

Testudinimycetes gracilis gen. nov, sp. nov. and *Astrotestudinimycetes divisus* gen. nov, sp. nov., two novel, deep-branching anaerobic gut fungal genera from tortoise faeces

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Abstract

The anaerobic gut fungi (AGF, *Neocallimastigomycota*) represent a basal zoosporic phylum within the kingdom *Fungi*. Twenty genera are currently described, all of which were isolated from the digestive tracts of mammalian herbivores. Here, we report on the isolation and characterization of novel AGF taxa from faecal samples of tortoises. Twenty-nine fungal isolates were obtained from seven different tortoise species. Phylogenetic analysis using the D1/D2 region of the LSU rRNA gene, ribosomal internal transcribed spacer 1, and RNA polymerase II large subunit grouped all isolates into two distinct, deep-branching clades (clades T and B), with a high level of sequence divergence to their closest cultured relative (*Khoyollomyces ramosus*). Average amino acid identity values calculated using predicted peptides from the isolates' transcriptomes ranged between 60.80–66.21 % (clade T), and 61.24–64.83 % (clade B) when compared to all other AGF taxa; values that are significantly below recently recommended thresholds for genus (85%) and family (75%) delineation in the *Neocallimastigomycota*. Both clades displayed a broader temperature growth range (20–45 °C, optimal 30 °C for clade T, and 30–42 °C, optimal 39 °C for clade B) compared to all other AGF taxa. Microscopic analysis demonstrated that strains from both clades produced filamentous hyphae, polycentric rhizoidal growth patterns, and monoflagellated zoospores. Isolates in clade T were characterized by the production of unbranched, predominantly narrow hyphae, and small zoospores, while isolates in clade B were characterized by the production of multiple sporangiophores and sporangia originating from a single central swelling resulting in large multi-sporangiated structures. Based on the unique phylogenetic positions, AAI values, and phenotypic characteristics, we propose to accommodate these isolates into two novel genera (*Testudinimycetes* and *Astrotestudinimycetes*), and species (*T. gracilis* and *A. divisus*) within the order *Neocallimastigales*. The type species are strains T130A^T (*T. gracilis*) and B1.1^T (*A. divisus*).

INTRODUCTION

Anaerobic gut fungi (AGF) are important members of the gastrointestinal microbiome in herbivores. AGF are encountered in hosts with long feed retention times and digestive chambers with a relatively neutral pH. In such hosts, AGF often play a critical role in plant biomass degradation by breaking down complex polysaccharides [1]. The strict anaerobic nature and overall fastidiousness of AGF render them difficult to culture and maintain. Since their recognition as members *Fungi* in 1975 [2], 20 genera have been described, all of which have been isolated from mammalian faeces, rumen contents, and forestomach digesta [3].

Tortoises are terrestrial herbivorous reptiles belonging to the family *Testudinidae* in the order *Testudines* [4]. Tortoises have an enlarged caecum and a long feed retention time (12–14 days) [5]. Evidence for hindgut fermentative capacities has been documented in some tortoise species, e.g. Galápagos [6] and gopher [7] tortoises. The anaerobic conditions and long feed retention times within the tortoise caecum render them candidates for harbouring AGF, albeit under conditions (temperature, substrate, etc.) that are markedly different from those prevailing in mammalian herbivores.

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Abbreviations: AAI, average amino acid identity; AGF, anaerobic gut fungi; DAPI, 4,6'-diamidino-2-phenylindole; ITS, internal transcribed spacer; LSU, large subunit; RF, rumen fluid; RFC, rumen fluid cellobiose; RPB1, RNA polymerase II large subunit. QO382931 (*Testudinimycetes gracilis* ITS1, 5.8S rDNA, ITS2, D1–D2 28S rRNA locus). QO382915 (*Astrotestudinimycetes divisus* ITS1, 5.8S rDNA, ITS2, D1–D2 28S rRNA locus).

A supplementary figure is available with the online version of this article.

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We recently reported on the occurrence of AGF in faecal samples of tortoises [8]. Here, we report on the morphological and phenotypic characterization, as well as the phylogenetic position of multiple novel tortoise-sourced AGF isolates. We highlight key differences between these isolates and all currently described mammalian-sourced AGF. We argue that such differences justify the proposal of two novel genera (*Testudinimycetes* and *Astrotestudinimycetes*) to accommodate these isolates.

METHODS

Samples

Fresh faecal samples were obtained from seven species of tortoises kept in captivity. Six of these species were housed at the Oklahoma City Zoo (Oklahoma City, OK, USA, 35.5235417, -97.472475), and were sampled in November 2020: Egyptian tortoise (*Testudo kleinmanni*), Galápagos tortoise (*Chelonoidis niger*), Indian star tortoise (*Geochelone elegans*), pancake tortoise (*Malacochersus tornieri*), ploughshare tortoise (*Astrochelys yniphora*), and Burmese star tortoise (*Geochelone platynota*). The seventh species, sulcata tortoise (*Centrochelys sulcata*), was housed at Hawk Hill Farm, near Walters, OK, USA (34.4786944, -98.2258333) and was sampled in March 2022. Freshly deposited samples were collected on-site in 15–50 ml conical centrifuge tubes and transferred on ice to the laboratory within 24 h of collection, where they were frozen at -20°C . Every effort was made to minimize oxygen exposure and prevent dryness of the samples. Isolation efforts were conducted on samples 4–18 months post-collection.

Isolation

Samples (0.5–1 g) were serially diluted into rumen fluid cellobiose (RFC) media [9] supplemented with antibiotics ($50\text{ }\mu\text{g ml}^{-1}$ chloramphenicol, $20\text{ }\mu\text{g ml}^{-1}$ streptomycin, $50\text{ }\mu\text{g ml}^{-1}$ penicillin, $50\text{ }\mu\text{g ml}^{-1}$ kanamycin, and $50\text{ }\mu\text{g ml}^{-1}$ norfloxacin) and 0.1 g ml^{-1} switchgrass, and incubated at both 30°C and 39°C . Efforts to enrich AGF at both temperatures were conducted to account for the ectothermic nature of their hosts, which contributes to lower and variable temperatures in the hosts' digestive tracts. Dilutions displaying visible signs of growth (plant materials clumping and floating, visible production of fungal biomass, and production of gas bubbles) were subcultured into fresh media. In addition, 1 ml of the enrichment was rolled on RFC agar roll tubes [10]. Roll tubes were incubated at the corresponding isolation temperature (30°C or 39°C) until colonies were visible. Single colonies were picked into liquid RFC media to ensure purity. Strains were maintained by weekly or biweekly subculturing.

Temperature and substrate preferences

Isolates obtained (regardless of the isolation and maintenance temperature) were grown at 20°C , 25°C , 30°C , 35°C , 37°C , 39°C , 42°C , and 45°C in RFC media with antibiotics ($50\text{ }\mu\text{g ml}^{-1}$ chloramphenicol, $20\text{ }\mu\text{g ml}^{-1}$ streptomycin, $50\text{ }\mu\text{g ml}^{-1}$ penicillin). Growth was assessed both visually, as well as by measuring headspace accumulated gas pressure using a digital pressure gauge (MediaGauge, SSI Technologies) as previously suggested [11]. The same media to headspace ratio (12 ml liquid culture:15 ml headspace) was used in all experiments. Formation of biofilm in one type strain (B1.1^T) was also recorded as a morphological indicator of growth. *Orpinomyces joyonii* strain AB.5, isolated from an American bison (*Bison bison*), was used as a control. Both the capacity to grow at a certain temperature, as well as the long-term viability of strains upon repeated subcultures at that temperature were assessed.

