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Monoblepharidomycetes diversity includes new parasitic and saprotrophic species with highly intronized rDNA

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ABSTRACT

The *Monoblepharidomycetes* is the sister class to the *Chytridiomycetes* in the phylum *Chytridiomycota*. The six known genera have thalli that are either monocentric and without rhizoids or produce hyphae with an independent evolutionary origin from the hyphae of higher fungi. On the basis of morphological characters and phylogenetic evidence from the small and large subunits of nuclear ribosomal RNA, we established two new genera, *Sanchytrium* and *Telasphaerula*, each with a single species. We re-analyzed intergeneric relationships within the monoblephs, and established two new families. The new genera significantly expand the known morphological and ecological diversity of the *Monoblepharidomycetes* by adding a monocentric, epibiotic, algal parasitic species and a rhizomycelial, saprotrophic species. Based on the presence of environmental sequences related to *Sanchytrium* strains, the *Monoblepharidomycetes* contain previously unsuspected diversity. The ribosomal DNA of the new genera contains an unusually high density of group I introns. We found 20 intron insertion positions including six that are new for rRNA genes (S1053, L803, L829, L961, L1844, and L2281).

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Introduction

The *Monoblepharidales* is the single order in the *Monoblepharidomycetes* (monoblephs), a class generally accepted as sister to the *Chytridiomycetes* (chytrids) in the Phylum *Chytridiomycota* (Dee *et al.* 2015; Hibbett *et al.* 2007). Some authors recognize the monoblephs as a separate phylum *Monoblepharidomycota* containing the *Monoblepharidomycetes* and the *Hyaloraphidiomycetes* (Doweld 2001; Powell & Letcher 2014), but herein we consider them at the class level. The known monoblephs are saprotrophs and have either flagellated zoospores or, in the case of *Hyaloraphidium curvatum* Korshikov, autospores as a dispersal stage (Powell & Letcher 2014). In Sparrow's (1960) monograph of the 'Phycomycetes', only three members, all mycelial, were classified in the *Monoblepharidales*: *Monoblepharis* Cornu, *Gonapodya* Fischer, and *Monoblepharella* Sparrow. These genera lack a typical Spitzenkörper, and their hyphae seem to have an independent evolutionary origin from hyphae in the Dikarya (Dee *et al.* 2015). Subsequently, *Harpochytrium* Lagerheim and the rod-shaped *Oedogoniomyces* Kobayashi & Okubo were found to be in the class. This was based for *Harpochytrium* (many species of which had formerly been considered to be algae) on mitochondrial DNA (Paquin *et al.* 1997) and for *Oedogoniomyces* on the ultrastructure of its zoospore (Reichle 1972; Barr 1990). Another species, *Hyaloraphidium curvatum*, with its crescent-shaped, planktonic thalli that reproduce with non-flagellated spores, was also considered to be an alga; but, on the basis of DNA sequence information, it is now considered a member of the *Monoblepharidomycetes* (Ustinova *et al.* 2000). In all, the class contains six genera and about 30 species. Some authors have proposed, or accepted, additional classes and orders in the *Monoblepharidomycetes* (Doweld 2001; Powell & Letcher 2014), but until descriptions of many more genera and species make additional higher level taxonomy necessary, we prefer to retain the diverse clades in the class as families within the single order *Monoblepharidales*.

In a recent phylogenetic study based on concatenated small subunit (SSU) and large subunit (LSU) nuclear ribosomal RNA sequences, each of the three mycelial genera is monophyletic and they all group together; *Hyaloraphidium* is basal to all other members of the monobleph clade and *Oedogoniomyces* is sister to a *Harpochytrium* isolate within a poorly supported *Harpochytrium* lineage (Dee *et al.* 2015). Although the *Monoblepharidomycetes* lineage appears to be as ancient as the chytrid lineage, it contains considerably less described genetic and morphological diversity compared with that of the *Chytridiomycetes*. Our purpose is to describe two new genera and species recovered during surveys of zoosporic fungi. These species fall into two new families and expand the genetic, morphological, and ecological diversity of the *Monoblepharidomycetes* by adding a monocentric, epibiotic parasite of the xanthophyte alga *Tribonema gayanum* and a rhizomycelial species with unknown, saprotrophic substrate preference. The rDNA of the new taxa has numerous Group I introns, which we discuss and compare with those in the *Chytridiomycetes*.

