pennsylvania). 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).

For Alpaca isolates, light microscopy was performed using a Nikon® Eclipse E200 with a DFK 23U274 camera (Imaging Source®), while fluorescence microscopy (to visualize DAPI staining) was performed using a Zeiss® Axio Imager Z1 at an excitation wavelength of 353 nm. Differential interference contrast microscopy (DIC) was used for generating image overlay.

**Substrate utilization.** Growth of the type strain (R4) obtained from aoudad sheep was assessed by replacing the cellobiose in RFC medium with glucose, xylose, mannose, fructose, glucuronic acid, arabinose, ribose, galactose, sucrose, maltose, trehalose, lactose, cellulose, xylan, starch, inulin, raffinose, polygalcturonate, chitin, alginate, pectin, peptone, or tryptone at a final concentration of 0.05% w/v [13, 14]. To assess substrate utilization in the Alpaca isolate, the strain was grown in defined rumen-free media adapted from [12] with omission of clarified rumen fluid and addition of trace metal (prepared according to [27]) and vitamin solution (prepared according to [28]) and replacement of the cellobiose with 0.05% of hemicellulose, xylan, starch, crystalline cellulose, inulin, chitin, pectin, cellobiose, maltose, trehalose, lactose, sucrose, glucose, xylose, mannose, fructose, arabinose, ribose, galactose, or glucuronic acid, or 0.5% of wheat straw. The ability of a strain 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.** For the aoudad sheep isolates, DNA was extracted from 10 ml of 2-3 d old RFC-grown cultures of five strains using DNeasy PowerPlant Pro Kit (Qiagen Corp., Germantown, MD) according to the manufacturer's instructions. For the alpaca isolate, DNA was extracted from 1-week old cultures grown in rumen-free medium supplemented with 0.05% w/v cellobiose using the Quick-DNA Fecal/Soil Microbe DNA Miniprep Kit (Zymo Research). 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 protocols: For the aoudad sheep samples: 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, and a final elongation step at 72º C for 10 min. For the alpaca samples: Initial denaturation at 98ºC for 30 sec followed by 30 cycles of denaturation at 98º C for 10 sec, annealing at 62º C for 30 sec, and elongation at 72º C for 90 sec, and a final elongation step at 72º C for 2 min. PCR amplicons were cloned into TOPO-TA cloning vector (USA) (Life Technologies®, Carlsbad, CA), or PCR Cloning Kit (NEB) following the manufacturers' instructions, and were Sanger-sequenced at the Oklahoma State University DNA sequencing core facility (22 clones from 5 aoudad sheep strains), or Eurofins Genomics (14 clones from 1 alpaca strain). For every clone sequence obtained, the ITS-1, and the D1/D2-LSU regions were extracted in Mega7 [29] by trimming using the sequence of the ITS1 reverse primer MNGM2, and the sequence of the LSU forward primer NL1, respectively. The trimmed sequences were aligned to anaerobic fungal reference ITS-1 and D1/D2-LSU sequences using MAFFT v7.471 [30] and the alignments were manually curated in BioEdit [31]. The refined alignments were used to construct maximum likelihood trees to assess the phylogenetic position of the obtained sequences using

IQ-TREE v2.0.3 [32]. The best model was selected using ModelFinder [33] and 1000 ultrafast bootstraps [34] were applied. *Gonopodya prolifera* was used as the outgroup (NR_132861 for ITS-1, JN874506 for 28S).

To assess the ecological distribution of this novel lineage, we queried the trimmed ITS-1 sequences against a manually curated Neocallimastigomycota ITS-1 database encompassing all known cultured genera, as well as yet-uncultured taxa previously identified in culture-independent studies [20, 21, 23, 24, 35, 36] using blastn. Hits with significant sequence similarity (>87%) were evaluated by insertion into ITS-1 phylogenetic trees. We also queried the D1/D2 LSU 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 for the Aoudad sheep strains R1-R5, and MW049132-MW049145 for the Alpaca strain A252.