Substrate preferences were monitored by growing strains on different substrates (0.5% w/v) in rumen fluid media without cellobiose (RF) with antibiotics ($50\text{ }\mu\text{g ml}^{-1}$ chloramphenicol, $20\text{ }\mu\text{g ml}^{-1}$ streptomycin, $50\text{ }\mu\text{g ml}^{-1}$ penicillin). Growth of each strain was assessed by measuring gas pressure buildup as described above and confirmed visually compared to the substrate-unamended control. Substrates were scored as supportive if growth was sustained after four subcultures, as previously recommended [12]. RF media with cellobiose served as the positive control, while RF substrate-unamended media served as the negative control. Incubations were conducted at the isolation temperatures for each strain. A scale based on maximum gas pressure production compared to cellobiose was used: ++++>100%, +++>75%, ++>50%, +>25%.

Phylogenetic analysis

Fungal biomass was separated by vacuum filtration and DNA was extracted using a DNeasy PowerPlant Pro Kit (Qiagen Corp., Germantown, MD, USA) according to the manufacturer's instructions. The identity of different isolates was first determined by amplification of a region of the 28S rRNA gene corresponding to the D1/D2 domains using the primers NL1 (5'-GCATATCA ATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3'). This was followed by amplicon cleanup using a PureLink PCR Purification Kit (ThermoFisher Scientific, Waltham, MA, USA) and direct Sanger sequencing. For type strains, the region corresponding to ITS1-5.8S rRNA-ITS2-D1/D2 domains of 28S rRNA gene was amplified using the primers ITS5 (5' -GGAAGTAAAAGTCGTAACAAGG-3') and NL4 (5'-TCAACATCCTAAGCGTAGGTA-3') as described previously [13], and amplicons were cloned into a pCR-XL-2-TOPO cloning vector according to the manufacturer's instructions (ThermoFisher Scientific, Waltham, MA, USA). At least twelve clones were Sanger sequenced for each type strain to examine intra-strain variability, as recently recommended [14]. Regions corresponding to the ITS1 and D1/D2 LSU from the obtained amplicons were extracted using the primer.seqs command in mothur [15], and aligned to reference ITS1 and D1/D2 LSU sequences using MAFFT

[16]. Maximum likelihood phylogenetic trees were reconstructed in FastTree using *Chytriumyces* sp. WB235A isolate AFTOL-ID 1536 as an outgroup. Bootstrap values were calculated on the basis of 100 replicates. Sanger sequencing was conducted at the Oklahoma State University DNA Protein Core Facility (Stillwater, OK, USA) or Eurofins Genomics (Louisville, KY, USA).

For single-copy protein trees, amino acid sequences of the RNA polymerase II large subunit gene (RPB1) were extracted from the isolates' transcriptomes (see below) and aligned using MAFFT [16]. The alignments were used as inputs to IQ-TREE [17, 18] to predict the best substitution model and to generate maximum-likelihood trees under the predicted best model. We added the '-alrt 1000' option for performing the Shimodaira-Hasegawa approximate-likelihood ratio test (SH-aLRT), the '-abayes' option for performing approximate Bayes tests, and the '-bb 1000' option for ultrafast bootstrap (UFB) to the IQ-TREE command line. IQ-TREE analysis resulted in the generation of phylogenetic trees with three support values (SH-aLRT, aBayes, and UFB) on each branch.

Transcriptomic sequencing and AAI determination

Transcriptomic sequencing of seven representative isolates was conducted as described previously [8]. Briefly, cultures grown in RFC media were vacuum filtered and total RNA was extracted using an Epicentre MasterPure Yeast RNA Purification Kit according to manufacturer's instructions. RNA-seq was conducted on an Illumina HiSeq2500 platform using a 2×150 bp paired-end library at the Oklahoma State University Genomics and Proteomics Core Facility (Stillwater, OK, USA). Quality trimming and *de novo* assembly of RNA-seq data was conducted using Trinity (version 2.6.6) and default parameters. CD-HIT [19] was then used on the assembly to cluster redundant transcripts with an identity parameter of 95% ($-c$ 0.95). Peptide and coding sequence predictions were conducted using TransDecoder (version 5.0.2) (<https://github.com/TransDecoder/TransDecoder>) with a minimum peptide length of 100 amino acids. The predicted peptides were used for average amino acid identity (AAI) calculations, as well as for extracting the single copy protein-coding gene, RPB1.

For AAI calculations, we included predicted peptides from other AGF transcriptomes ($n=52$ [20]). AAI values were calculated for all possible pairs in the dataset (predicted peptides from 52 previous transcriptomes, plus the seven newly generated ones) using the *aai.rb* script available as part of the Enveomics collection [21].

Morphological and microscopic characterization

Preliminary investigation suggested that isolates' microscopic features (hyphae, sporangia, sporangiophore, and zoospore morphology) did not vary when grown on different substrates or at different growth temperatures, and hence isolates were examined when grown in RFC media incubated at their isolation temperatures. Roll tube and liquid cultures grown in RFC media were used to examine colony morphology and liquid growth patterns. Isolates were examined at different growth phases with a focus on zoospores mostly during log phase and sporangia and hyphae development at late-log through early stationary phases. Microscopic features and growth patterns of various isolates were examined with light microscopy and scanning electron microscopy (SEM). Light microscopy was performed with an Olympus BX51 instrument equipped with a DP71 digital camera (Olympus). SEM was performed with an FEI Quanta 600 field-emission gun environmental scanning electron microscope) with a Bruker EDS X-ray microanalysis system) and an HKL EBSD system (Oxford Instruments). For SEM, samples were fixed and critical point dried as previously described [12]. Confocal microscopy using a Zeiss LSM 980 Airyscan 2 confocal laser scanning microscope was used to examine nuclei localization in samples stained with 4,6'-diamidino-2-phenylindole (DAPI; $10\ \mu\text{g ml}^{-1}$) as previously described [12]. Sizes of various microscopic structures were measured with Fiji software [22].

Data and culture accession

Sequences generated in this study are deposited in GenBank under the accession numbers OQ382931–OQ382943 (for strain T130A^T) and OQ382915–OQ382930 (for strain B1.1^T). Cultures are available at Oklahoma State University, Department of Microbiology and Molecular Genetics (Stillwater, OK, USA).

RESULTS

Isolation

Enrichments in RFC media amended with switchgrass and antibiotics yielded 29 different fungal isolates from seven tortoise species. Sequences of the LSU rRNA D1/D2 region grouped the isolates into two clades (henceforth clade T and clade B). Clade T representatives were recovered from Galápagos ($n=6$), Indian star ($n=5$), ploughshare ($n=3$), pancake ($n=2$), Burmese star ($n=2$), and Egyptian tortoises ($n=1$). Clade B representatives were recovered from sulcata ($n=7$) and Burmese star tortoises ($n=3$). Interestingly, no isolates belonging to any of the previously described 20 AGF genera were recovered from tortoise samples in this effort. The observed morphological and microscopic similarities between isolates in each clade led us to choose one strain from each lineage for in-depth characterization, according to the criteria put forth by [14]. Strain T130A^T, isolated from a ploughshare tortoise, was chosen as the type for clade T, while strain B1.1^T, isolated from a Burmese star tortoise, was chosen as the type for clade B.

Phylogenetic analysis

Phylogenetic analysis using the D1/D2 LSU region placed all isolates in two deeply branching, bootstrap-supported clades (clade T and clade B, Fig. 1a). D1/D2 LSU sequence divergence estimates ranged from 10.1 (*Khoyollomyces ramosus*) to 17.04% (*Aestipascuomyces dupliciliberans*) for the type strain of clade T and 13.02 (*Khoyollomyces ramosus*) to 17.65% (*Aestipascuomyces dupliciliberans*) for the type strain of clade B. D1/D2 LSU sequence divergence estimates between the type strains of clade T and B was 12.93%. Isolates within both clades displayed extremely low levels of inter-strain D1/D2 LSU sequence divergence (0–0.46% for clade T strains and 0–0.19% for clade B strains) regardless of the tortoise species from which they were isolated.