Materials and methods

Collection and isolation

We isolated JEL762 on Cd agar (peptonized milk, 0.2 g L^{-1}; tryptone 0.4 g L^{-1}; cellobiose, 2 g L^{-1}; soluble starch, 2 g L^{-1}; agar, 10 g L^{-1} in distilled water with 200 mg L^{-1} penicillin and 200–500 mg L^{-1} streptomycin sulphate added after autoclaving). We grew the isolate on Cd agar without antibiotics and maintained it in PmTG liquid medium (peptonized milk, 1 g L^{-1}; tryptone, 1 g L^{-1}; glucose, 5 g L^{-1}; Barr 1986) at 4 C after growth at room temperature for ~ 1 m and transferred it approximately every 100 d. We cryopreserved it in PmTG (80 %), FBS (10 %) and DMSO (10 %) according to Boyle *et al.* (2003) with long-term storage in liquid nitrogen. For photography (Nikon Eclipse E400 and Spot RT3 digital camera), we grew the fungus in PmTG broth and transferred bits of rhizomycelium to distilled water to initiate production of zoospores. Structures were measured with the Spot software program.

All three parasitic strains X-126, X-127, and X-128 were isolated by M. Mamkaeva in 2014. Strain X-126 was isolated from a roadside ditch in the vicinity of Kotka, Finland; strain X-127 from a roadside ditch of the village Zaborka of Novgorod region, Russia and strain X-128 from a pond in the village Zaytsevo of Novgorod region, Russia. All strains were transferred and maintained in culture on *Tribonema gayanum* Pasch. (strain 20 of Collection of Algae of Leningrad University (CALU)) as the host. The host was grown in mineral medium (KNO_3, 2 g L^{-1}; KH_2PO_4, 0.3 g L^{-1}; $MgSO_4$, 0.15 g L^{-1}; EDTA, 10 mg L^{-1}; $FeSO_4$, 5 mg L^{-1}; $NaBO_3$, 1.4 mg L^{-1}; $(NH_4)6Mo_7O_2$, 4.1 mg L^{-1}; $CaCl_2$, 0.6 mg L^{-1}; $ZnSO_4$, 0.1 mg L^{-1}; $CuSO_4$, 50 mg L^{-1}, $Co(NO_3)_2$, 20 mg L^{-1}) at room temperature in the presence of white light. After inoculation with a strain of parasite, the coculture was incubated for 1–2 weeks to reach the maximum infection of host cells. Cells were then harvested by centrifugation and used directly for DNA extraction. Light and DIC microscopy observations of living cultures were carried out on a Zeiss Axioplan microscope equipped with a Mrm Axiocam colour camera. We used the same culture methods to test the ability of the parasitic monoblephs to infect strains of the green algae *Cosmarium* sp. (VN 90-2, VN 92, VN81, VN 570, and Mn 101); *Closterium* (Ur 172, Mn DZ-2015 18K, 139, UR 84, and UR 100); and *Chlorococcum oleofaciens* (MZ-Ch-4); all provided by the Algae Culture Collection BOROK (WDCM 602).

FISH probe design

To confirm that the thalli seen by light microscopy were *Monoblepharidomycetes*, we used a standard *in situ* hybridization procedure (Pernthaler *et al.* 2001) with 35 % formamide in the hybridization buffer. Two 3′-end labelled by 6-fluorescein oligonucleotides F1_V9 AACCATTGCGTCGATCCGAAAA and R.m.1.8_V7 CGAGCCGCCTACCAAAG were designed to variable V9 and V7 regions of SSU rRNA and used as tracers for hybridization. For positive and negative controls we used Eub338 and Euk516 probes and cultures of *Paramecium caudatum* (data

not shown). We identified FISH results on a Zeiss Axio Imager 1 Research Microscope and recorded images with Axio Cam MRc5 camera.