## 3. Results

3.1. **Isolation.** Five rumen isolates (R1-R5) were obtained from a single wild aoudad sheep in Texas, USA. Concurrently one isolate, A252, was obtained from fecal samples of Alpaca in Baden-Württemberg, Germany. These six isolates were not phylogenetically affiliated with any of the previously cultured genera. Preliminary morphological and microscopic characterization as well as phylogenetic analysis showed identical attributes for strains R1-R5 and only minimal differences between R strains and strain A252. One isolate (strain R4) was chosen as the type strain for detailed characterization. Below, we present detailed characterization of the putative novel genus morphology and phylogenetic affiliation, highlighting differences between R4 and A252 when appropriate.

3.2. **Colony morphology and liquid growth pattern.** On solid media, strain R4 formed circular, white filamentous colonies with a white center of sporangia (Figure 1a). Colony size ranged from 2-5 mm. In liquid media, strain R4 produced a heavy fungal biofilm-like growth that loosely attaches to the tube’s glass surface (Figure 1b).

3.3 **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 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). Strain A252 produced slightly larger spherical zoospores (10–20 µm, average 14 µm) with slightly longer flagella (35–49 µm, average 42 µm) (Figure 3a).

- *Thalli and sporangia.* Zoospores germination in strain R4 resulted in monocentric thalli with highly branched anucleated rhizoids (Figure 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 2f), or two adjacent (Figure 2g) rhizoidal systems. Endogenous sporangia displayed different shapes and sizes including ovoid (20–70 µm L x 15–45 µm W) (Figure 2f), rhomboid (30–70 µm L x 40–85 µm W) (Figure 2g), and elongated (25–90 µm L x 15–40 µm W) (Figure 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 2i-j). Wide flattened sporangiophores (Figure 2i) and sporangiophores ending with sub-sporangial swellings (Figure 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 2i), ellipsoid (Figure 2j), globose (Figure 2k), ovoid (Figure 2m), and constricted ellipsoid (Figure 2 n). Sporangial necks (point between sporangia and rhizoids) were either tightly constricted (Figures 2f, 2k, and 2q) or broad (Figure 2j, 2l, 2n, and 2o). The neck opening, port, was either narrow (Figure 2k), or wide (Figure 2l).

On the other hand, only endogenous sporangia were observed in strain A252, with one (Figure 3b), or, less frequently, two adjacent (Figure 3c) rhizoidal systems. Endogenous sporangia were mainly globose (diameter up to 145 µm) (Figure 3d), and ellipsoidal (up to 76 µm L and 48 µm W) (Figure 3e).

- *Zoospore release.* Zoospore release in strain R4 was achieved through two mechanisms, either from an apical pore (Figure 2o) as previously observed in *Feramyces* [14],) or through rapturing of the sporangial wall (Figure 2p) as commonly 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 2o) or completely disintegrated after zoospore discharge (Figure 2q).

**3.4. Substrate utilization.** Strain R4 utilized a wide range of substrates as the sole carbon and energy source. 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, polygalacturonate, peptone, or tryptone.

On the other hand, strain A252 grew on polysaccharides including wheat straw, hemicellulose, xylan, starch, inulin, but did not grow on chitin, pectin, or crystalline cellulose. The disaccharides cellobiose, maltose, lactose, and sucrose supported the growth of strain A252, but trehalose was not utilized. Strain A252 was capable of utilizing the monosaccharides glucose, xylose, and fructose, but not mannose, arabinose, ribose, galactose, or glucuronic acid.

**3.5. 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.

### 3.6. **Figure Legends**

**Figure 1.** Macroscopic features of *Aestipascuomyces dupliciliberatus* 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.