In addition to D1/D2 LSU, RPB1 was recently proposed as an additional phylogenetic marker in the *Neocallimastigomycota* [20]. Phylogenetic trees reconstructed using the amino acid sequences of RPB1 also demonstrated a similar distinct, deeply branching, bootstrap-supported position for both clades (Fig. 1b). High levels of sequence divergence were observed between the clade T type strain and all other AGF genera, ranging from 8.39 (*Piromyces communis*) to 10.77% (*Caecomycetes communis*). Similarly, sequence divergence between the clade B type strain and other AGF genera ranged from 12.06 (*Piromyces communis*) to 15.79% (*Neocallimastix cameroonii*). A high level of RPB1 sequence divergence (10.76%) was also observed between the clade T and B type strains. Finally, negligible intra-clade RPB1 sequence divergence was observed (0–0.23% and 0–1.03% for clades T and B, respectively) (Fig. 1b).

Significant within-strain ITS1 sequence variability is often observed in AGF taxa [14]. To quantify such variability, we cloned and sequenced 13 and 16 clones from the type strains of clades T and B, respectively. Minimal within-strain divergence was observed for both type strains (0% for clade T and 0–0.92% for clade B) (Fig. 1c).

Ecologically, strains T130A^T and B1.1^T from clades T and B represent the first cultured representatives of the uncultured genera NY54 and NY36, respectively. These two then-uncultured genera were recently proposed in a global culture-independent survey of AGF in herbivorous mammals [23]. Subsequently, a culture-independent study of the tortoise gut has shown that, in contrast to their relative rarity in the mammalian gut, NY54 and NY36 represent a significant fraction of the AGF community in the tortoise gastrointestinal tract [8].

AAI values

Predicted peptides from transcriptomic datasets were used for AAI calculations. High levels of AAI similarity were observed between isolates belonging to the T clade (97.63–99.47%) as well as between isolates belonging to the B clade (99.4%) (Table 1). In contrast, consistently low AAI values were observed when comparing clades T and B isolates to all other AGF genera. AAI values between the clade T type strain and other AGF genera ranged from 62.45 (*Piromyces* sp. B4) to 66.15% (*Khoyollomyces ramosus*) (Table 1). Similarly, AAI values between the clade B type strain and other AGF genera ranged from 61.24 (*Piromyces* sp. B4) to 64.29% (*Khoyollomyces ramosus*). Finally, low AAI values were also observed when comparing clade T to clade B isolates (64.46±0.61%).

Temperature and substrate preferences

Strain T130A^T grew at a wide range of temperatures (20–45 °C) in initial subculture (Figs 2 and S1, available in the online version of this article). Growth rate was temperature-dependent, with the fastest growth observed at higher temperatures (35–45 °C). However, upon subculturing at temperatures higher than 35 °C, cultures quickly lost viability and adhesion capacity and exhibited a near cessation of gas production by the third subculturing event (every 11 days) (Fig. 2b).

While slower growth was observed at 30 °C (highest gas pressure after 8–10 days, Fig. 2a), all strains belonging to the T clade retained viability and have been successfully maintained via repeated weekly subculturing at 30 °C. Slowest growth was observed at 20 and 25 °C (Fig. S1).

Growth of strain B1.1^T was generally faster than T130A^T at comparable temperatures but showed a narrower temperature growth range (30–42 °C) in initial subcultures (Fig. 2c). After continuous subculturing (every 7 days), cultures incubated at 42 °C quickly lost viability and adhesion capacity and exhibited a near cessation of gas production by the third subculturing event (Fig. 2d), while cultures at 30, 35, 37, and 39 °C retained viability after repeated subculturing. Growth patterns were very similar for cultures incubated at 35, 37, and 39 °C, where peak gas production was detected between days two and three. Growth at 30 °C was slightly slower and peak gas production was detected on day four. Currently, strain B1.1^T is maintained by biweekly subculturing at 39 °C.

As a control, we evaluated the temperature growth range of a mammalian-sourced AGF strain (*Orpinomyces* sp. strain AB.5). Strain AB.5 grew in the initial subculture in a much narrower range (35–42 °C, Fig. 2e). Further, by the third subculture, only cultures at 39 °C retained viability, confirming the extremely narrow temperature growth range of mammalian-sourced AGF (Fig. 2f).

Substrate utilization

Strain T130A^T grew on a wide range of monosaccharides, with the strongest growth observed on glucose and fructose (Table 2). Most disaccharides tested (cellobiose, maltose, lactose, and sucrose) as well as several polymers (cellulose, starch, and inulin)