DNA amplification sequencing and analysis

We amplified the JEL762 SSU rRNA gene in two fragments with primer combinations SR1.5 and NS4 and BMB-BR and SR6.1 (James *et al.* 2000; Parrent & Vilgalys 2009). PCR of the LSU rRNA region was also amplified in two fragments with primer pairs LR0R and LR5 and LR3R and LR9 (Rehner & Samuels 1994; Hopple & Vilgalys 1999). PCR was conducted with GoTaq Green (Promega) using standard PCR cycle settings and 50° C annealing temperature. Following clean-up with ExoSAP-IT (Promega), amplicons were sequenced at the U. Michigan Sequencing Core.

SSU and LSU rDNAs of X-126, X-127, and X-128 were amplified as overlapping fragments using Encyclo PCR kit (Evrogen, Moscow) and a set of primers (Table S1) (van der Auwera *et al.* 1994; Medlin *et al.* 1988). Specific forward and reverse primers were designed to 3′-end region of the SSU rRNA gene to avoid amplification of the host DNA. They included motifs specific for opisthokonts (Aleshin *et al.* 2007). Fungal SSU rRNA amplified from the specific reverse primer and fungal ITS-containing DNA fragment amplified from the forward specific primer overlap each other on the hypervariable V9 region, which provides sufficient evidence of their conspecificity, if any. Several specific primers were also constructed (Table S1). We used PCR cycles with varying annealing temperatures and elongation times. PCR products were separated with agarose gel electrophoresis and purified using Cleanup Mini kit (Evrogen, Moscow). Amplicons were sequenced directly with an Applied Biosystems 3730 DNA Analyzer.

Intron localization was defined by comparing assembled contigs with other species from NCBI *nr* database using BLAST (Altschul *et al.* 1990). Insertion positions were identified using the 16S and 23S gene reference of *Escherichia coli* (Cannone *et al.* 2002; http://www.rna.icmb.utexas.edu/SAE/2C/rRNA_Introns). Intron type and homology was determined using GISSD database (Zhou *et al.* 2008; http://www.rna.whu.edu.cn/gissd/). Secondary structures of introns were constructed manually with help from Mfold (Zuker 2003) and drawn using RnaViz (De Rijk & De Wachter 1997). We searched for introns among *Fungi* and *Viridiplantae* (without *Embryophyta*, which are poor in group I introns and are not the usual hosts for parasitic *Chytridiomycetes*) with NCBI *nr* database using BLAST for sequences more than 600 bp in length. Insertion positions were identified by alignment with novel sequences with MUSCLE (Edgar 2004).

Phylogenetic analysis

Additional LSU and SSU sequences were downloaded from GenBank. Sequences were aligned with MUSCLE and manually adjusted in BioEdit (Hall 1999). RAxML v.7.2.8 was used to construct a maximum likelihood (ML) tree with general time reversible (GTR) + F + G + I model of site substitution including estimation of Gamma-distributed rate heterogeneity with four categories and a proportion of invariant sites (Stamatakis 2006). Branch support was evaluated by bootstrapping using 100 replicates (Hillis & Bull 1993). Phylogenetic trees were visualized with MEGA 6.0 (Tamura *et al.* 2013). Bayesian inference was calculated with MrBayes 3.1.2 with a GTR model of DNA substitution and a Gamma distribution of rate variation across sites with eight categories (Ronquist & Huelsenbeck 2003). Four Markov chains were run for two runs from random starting trees for five million generations and trees were sampled every 1000 generations. The first one-half generations were discarded as burn-in. Alternative topologies were tested with the approximately unbiased (AU) test and Kishino-Hasegawa test (KH) (Kishino & Hasegawa 1989) implemented by the CONSEL (Shimodaira 2002) program, and Expected Likelihood Weight (ELW) (Strimmer & Rambaut 2002) implemented by the TREE-PUZZLE (Schmidt *et al.* 2002). Alternative topologies were constructed using MEGA 5.2.2, and corresponding site-wise log likelihood values for them were computed with TREE-PUZZLE under the GTR model. Substitution rate parameters of the GTR model were taken from the results of previous Bayesian inference calculated with MrBayes program.