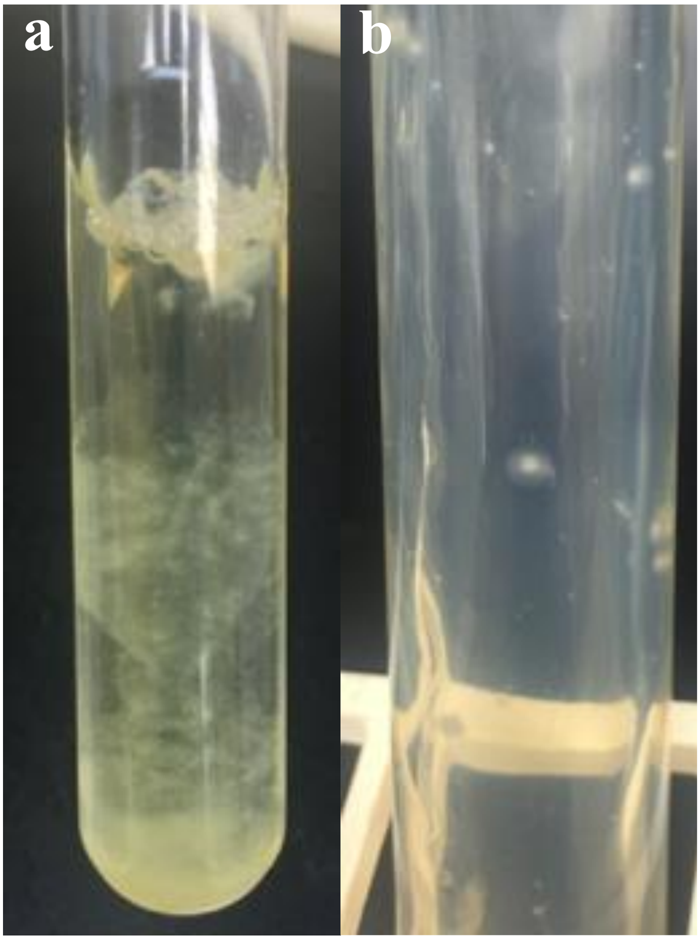
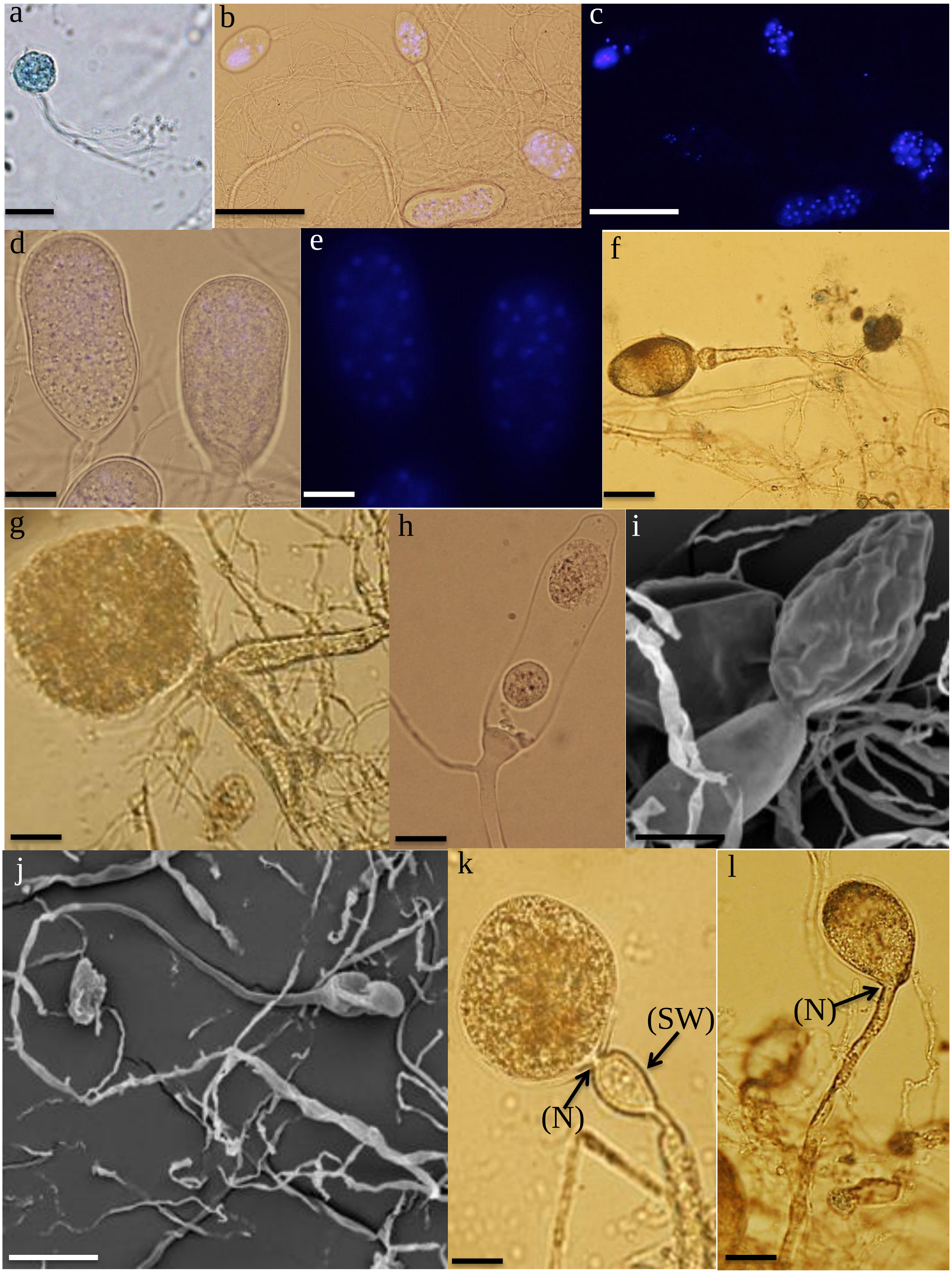