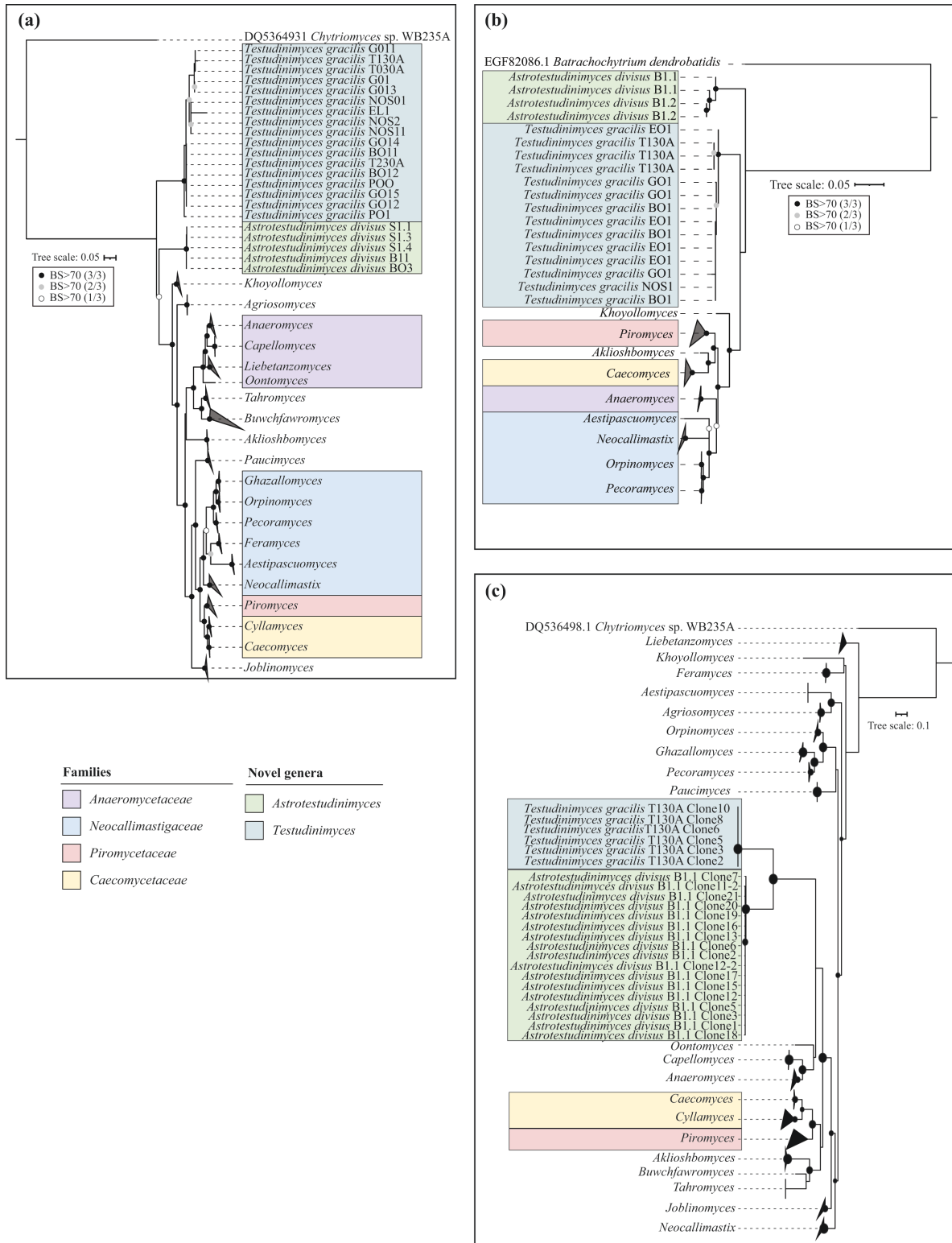


Fig. 1. Assessment of the phylogenetic position of *Testudinimycetes* gen. nov. and *Astrotetudinimycetes* gen. nov. using D1/D2 LSU (a), RPB1 (b), and ITS1 (c) as phylogenetic markers. The tree was reconstructed using the maximum-likelihood approach implemented in the iq-TREE package. Scale bars indicate the number of substitutions per site. The three bootstrap support values in (a) and (b) (SH-aLRT, aBayes, and UFB) are shown as coloured dots as follows: all three support values >70%, black; 2/3 support values >70%, light grey; 1/3 support values >70%, white. Bootstrap values in (c) are shown for nodes with >70% support as black spheres.

Table 1. Average amino acid identity (AAI; %) between *Testudinimyces gracilis* and *Astrotestudinimyces divisis* strains with sequenced transcriptomes and taxa belonging to currently described anaerobic gut fungal genera

	<i>Astrotestudinimyces divisis</i> B1.1 [†]	<i>Astrotestudinimyces divisis</i> B1.2	<i>Testudinimyces gracilis</i> BO1	<i>Testudinimyces gracilis</i> EO1	<i>Testudinimyces gracilis</i> GO1	<i>Testudinimyces gracilis</i> NOS1	<i>Testudinimyces gracilis</i> T130A [†]
<i>Astrotestudinimyces divisis</i> B1.1 [†]							
<i>Astrotestudinimyces divisis</i> B1.2	99.40						
<i>Testudinimyces gracilis</i> BO1	64.48	64.63					
<i>Testudinimyces gracilis</i> EO1	63.64	63.99	97.97				
<i>Testudinimyces gracilis</i> GO1	65.08	65.39	99.33	98.10			
<i>Testudinimyces gracilis</i> NOS1	64.51	64.78	99.26	98.17	99.47		
<i>Testudinimyces gracilis</i> T130A [†]	64.73	64.99	99.05	97.92	99.00	99.07	
<i>Aestipascuomyces</i> (1)	63.43	63.90	64.75	64.00	65.13	64.68	65.04
<i>Feramyces</i> (2)	62.55–62.85	62.87–63.12	63.66–63.91	63.06–63.15	64.32–64.49	63.52–63.78	64.34–64.42
<i>Neocallimastix</i> (6)	61.85–63.74	62.02–63.94	63.03–64.77	62.12–63.56	63.48–65.22	62.57–63.99	63.43–65.27
<i>Pecoramyces</i> (9)	62.62–63.83	62.77–64.12	63.64–65.23	62.73–64.35	64.07–65.7	62.88–65.36	64.29–65.61
<i>Orpinomyces</i> (7)	62.45–63.47	62.7–63.96	63.71–64.91	62.89–64.13	64.23–65.14	63.58–64.31	64.12–65.52
<i>Paucimyces</i> (1)	62.65	63.10	64.15	63.12	64.46	63.55	64.61
<i>Caecomyces</i> (5)	61.83–63.67	62.07–63.79	62.98–64.54	62.28–63.62	63.5–64.95	62.39–63.87	63.5–64.93
<i>Cyllamyces</i> (1)	62.52	62.98	64.10	63.26	64.56	63.34	64.79
<i>Akkioshomyces</i> (1)	62.91	62.99	63.98	63.18	64.33	63.05	64.77
<i>Piromyces</i> (8)	61.24–63.69	61.65–64.01	62.02–64.73	61.32–63.73	62.41–65.25	62.14–64.06	62.45–65.29
<i>Anaeromyces</i> (7)	62.65–63.35	62.95–63.53	64.02–64.86	63.17–64.03	64.15–64.99	63.43–64.38	64.44–65.23
<i>Capallomyces</i> (2)	63.53–64.62	63.81–64.83	64.71–65.75	63.94–64.52	65.01–66.21	64.35–65.05	65.16–66.03
<i>Liebetanzomyces</i> (1)	63.48	63.88	64.94	64.07	65.08	64.36	65.66
<i>Khoyollomyces</i> (1)	64.29	64.77	65.71	65.06	66.07	65.42	66.15