Results

Morphology

Unlike monoblephs with approximately isodiametric hyphae, the thallus of JEL762 consists of swellings connected by narrow filaments (Fig 1A–C) and only asexual reproduction has been seen (Fig 1D–L). Zoosporangia are terminal, ephemeral and, unique for the class, seem to release a single zoospore (Fig 1J). The monocentric, epibiotic morphology of the algal parasites X-126, X-127, and X-128 (Fig 2A–H) is more like that of monocentric members of the *Chytridiomycetes* than like that of other members of the *Monoblepharidomycetes*. Like some chytrids, zoospores exit through discharge papillae and the rhizoidal system is reduced (Fig 2C and D). Discharge papillae can be apical or lateral (Fig 2A and D), which may suggest the presence of more than one papilla per sporangium. Papillae are not visible on young sporangia and seem to develop in mature ones shortly before zoospore discharge. Shape of sporangia varies from spherical to ovate or irregular (Fig 2A, C–H), but the diameter is always ~10 μm. *In situ* hybridization (FISH) unambiguously showed that all stages of development (cysts, young and mature sporangia of each strain; shown here for X-126; Fig 2F) belong to the sequenced strains.

Molecular phylogeny

The Bayesian tree of combined SSU and LSU rDNA sequences (Fig 3) differs from the SSU tree (Fig S1) in the position of isolate JEL762 and some details inside the main *Monoblepharidomycetes* groups. To resolve these mismatches we tested alternative topologies with the Kishino-Hasegawa tests, and confidence value (ELW). Also we tested some possible alternative positions of JEL762 and the group including X-126, X-127, and X-128 strains. In the overwhelming majority cases all three tests gave the same results in every topology. Note that the combined and separate LSU trees have almost the same topologies.