**Figure 2.** Microscopic features of *Aestipascuomyces dupliciliberatus* type strain R4. Light (a-h, k-n and p-q), fluorescence (c and e), and scanning electron (i, j and o) micrographs are shown. (b-c), and (d-e) each depict the same field with c, and e showing the fluorescence field and b and d showing 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).



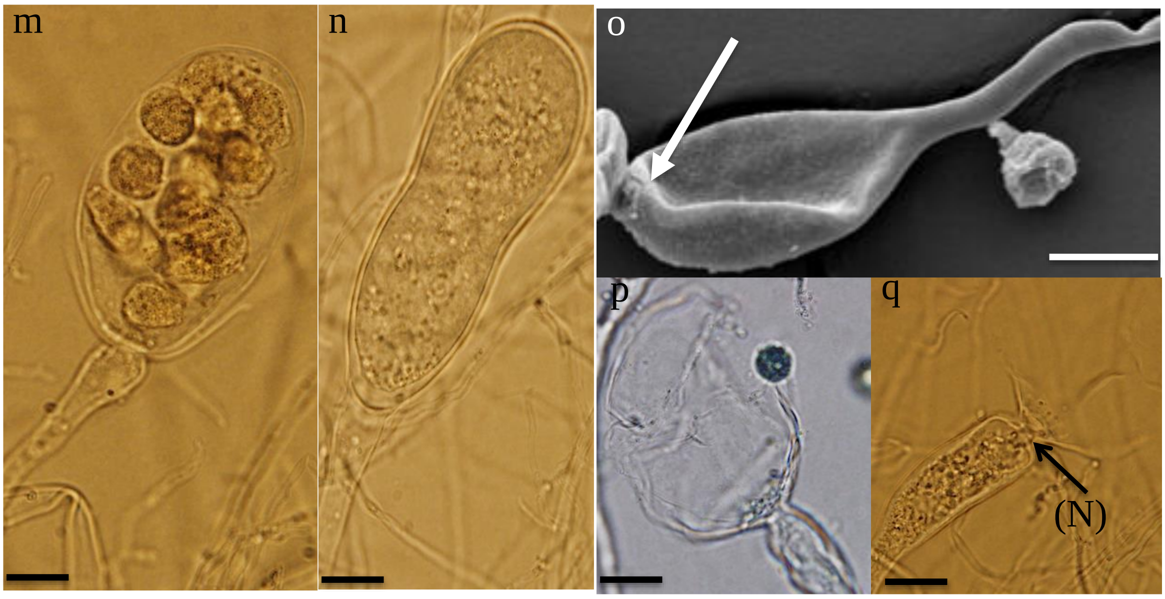

m
n
o
p
q
(N)

**Figure 3.** Microscopic features of *Aestipascuomyces dupliciliberatus* strain A252. Light (a-e), and DIC (f) micrographs. (a) A spherical polyflagellated zoospore with long flagella. (b-e) Endogenous sporangia: (b) young globose sporangium with a single rhizoidal system, (c) ovoid sporangium with two rhizoidal systems, (d) large globose sporangium with an apical pore for zoospore release, (e) sporangium during zoospore release, (f) DAPI stained mature ellipsoid sporangia. All scale bars are 100 µm.