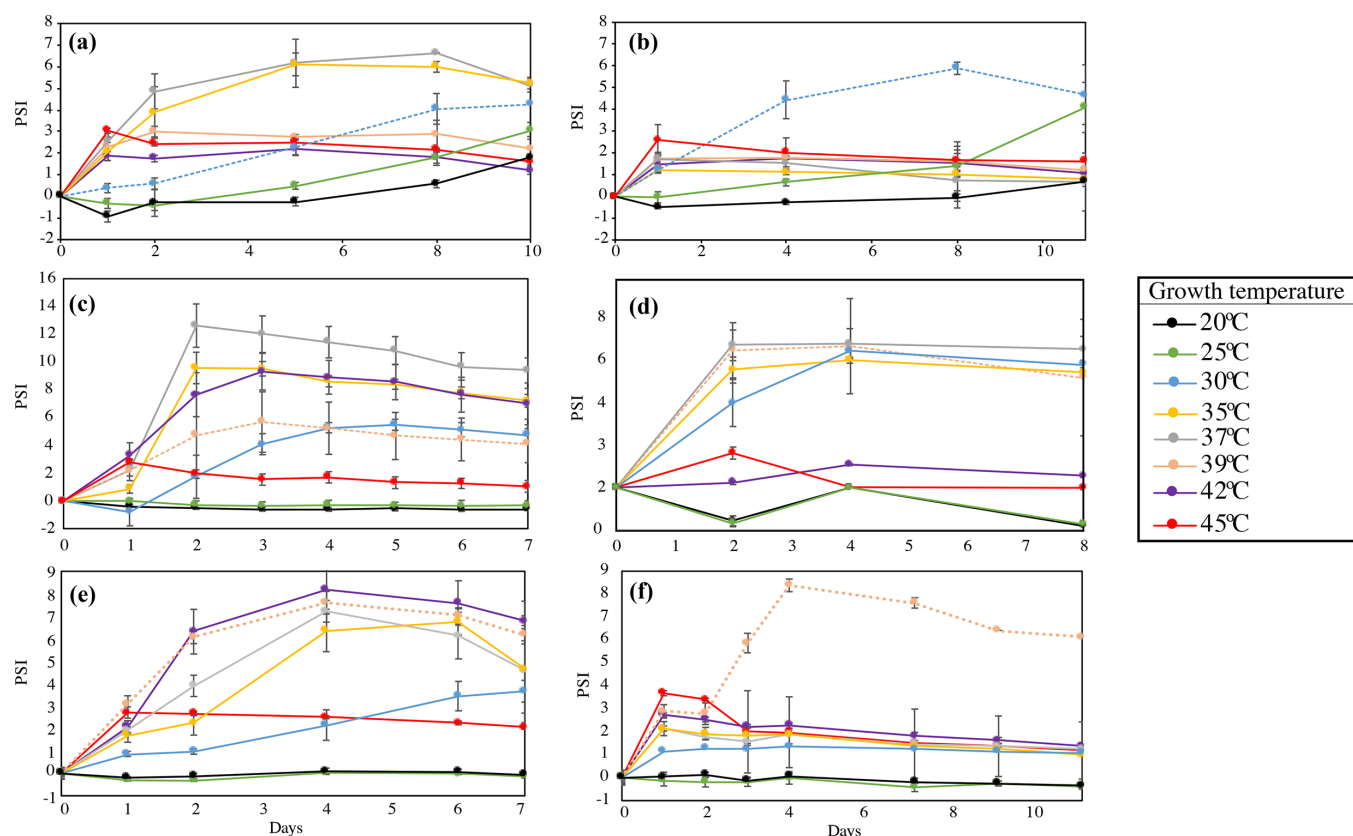


Fig. 2. Temperature growth preferences of strains T130A^T (a, b), B1.1^T (c, d), and AB.5 (e, f). Gas pressure in PSI (as a proxy for growth) is shown on the y-axis. During the first subculture (A, C, and E), strains were grown in triplicates at different temperatures (colour-coded as shown in the figure legend) for extended periods of time (shown in days on the x-axis) and the gas pressure buildup was measured. Averages±standard deviation are shown for PSI values. Tubes were subcultured at 11, 7, and 7 days for strains T130A^T, B1.1^T, and AB.5, respectively, and tubes were incubated for another 11, 7, and 7 days, after which the third subculture started. Measurements during the third subculture are shown in B, D, and F.

also supported strong growth (Table 2). On the other hand, growth of strain B1.1^T was supported by relatively fewer substrates. Of the substrates tested, fructose and mannose were the only monosaccharides that supported B1.1^T growth. Most disaccharides tested and the polymers inulin and starch supported growth. Remarkably, although starch and the glucose dimers cellobiose and maltose supported growth, we were unable to grow strain B1.1^T, or any other strain from the B clade, on glucose or cellulose. This discrepancy is puzzling, since all known AGF can readily grow on both substrates. The possible rationale for this unexpected result is currently under evaluation, but potentially points to a lack of glucose transporting ability, coupled to possible sole production of disaccharide hydrolases intracellularly in clade B isolates.

Macroscopic and microscopic characteristics

Strain T130A^T

In liquid RFC media, strain T130A^T formed large, dense, granular beige biomass (Fig. 3a), occasionally coalescing into a biofilm. On agar roll tubes (Fig. 3b), strain T130A^T formed small, beige, circular colonies (up to 2.5 mm). Colonies exhibited a dense (darker) centre of sporangial structure and a diffuse outer ring of new sporangia (Fig. 3c). Strain T130A^T produced globose zoospores (Fig. 3d–g) with a rough external texture (Fig. 3f, g). Zoospores exhibited an average size of $5.1 \pm 1.15 \times 4.49 \pm 1.17 \mu\text{m}$ ($n=39$) and were unflagellated (i.e. exclusively monoflagellated), with an average flagella length of $10.54 \pm 8.72 \mu\text{m}$ ($n=5$, Fig. 3d, e). In addition to released spores, internal zoospores could be observed within sporangia (Fig. 3h, i).

Strain T130A^T displayed a polycentric thallus development pattern, with DAPI staining demonstrating the presence of nuclei in both the hyphae and the sporangia (Fig. 3j, k). Strain T130A^T was characterized by a filamentous hyphal growth pattern that extensively wrapped around the sporangia and displayed minimal branching (Fig. 3l, m). Remarkably, strain T130A^T hyphae were predominantly narrow (Fig. 3j–m) with wide hyphae less frequently encountered (Fig. 3j). In contrast, most polycentric AGF taxa display a significantly larger proportion of wide hyphae in cultures [3].

Table 2. Substrate utilization patterns of *Testudinimycetes gracilis* T130A^T and *Astrotestudinimycetes divisus* strain B1.1^T

Substrate	<i>Testudinimycetes gracilis</i> T130A ^T	<i>Astrotestudinimycetes divisus</i> B1.1 ^T
Monomers:		
Glucose	++++	–
Fructose	++++	++++
Mannose	++++	+++
Galactose	++	–
Gluconic acid	+	–
Ribose	++	–
Xylose	+	–
Arabinose	+	+
Dimers:		
Cellobiose	++++	++++
Lactose	++++	+++
Maltose	++++	++++
Sucrose	+++	++
Trehalose	+	–
Trimer:		
Raffinose	–	–
Polymers:		
Cellulose	++++	–
Xylan	–	–
Inulin	+++	+++
Starch	++++	++++
Alginic acid	–	–
Chitin	–	–
Pectin	–	–
Polygalacturonic acid	+	+
Undefined substrates:		
Peptone	++	+
Yeast extract	++	++

Individual sporangia developed terminally and an intercalary sporangial development pattern was not observed. Due to the extensive wrapping of hyphae around sporangia, observing sporangiophore shapes was not always straightforward. However, wide, flattened sporangiophores (Fig. 3l) were encountered. Strain T130A^T sporangia ranged in size between 123.88±38.18×86.17±33.17 µm ($n=9$). Sporangia were mostly globose, subglobose, and ovoid (Fig. 3h, i), while elongated and irregularly shaped sporangia (Fig. 3n) were less common. Zoospore release appeared to occur through an apical pore (Fig. 3o) followed by the dissolution of the sporangial wall (Fig. 3p).

Strain B1.1^T

Strain B1.1^T exhibited granular beige growth in liquid RFC media (Fig. 4a) and formed a thin biofilm that clung to the sides of the tube and spanned across to the surface of the liquid (Fig. 4b). On agar roll tubes the strain produced minute, beige, pinpoint colonies (Fig. 4c). Strain B1.1^T produced globose zoospores that were monoflagellated (i.e. predominantly unflagellated) (Fig. 4d, e), with an average dimension of 6.89±2.16×5.71±1.68 µm ($n=60$). Zoospore flagella averaged 34.45±6.07 µm ($n=23$).