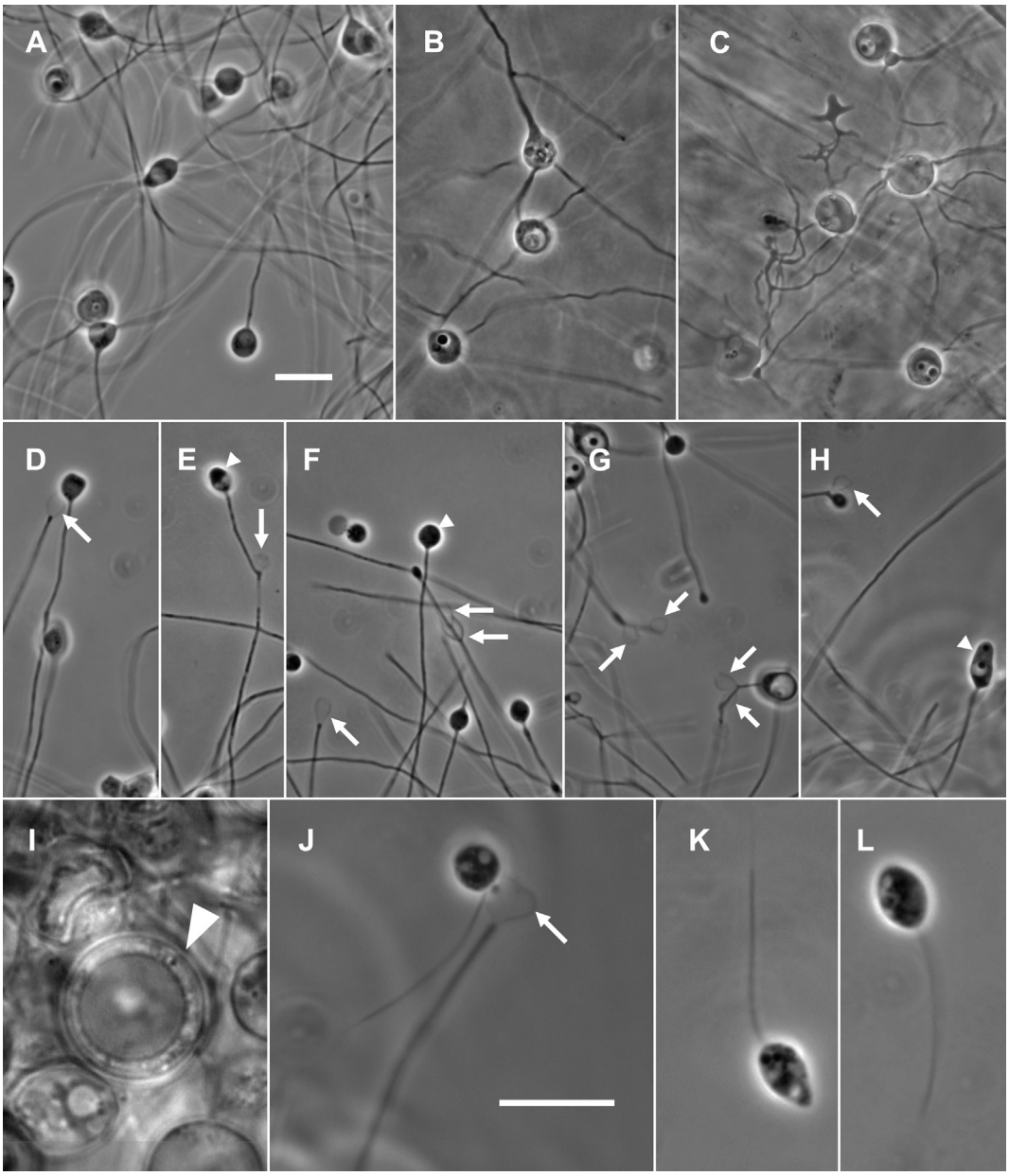


Fig 1 – Morphology of *Telasphaerula gracilis*. (A–C). Polycentric thallus consisting of swellings connected by fine isodiametric tubes. (A). growth on mPmTG agar. (B). Growth in Cd broth. (C). Irregular intercalary rhizoidal system growth on onion-skin placed in PmTG broth lacking glucose. (D–H). Examples of thin-walled zoosporangia large enough to produce a single zoospore; arrows indicate empty zoosporangia. Terminal swellings (arrow heads) seem to be able to either continue polycentric growth or develop into zoosporangia. (I). Older, dense growth in Cd broth; arrowhead indicates a rare, thick-walled, probable resting spore. (J). Highly magnified zoospore recently emerged from adjacent zoosporangium (arrow). (K and L). Motile zoospores with typical shape for *Monoblepharidales*. Scale bar in A = 10 μm for A–H. Scale bar in J = 10 μm for I–L.

The combined SSU – LSU tree placed JEL762 strain as sister to the clade that contains the two well-known, sexually reproducing hyphal genera, *Monoblepharis* and *Monoblepharella* with posterior probability of 1.00 (Fig 3). The SSU tree placed JEL762 more basally, as the sister to the clade uniting all mycelial genera, *Monoblepharis*, *Monoblepharella*, and *Gonapodya*. The results of the hypotheses testing using SSU data (Fig S1) do not contradict the position of JEL762 in the combined tree. Therefore, the main group of the *Monoblepharidomycetes* includes hyphal and rhizomycelial species.

Both the combined tree and the SSU tree grouped X-126, X-127, and X-128 strains with a set of uncultured environmental clones on a long branch sister to the main group of the *Monoblepharidomycetes*. Two alternative placements (sister to the *Hyaloraphidium* clade as in the LSU tree or to the monoblephs as a whole) were not rejected (Fig 3 and Fig S1). The Bayesian

tree of combined data unites all five *Gonapodya* isolates into a monophyletic clade while in the SSU tree they form two independent branches. In the combined tree the *Harpochytrium* clade includes two *Oedogoniomyces* isolates. The SSU tree placed *Oedogoniomyces* outside the *Harpochytrium* clade, but divided the *Harpochytrium* clade into two smaller clades. In both the *Gonapodya* and *Harpochytrium/Oedogoniomyces* clades, the combined LSU and SSU topologies are not significantly worse than the SSU Bayesian topology, whereas the SSU topologies are rejected by the tests of SSU – LSU combined data (Fig 3 and Fig S1).