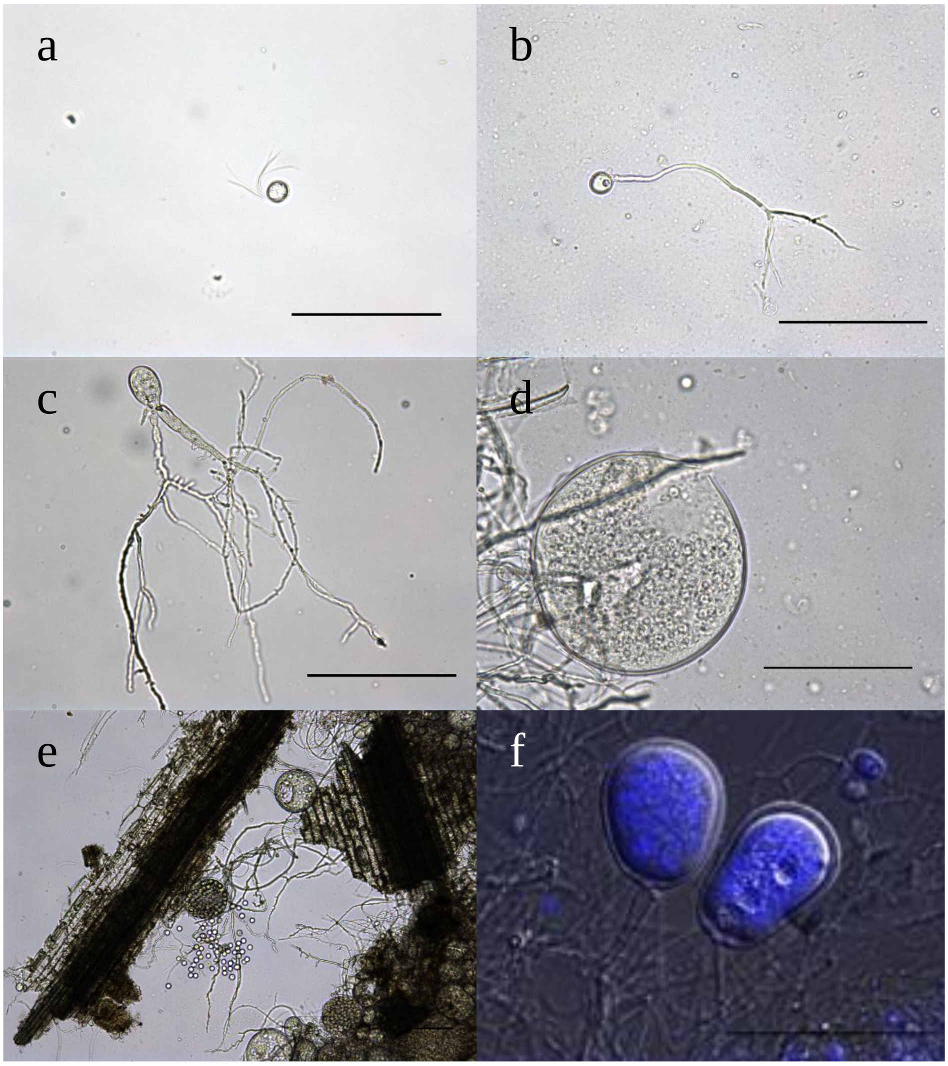