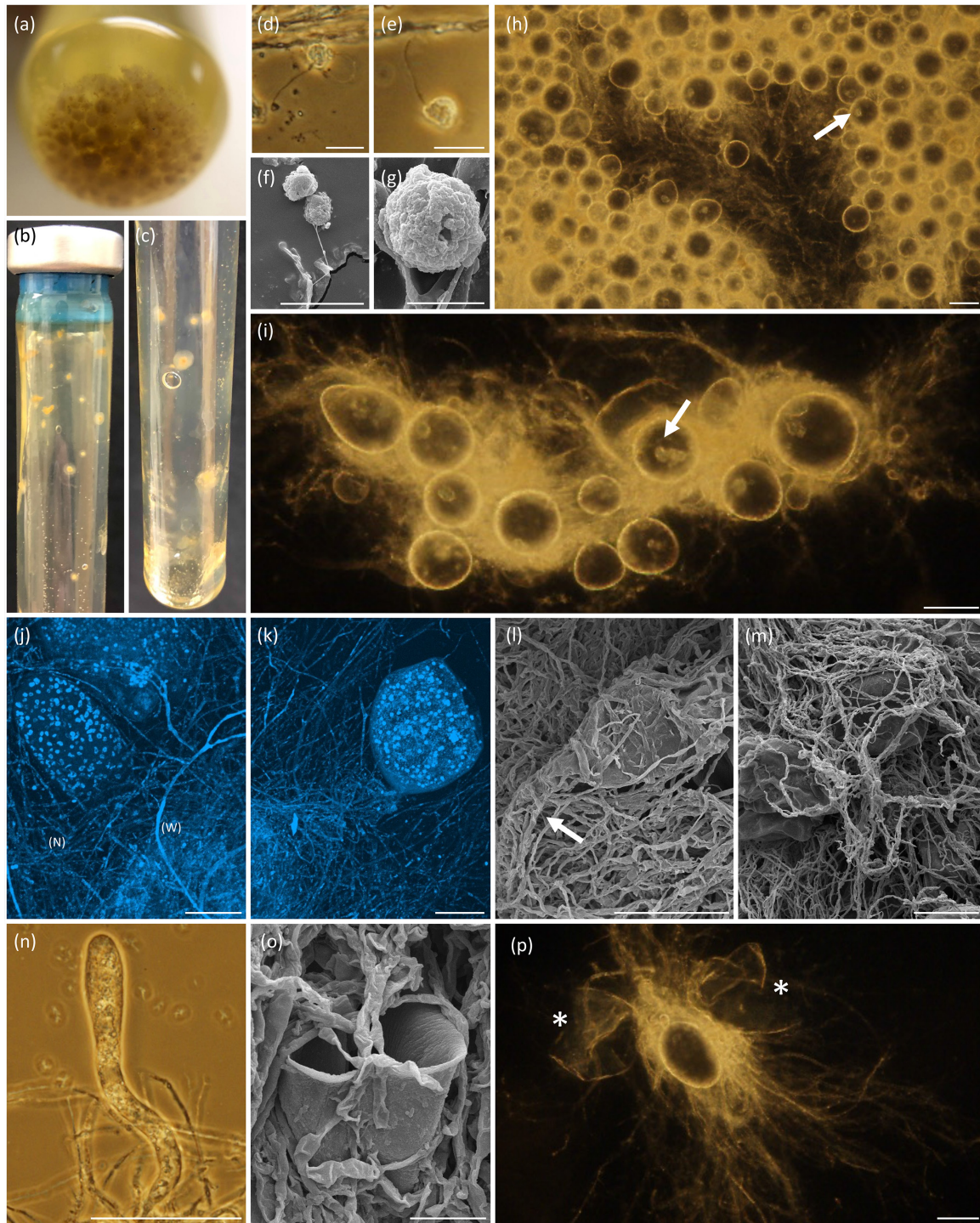


Fig. 3. Macroscopic and microscopic features of *Testudinimycetes gracilis* strain T130A^T, observed using light (a–e, h, i, n, and p), confocal (j, k), and scanning electron (l, m, and o) microscopy images showing (a) large, dense, granular, beige biomass in liquid media, (b, c) small (~2.5 cm), beige, circular colonies on roll tubes exhibiting a dense (darker) centre of sporangial structure and a diffuse outer ring of new sporangia. (d–g) Monoflagellated zoospores (d, e) displaying rough surface textures (f, g). (h, i) Globose, subglobose, and ovoid sporangia with zoospores appearing inside (arrows). (j, k) Polycentric thalli, with nuclei observed throughout both the sporangia and hyphae, with both wide (w) and more predominantly narrow (n) hyphae. (l, m) Minimally branched extensive filamentous hyphal growth tightly wrapping around sporangia. (l) Wide flattened sporangiophore. (n) Less commonly observed elongated and irregularly shaped sporangia. (o–p) Zoospores release through an apical pore followed by the dissolution of the sporangial wall (asterisks in p). Bars, 3 µm (g), 10 µm (d–f, o), and 50 µm (h–n, p).