Screening of the GenBank database revealed several partial SSU and LSU rDNA sequences of unidentified monobleph clones (Fig 3, Figs S1 and S2). Some of them belong to the genera *Monoblepharella*, *Harpochytrium*, and *Hyaloraphidium*. Others clustered with the X-126, X-127, and X-128 clade (Fig S3). Interestingly, these fragments of environmental rDNA lack introns, whereas the corresponding fragments of studied strains are intronized. The monoblephs detected from environmental DNA are geographically widespread and found from different substrates, usually soil (Fig S3). Other environmental DNA sequences (GenBank accession numbers DQ244008, LC165109, FJ354068, and EF024210) grouped together as a sister clade to all monoblephs with posterior probability of 1.00 (Fig 3).

Introns in rRNA genes

We amplified SSU and LSU rDNAs of X-126, X-127, X-128, and JEL762 as sets of overlapping fragments and when assembled, the contigs were longer than in the majority of rRNA genes in other fungi — they include up to six (SSU) or nine (LSU) group I introns that occupy up to 50 % of amplified lengths (Tables 1 and 2). Each sequenced strain has only one SSU intron pattern; but in the LSU rRNA, X-127 has three intron patterns (L, S1, and S2), which differ by intron number and localization. In total, SSU rDNA (Fig 4) had seven insertion positions (S323, S516, S896, S943, S1053, S1199, and S1389) and LSU (Fig 5) had 13 (L803, L829, L961, L1066, L1844, L1921, L1951, L2066, L2281, L2449, L2499, L2563, and L2584). Position numbers are according to *Escherichia coli* reference (Cannone *et al.* 2002) and introns are classified into IC1, IC2, IE2, and IE3 subgroups according to the GISSD database (Zhou *et al.* 2008) (Figs 4 and 5). Homing endonuclease genes are absent in all described introns.