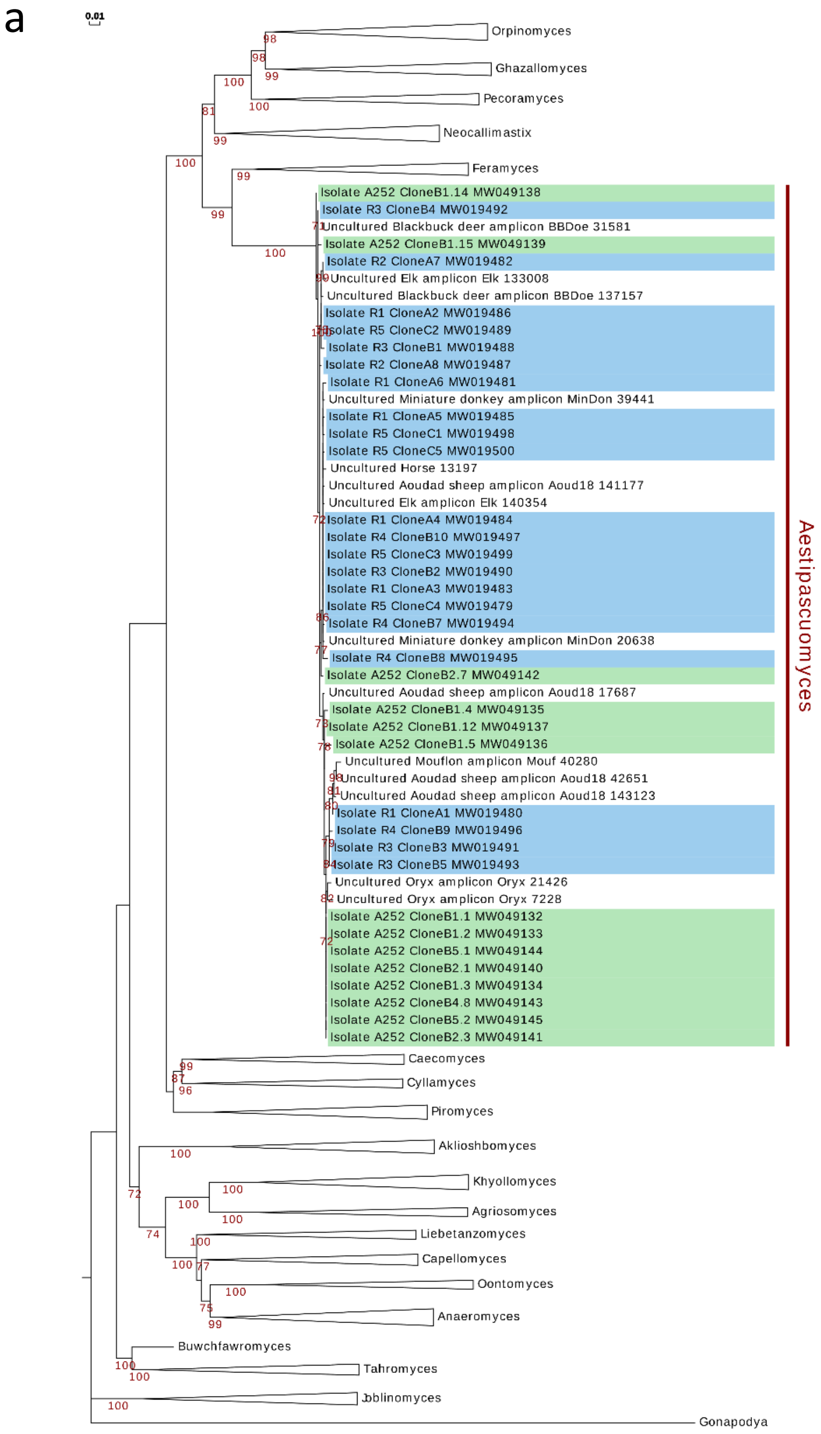