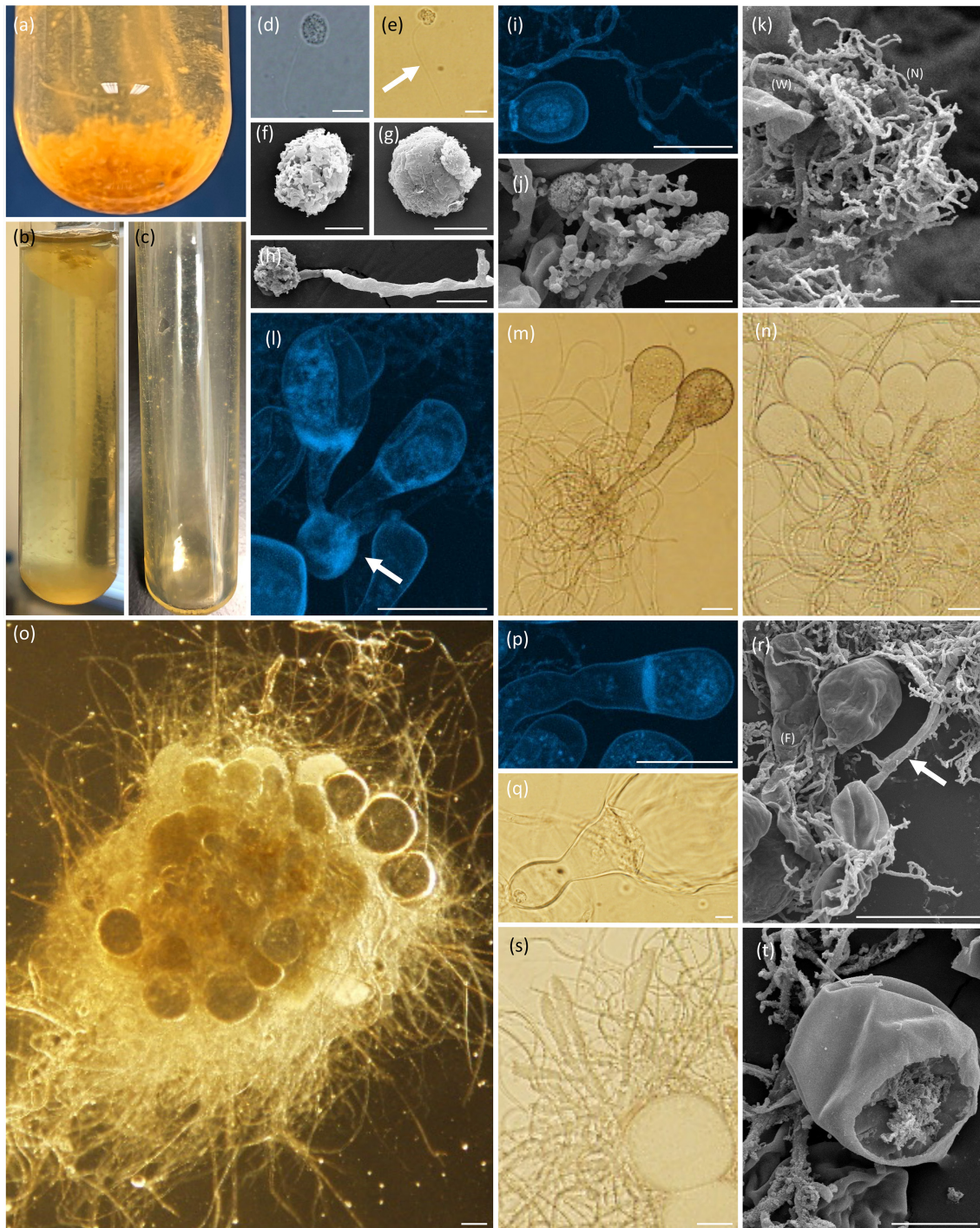


Fig. 4. Macroscopic and microscopic features of *Astrotestudinimycetes divisus* strain B1.1^T. Light (a–e, m–o, q, and s), confocal (i, l, and p), and scanning electron (f–h, r, and t) microscopy images showing (a) granular growth in liquid media that develops into (b) a thin biofilm clinging to the side of the tubes and spanning across to the liquid surface. (c) Minute, beige, pinpoint colonies on roll tubes. (d–g) Globose monoflagellated zoospores (d, e) displaying rough surface textures (f, g). (h) Germinating zoospores producing a single germ tube. (i) Polycentric thalli with nuclei observed throughout both the sporangia and hyphae. (j, k) Blunted rhizoids (j) and extensive hyphal branching (k) with both narrow (n) and wide (w) hyphae. (l–n) Swellings forming on nucleated rhizomycelium (arrow in l) from which multiple sporangiophores develop (three in l, two in m, and six in n). (o) Dense concentration of predominantly globose and subglobose sporangia on sporangiophores. (p–r) Cup-shaped sporangiophore (p) with subsporangial swelling (q), long, narrow sporangiophore (white arrow in q), and thick, flattened (f) sporangiophore (r). (s) Less commonly observed elongated irregular sporangia alongside typical globose sporangia. (t) Zoospore release through an apical pore. Bars, 5 µm (f, g), 10 µm (d, e, h, j, k, q, t), and 50 µm (i, l–p, r, s).

in length and displayed rough external texture (Fig. 4f, g). Zoospores were observed to form a germ tube upon germination (Fig. 4h) and developed into polycentric thalli with nucleated rhizomycelia (Fig. 4i). Filamentous hyphae in strain B1.1^T were characterized by the formation of blunted rhizoids (Fig. 4j) and extensive branching (Fig. 4k). Both narrow and wide hyphae were observed (Fig. 4k). Nucleated rhizomycelia formed swellings from which multiple sporangiophores developed (Fig. 4l–n). This pattern resulted in a dense concentration of sporangiophores on examined thalli (Fig. 4o). Sporangiophores were predominantly cup-shaped (Fig. 4p) sometimes with subsporangial swelling (Fig. 4q) with occasional occurrences of long and relatively narrow (length: $115.94 \pm 24.84 \mu\text{m}$, $n=21$) sporangiophores (Fig. 4r, arrow), as well as thick and flattened (Fig. 4r and (F) symbol) sporangiophores. Terminal sporangia developing at the end of these sporangiophores were fairly uniform in shape (globose and subglobose) (Fig. 4o–s) and size ($64.82 \pm 32.16 \times 35.3 \pm 10.04 \mu\text{m}$, $n=21$). Additional less common sporangial shapes were elongated irregular sporangia (Fig. 4s). Zoospore release appeared to occur through an apical pore (Fig. 4t).

DISCUSSION

Members of the *Neocallimastigomycota* have so far been exclusively recovered from mammalian herbivores. Our recent study [8] has identified their occurrence in tortoises and we present here a detailed characterization of their phylogenetic affiliation, microscopic features, and phenotypic preferences. We are not aware of any prior efforts for AGF detection or isolation from the *Testudinidae*. However, efforts to document the presence of AGF in the gastrointestinal tract of other non-mammalian herbivores using culture-independent techniques (microscopic observation and amplicon-based molecular characterization) have previously been reported. For example, microscopic observation of structures similar to AGF zoospores in faecal samples from land (*Conolophus pallidus*) and marine (*Amblyrhynchus cristatus*) iguanas was reported in [24]. Further, the occurrence of an AGF community in the faeces of a captive green iguana (*Iguana iguana*) using ITS1 amplification and sequencing has been reported, with a community putatively identified as the genera *Neocallimastix*, *Piromyces*, *Anaeromyces*, *Khoyollomyces*, *Joblinomyces*, and the uncultured genus AL3 [25].

We acknowledge that all isolates were obtained from only two locations (Oklahoma City Zoo and Hawk Hill Farm, OK, USA) and that all tortoises sampled were not free-living in their natural geographic range. Obtaining samples from tortoises residing in their natural habitats could provide better insights into the wide distribution of these lineages in the family *Testudinidae* and potentially lead to the isolation of additional taxa. However, it should be noted that access to these taxa in their natural habitat is not trivial, as many of these species (e.g. ploughshare tortoise) are critically endangered [26].

Interestingly, we successfully isolated highly similar AGF isolates from faecal samples of multiple different tortoise taxa (Figs 1 and 2). For example, isolates belonging to clade T were recovered from Burmese star, Egyptian, Galápagos, Indian star, pancake, and ploughshare tortoises. These species belong to five different tortoise genera. Such a pattern of ubiquity in the tortoises we sampled is in stark contrast to their extreme rarity (clade B) or universally low relative abundance (clade T) in hundreds of recently examined mammalian faecal samples [23].

Phylogenetic analysis using ribosomal loci (D1/D2 LSU, ITS1) and the protein-coding genes RPB1 supported the placement of the T and B clades as two distinct, basal, deep-branching lineages in the *Neocallimastigomycota*. As recently proposed, a minimum sequence divergence of 3% in D1/D2 LSU sequences from the closest cultured, validly-described taxa is required for novel, genus-level delineation [14]. Values obtained here (10.10% for clade T and 13.02% for clade B to *Khoyollomyces ramosus*) far exceed this threshold. As such, we propose two novel genera to accommodate clades T and B. Further, a recent study proposed four novel families within the *Neocallimastigomycota*, with AAI values as quantitative measures for assigning intermediate ranks in the phylum [20]. The study proposed 85% AAI identity as a cutoff for genus-level delineation and 75% AAI as a cutoff for family-level delineation. Here, significantly lower AAI values (<64%) were observed when comparing clades T and B to all previously described AGF taxa (Table 1). Similarly, low AAI values (<65%) were also observed when comparing clade B to clade T (Table 1). This information putatively justifies accommodating clades T and B as two additional distinct novel families in the order *Neocallimastigales*. However, as proposed earlier, proposition of novel families based on a solitary genus and species is unadvisable [20], and solitary genera should be designated as ‘genera *incertae sedis*’ pending the description of additional genera and species.

In addition to phylogenetic distinction, type strains for clades T and B exhibit unique characteristics that differentiate them from previously described AGF taxa. Of note, the clade T type strain exhibited a broader growth temperature range (20–45 °C) and a lower optimum growth temperature (30 °C). This wider range and lower optimum has not been reported before in the *Neocallimastigomycota*. By comparison, AGF cultures are typically isolated and maintained at 39 °C [3], a temperature that mimics the prevailing conditions in the mammalian gut. However, we are not aware of prior studies that systematically evaluated temperature growth range of AGF strains, although prevailing opinion and empirical evidence (e.g., from accidental incubator malfunction or human error) points to a very narrow temperature growth range for currently described AGF taxa. To this end, we conducted a control experiment using strain AB.5, belonging to the ubiquitous and highly successful genus *Orpinomyces*. Our results confirm the narrow temperature growth range of AGF mammalian-sourced taxa and contrast such a pattern to that observed with the clade T and B type strains.

Finally, in addition to physiological preferences, both type strains exhibit macroscopic and morphological features that readily differentiate them from each other, as well as from other members of the *Neocallimastigomycota*. Both strains demonstrate polycentric thallus development, where nuclei migrate throughout both the sporangia and rhizoids (Figs 3 and 4). Polycentric thallus development in the *Neocallimastigomycota* only occurs in two members of the *Neocallimastigaceae* (*Orpinomyces* and *Paucimyces*), one member of the *Anaeromycetaceae* (*Anaeromyces*), and one member of the *Caecomycetaceae* (*Cyllamyces*) [3, 27]. Given their deep-branching, early evolving position in the *Neocallimastigomycota* tree, polycentric growth could possibly be an ancient trait of the phylum that might have been lost multiple times during their evolution. Within the earlier evolving and closely related *Chytridiomycota*, the same pattern of co-existence of monocentric and polycentric genera occurs (e.g. the polycentric genus *Physocladia* and the monocentric genus *Chytriumyces* in the *Chytridiales* lineage [28]).