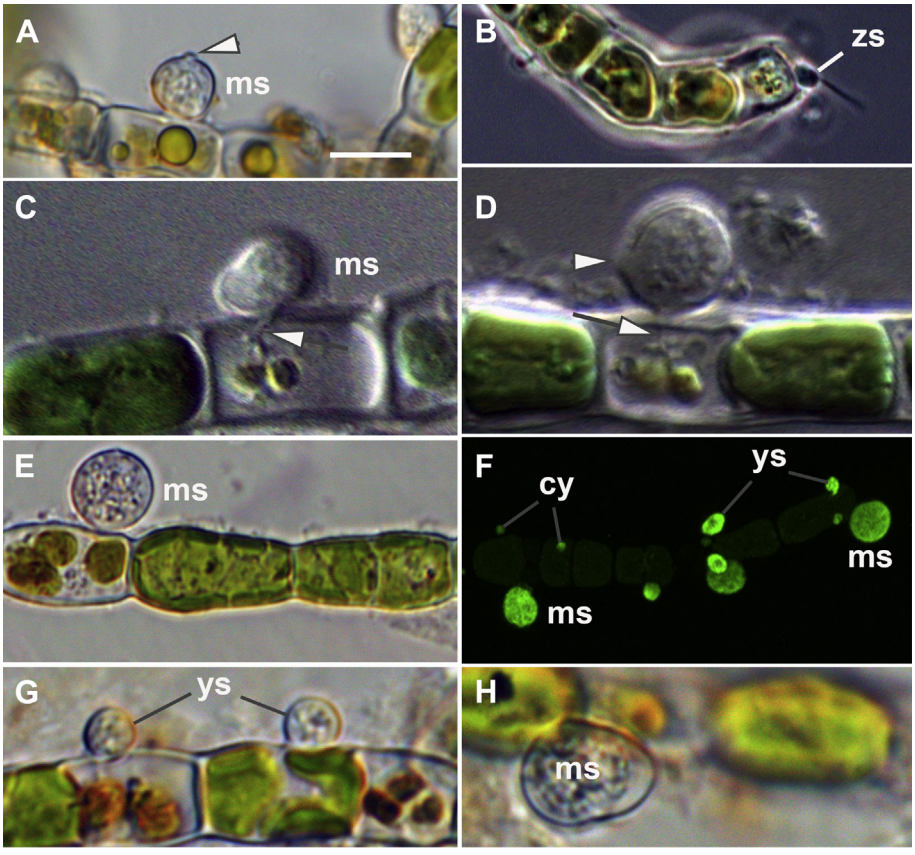


Fig 2 – Morphology of *Sanchytrium tribonematis*. (A–D). Strain X-128. (A). Mature sporangium (ms) with papilla (arrowhead). (B). Zoospore (zs) attached to the algal filament. (C, D). Sporangia with rhizoids in the infected cells. Arrows indicate rhizoids; arrowhead indicates papilla. (E, F). Strain X-126. (E). Mature sporangium. (F). Confocal image of cysts (cy), young (ys), and mature sporangium (ms) in *situ* hybridization with specific oligonucleotide (green). (G, H). Strain X-127. Sporangia of different shapes on the algal filament; Scale bar in A = 10 μm for A, B, E; =7 μm for C, D, H; =16 μm for F; =8 μm for G. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1 – Type, position, and length of SSU introns in *Telaspherula gracilis* and *Sanchytrium tribonematis* isolates.

Intron type	Insertion position relative to *E. coli*	Length, bp			
		X-126	X-128	X-127	JEL762
IC1	323	340	340	–	–
IE3	516	364	363	364	–
IC1	896	280	–	280	–
IC1	943	–	–	316	406 with 3′
IC1	1053	–	354	354	–
IC1/IE3	1199	285	–	285	447
IC1	1389	–	–	414	–

Six of the intron insertion positions are new—one in SSU rDNA (S1053) and five in LSU rDNA (L803, L829, L961, L1844, and L2281) in X-126, X-127, and X-128 strains. All of these introns have lengths and structure typical for group I introns and belong to IC1 (S1053, L829, L1844, and L2281) or IE2 (L803 and L961) subgroup. Intron insertion position S323 (Fig 4) was not previously found in Fungi, but occurs in X-126 and X-128 strains, and in many green algae species. Nucleotide sequences of introns occupying the same position in X-126, X-127, and X-128 strains are almost identical. For example, S516 introns differ between these strains by only four mismatches and indels in a total of 363 bp (Fig S4B). In contrast, JEL762 introns are dissimilar in their nucleotide sequences to homologous introns in X-126 and X-127 strains.