**Figure 4.** Phylogenetic affiliation of the *Aestipascuomyces* genus to other AGF genera based on the sequences of the D1–D2 domains of nuc 28S rDNA gene (a), and partial ITS-1 sequences (b). Sequences were aligned in MAFFT [30] and manually curated in BioEdit [31] . Curated alignments (LSU: 677 characters 209 sequences; ITS: 295 characters 126 sequences) were used to construct ML-trees using IQTREE with the predicted models TN+F+R2 (28S rDNA) or HKY+F+G4 (ITS) and –bb 1000. Bootstrap values are shown for nodes with more than 70 % bootstrap support. Background color indicates the origin of the isolate (blue: Texas, USA; green: Baden-Württemberg, Germany).


a
0.01
Orpinomyces
Ghazallomyces
Pecoramyces
Neocallimastix
Feramyces
Isolate A252 CloneB1.14 MW049138
Isolate R3 CloneB4 MW019492
Uncultured Blackbuck deer amplicon BBDoe 31581
Isolate A252 CloneB1.15 MW049139
Isolate R2 CloneA7 MW019482
Uncultured Elk amplicon Elk 133008
Uncultured Blackbuck deer amplicon BBDoe 137157
Isolate R1 CloneA2 MW019486
Isolate R5 CloneC2 MW019489
Isolate R3 CloneB1 MW019488
Isolate R2 CloneA8 MW019487
Isolate R1 CloneA6 MW019481
Uncultured Miniature donkey amplicon MinDon 39441
Isolate R1 CloneA5 MW019485
Isolate R5 CloneC1 MW019498
Isolate R5 CloneC5 MW019500
Uncultured Horse 13197
Uncultured Aoudad sheep amplicon Aoud18 141177
Uncultured Elk amplicon Elk 140354
Isolate R1 CloneA4 MW019484
Isolate R4 CloneB10 MW019497
Isolate R5 CloneC3 MW019499
Isolate R3 CloneB2 MW019490
Isolate R1 CloneA3 MW019483
Isolate R5 CloneC4 MW019479
Isolate R4 CloneB7 MW019494
Uncultured Miniature donkey amplicon MinDon 20638
Isolate R4 CloneB8 MW019495
Isolate A252 CloneB2.7 MW049142
Uncultured Aoudad sheep amplicon Aoud18 17687
Isolate A252 CloneB1.4 MW049135
Isolate A252 CloneB1.12 MW049137
Isolate A252 CloneB1.5 MW049136
Uncultured Mouflon amplicon Mouf 40280
Uncultured Aoudad sheep amplicon Aoud18 42651
Uncultured Aoudad sheep amplicon Aoud18 143123
Isolate R1 CloneA1 MW019480
Isolate R4 CloneB9 MW019496
Isolate R3 CloneB3 MW019491
Isolate R3 CloneB5 MW019493
Uncultured Oryx amplicon Oryx 21426
Uncultured Oryx amplicon Oryx 7228
Isolate A252 CloneB1.1 MW049132
Isolate A252 CloneB1.2 MW049133
Isolate A252 CloneB5.1 MW049144
Isolate A252 CloneB2.1 MW049140
Isolate A252 CloneB1.3 MW049134
Isolate A252 CloneB4.8 MW049143
Isolate A252 CloneB5.2 MW049145
Isolate A252 CloneB2.3 MW049141
Aestipascuomyces
Caecomyces
Cyllamyces
Piromyces
Aklioshbomyces
Khyollomyces
Agriosomyces
Liebetanzomyces
Capellomyces
Oontomyces
Anaeromyces
Buwchfawromyces
Tahromyces
Joblinomyces
Gonapodya