In summary, phylogenetic analysis, physiological preferences, and morphological characteristics of the isolated strains when compared to all other currently described AGF genera justify proposing two new genera to accommodate these isolates, for which the names *Testudinimycetes* and *Astrotestudinimycetes* are proposed.

DESCRIPTION OF *TESTUDINIMYCES* CARRIE J. PRATT, EMILY E. CHANDLER, NOHA H. YOUSSEF, AND MOSTAFA S. ELSHAHED GEN. NOV.

MycoBank ID: 847430

Typification: *Testudinimycetes* sp. Carrie J. Pratt, Emily E. Chandler, Noha H. Youssef and Mostafa S. Elshahed.

Etymology: *Testudinimycetes* (Tes.tu.di.ni.my'ces. L. fem. n. *testudo*, a tortoise; Gr. masc. n. *mykes*, a mushroom; N.L. masc. n. *Testudinimycetes*, a fungus from a tortoise).

An anaerobic filamentous fungus with polycentric thallus development. The fungus is characterized by the formation of predominantly thin, nucleated hyphae with minimal branching. Mature sporangia are most often globose, subglobose, and ovoid. Zoospores are monoflagellated. Forms large, dense, granular growth in cellobiose-containing liquid medium and circular, beige colonies on agar roll tubes. The clade is defined by the sequence OQ382931 (for the ITS1, 5.8S rDNA, ITS2, D1-D2 28S rRNA ribosomal locus).

DESCRIPTION OF *TESTUDINIMYCES GRACILIS* CARRIE J. PRATT, EMILY E. CHANDLER, NOHA H. YOUSSEF AND MOSTAFA S. ELSHAHED SP. NOV.

MycoBank ID: 847432

Typification: Isolated in February 2022 from the frozen, then thawed faeces of a ploughshare tortoise housed at the Oklahoma City Zoo (USA, Oklahoma, Oklahoma City, 35.5235417, -97.472475). A metabolically active ex-type strain T130A^T is maintained under anaerobic conditions by weekly subculture at 30 °C. It has been maintained at Oklahoma State University since its isolation. The holotype is stored at Oklahoma State University in 4% glutaraldehyde solution. DNA and RNA extractions from the ex-type strain are stored at -80 °C at Oklahoma State University. GenBank accession number OQ382931 (for ITS1, 5.8S rDNA, ITS2, D1-D2 28S rRNA locus).

Etymology: *Testudinimycetes gracilis* (gra'ci.lis. L. masc. adj. *gracilis*, thin).

An obligate anaerobic fungus with mature sporangia that are most often globose, subglobose, and ovoid, averaging 123.88±38.18×86.17±33.17 µm. The fungus produces small globose monoflagellated zoospores averaging 5.1±1.15×4.49±1.17 µm with flagella averaging 10.54±8.72 µm in length. Produces large, dense, granular growth in cellobiose-containing liquid media and circular, beige colonies (up to 2.5 mm) on agar roll tubes. This species is characterized by a broad temperature growth range that distinguishes it from other anaerobic fungi.

Additional specimens examined: Additional strains belonging to *Testudinimycetes gracilis* were isolated from faecal samples of Burmese star (B01.1, B01.2), Egyptian (E01), Galápagos (G01, G01.1, G01.2, G01.3, G01.4, G01.5), Indian star (N0S0.1, N0S0.3, N0S1.1, N0S1.3, N0S2), pancake (P00, P01), and ploughshare tortoises (T030A, T230A), all of which were collected from: USA, Oklahoma, Oklahoma City, 35.5235417, -97.472475).

DESCRIPTION OF *ASTROTESTUDINIMYCES* CARRIE J. PRATT, EMILY E. CHANDLER, NOHA H. YOUSSEF AND MOSTAFA S. ELSHAHED GEN. NOV.

MycoBank ID: 847431

Typification: *Astrotestudinimycetes* sp. Carrie J. Pratt, Noha H. Youssef and Mostafa S. Elshahed.

Etymology: *Astrotestudinimycetes* (Astro.tes.tu.di.ni.my'ces. Gr. masc. n. *astron*, a celestial body; L. fem. n. *testudo*, a tortoise; Gr. masc. n. *mykes*, a mushroom; N.L. masc. n. *Astrotestudinimycetes*, a fungus from a star tortoise).

An anaerobic fungus with a polycentric thallus developmental pattern. Produces filamentous hyphae characterized by extensive branching and the formation of blunted rhizoids. Formation of sporangiophores occurs predominantly from a central swelling. Produces monoflagellated zoospores. Forms thin biofilm growth in cellobiose-containing liquid media and minute, beige, pinpoint colonies on agar roll tubes. The clade is defined by the sequence OQ382915 (for the ITS1, 5.8S rDNA, ITS2, D1-D2 28S rRNA ribosomal locus).

DESCRIPTION OF *ASTROTESTUDINIMYCETES DIVISUS* CARRIE J. PRATT, EMILY E. CHANDLER, NOHA H. YOUSSEF AND MOSTAFA S. ELSHAHED SP. NOV.

MycoBank ID: 847433

Typification: Isolated in March 2022 from the frozen, then thawed faeces of a Burmese star tortoise housed at the Oklahoma City Zoo (USA, Oklahoma, Oklahoma City, 35.5235417, -97.472475). A metabolically active ex-type strain B1.1^T is maintained under anaerobic conditions by biweekly subculture at 39°C. It has been maintained at Oklahoma State University since its isolation. The holotype is stored at Oklahoma State University in 4% glutaraldehyde solution. DNA and RNA extractions from the ex-type strain are stored at -80°C at Oklahoma State University. GenBank accession number OQ382915 (for ITS1, 5.8S rDNA, ITS2, D1-D2 28S rRNA locus).

Etymology: *Astrotestudinimycetes divisus* (di.vi'sus. L. part. adj. *divisus*, divided).

An anaerobic fungus with polycentric thallus developmental pattern. Produces thick and thin nucleated hyphae characterized by extensive branching and formation of blunted rhizoids. Mature sporangia are most often globose and subglobose, averaging $64.82 \pm 32.16 \times 35.3 \pm 10.04 \mu\text{m}$. Sporangia often found on cup-shaped, thin, elongated ($115.94 \pm 24.84 \mu\text{m}$) or wide, flattened sporangiophores. Produces monoflagellated zoospores averaging $6.89 \pm 2.16 \times 5.71 \pm 1.68 \mu\text{m}$ with flagella averaging $34.45 \pm 6.07 \mu\text{m}$ in length. Granular growth and thin biofilm formation in cellobiose-containing liquid media and minute, beige, pinpoint colonies on agar roll tubes. Optimal growth temperature is similar to isolates obtained from mammalian hosts but can grow over wider temperature range.

Additional specimens examined: Additional isolates belonging to *Astrotestudinimycetes divisus* were isolated in March 2022 from frozen, then thawed faeces of a Burmese star tortoise (strains B1.2 and B.03) collected from the Oklahoma City Zoo (USA, Oklahoma, Oklahoma City, 35.5235417, -97.472475). Additional isolates belonging to *Astrotestudinimycetes divisus* were isolated in August 2022 from frozen then thawed faeces of a sulcata tortoise (strains S09, S19, S29, S1.1, S1.2, S1.3, and S1.4) which were collected from Hawk Hill Farm (USA, Oklahoma, Walters, 34.4786944, -98.2258333).

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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