## 4. Discussion

Here, we report on the isolation and characterization of the previously uncultured Neocallimastigomycota lineage SK4 from the rumen contents of a wild aoudad sheep and feces of a zoo-housed alpaca. Phylogenetic analysis using the D1/D2-LSU region showed that the six isolates obtained formed a single, monophyletic cluster within the *Orpinomyces*-

*Neocallimastix*-*Pecoramyces*-*Feramyces*-*Ghazallamyces* supragenus clade [24, 37]. 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 [37]), followed by a recent loss and reverting to zoospore monoflagellation for the relatively recently-evolved genus *Pecoramyces* (current estimates of emergence at 19.1 Mya, [37]). 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, the majority of *Neocallimastix* genus members are known to release zoospores through complete rupturing and lysis of the sporangial wall (Figure 25 in [16], with only a few exceptions (e.g. [38, 39]), 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 [40-43]. Within the anaerobic fungal phylum Neocallimastigomycota, multiple putative novel genera were identified in culture-independent studies [20, 21, 35]. 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, 44]. 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 up the majority (>90%) of the AGF community.

While information is currently lacking on the AGF community in the Alpaca sample that was used for isolation in Germany, we believe that the success of obtaining a cultured SK4 representative was largely dependent on its presence in high relative abundance in the samples used for isolation. Therefore, this study clearly demonstrates the value of the sequence-guided isolation strategy that was 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 dupliciliberatus* (to indicate the two zoospore release mechanisms exhibited by members of the clade), and the type strain is *Aestipascuomyces dupliciliberatus* strain R4.

## 5. Taxonomy

***Aestipascuomyces*** Marcus Stabel, Radwa Hanafy, Tabea Schweitzer, Meike Greif, Habibu Aliyu, Veronika Flad, Diana Young, Michael Lebuhn, Mostafa Elshahed, Katrin Ochsenreither &. Noha Youssef, gen. nov.

MycoBank ID: MB837524

*Typification: Aestipascuomyces dupliciliberatus* Marcus Stabel, Radwa Hanafy, Tabea Schweitzer, Meike Greif, Habibu Aliyu, Veronika Flad, Diana Young, Michael Lebuhn, Mostafa Elshahed, Katrin Ochsenreither &. 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 and MW049132-MW049145 (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 polyflagellated zoospores (7-30), monocentric thalli, and empty zoospore cysts that remain at the base of sporangiophores.

***Aestipascuomyces dupliciliberatus*** Marcus Stabel, Radwa Hanafy, Tabea Schweitzer, Meike Greif, Habibu Aliyu, Veronika Flad, Diana Young, Michael Lebuhn, Mostafa Elshahed, Katrin Ochsenreither &. Noha Youssef, sp. nov.

Mycobank ID: MB837526

*Typification*: The holotype is figure 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.

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

An obligate anaerobic fungus that produces 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 strain 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. Marcus Stabel strain A252 (MW049132-MW049145) (GenBank accession number of clones in parenthesis), isolated from the feces of an Alpaca (*Vicugna pacos*) from the Karlsruhe Zoo, Karlsruhe, Germany in August 2019.

**Author Contributions:** Conceptualization, M.S.E., N.H.Y., and K.O.; methodology and formal analysis, M.S. T.S., M.G., H. A., and R.H.; resources, M.S.E., N.H.Y., and K.O.; writing—original draft preparation, M.S. and R.H.; writing—review and editing, V.F., D.Y., M.L., M.S.E., N.H.Y., and K.O.; supervision, M.S.E, N.H.Y., D.Y., V.F., M.L., and K.O.; project administration, M.S.E., N.H.Y., M.L., and K.O. All authors have read and agreed to the published version of the manuscript.

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