ITS2 structures

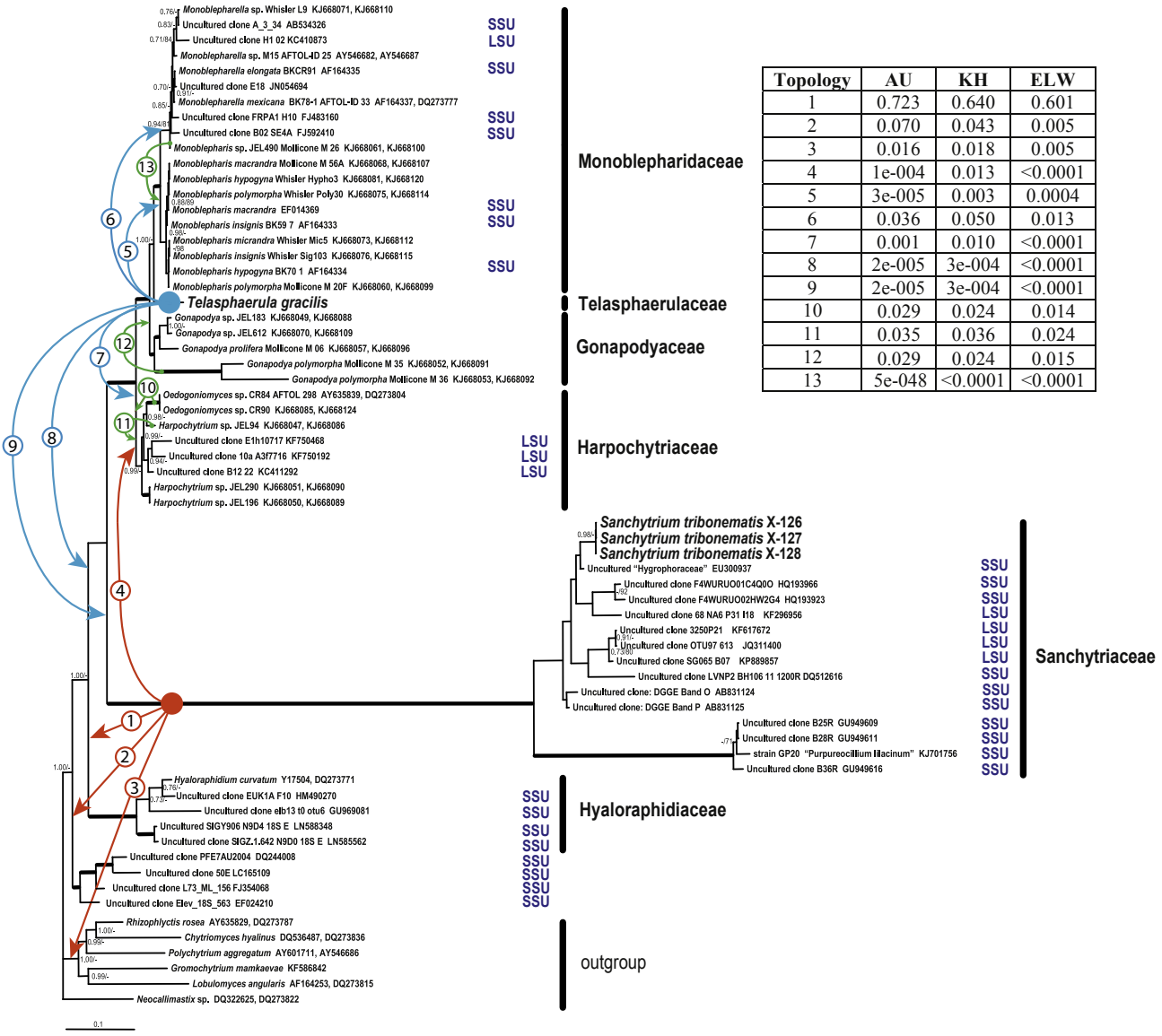
Predicted ITS2 structures of X-128 and X-126 strains have some typical features (Coleman 2007) such as: four easily recognizable helixes, a short unbranching helix II, and a longer helix III with highly conserved nucleotide sequence on the 5′ side (Fig S4A). Also some spacer features are typical for fungi, e.g., the ITS2 length and conserved nucleotide sequence on the 5′ side of helix III (Coleman 2007). However, they lack the typical pyrimidine–pyrimidine mismatch in helix II. Strains X-128 and X-126 differ by two point substitutions in ITS2. In both cases one strain is ambivalent, i.e. contains both versions of nucleotides in the site among its multiple genome copies. There are no compensatory base changes (CBCs) between ITS2 of X-128 and X-126 strains.

Table 2 – Type, position, and length of LSU introns in *Sanchytrium tribonematis* isolates.

Intron type	Insertion position relative to *E. coli*	Length, bp				
		X-126	X-128	X-127		
				S1	S2	L
IE2	803	–	–	358	–	358
IC1	829	294	293	294	294	294
IE2	961	–	355	–	355	–
IC1	1066	291	–	291	–	291
IC1	1844	–	–	–	–	372
IC1	1921	345	–	–	–	345
IC1	1951	335	335	335	335	–
IC1	2066	311	311	–	–	311
IC1	2281	–	–	–	–	293
IE2	2449	423	423	423	423	–
IC1	2499	377	–	–	–	–
IE2	2563	–	335	–	–	335
IC1	2584	–	–	401	401	401

Taxonomy

Class *Monoblepharidomycetes* Schaffn. 1909 emend.
Type: *Monoblepharis* Cornu 1871.
Thallus hyphal, rhizomycelial, or monocentric. Monocentric forms may be planktonic, epibiotic with basal holdfasts, or epibiotic with branched rhizoids inside of algal host. Asexual reproduction by zoospores or autospores; sexual reproduction, when present, oogamous by means of posteriorly uniflagellate antherozoids borne in antheridia and nonflagellate female gametes borne in oogonia. Sister clade to *Chytridiomycetes*.
Order *Monoblepharidales* J. Schröt. 1893 emend.
Diagnosis as for the class *Monoblepharidomycetes*.
Telasphaerulaceae Longcore et T.Y. James **fam. nov**.
MycoBank MB 818658.
Delicate rhizomycelial thallus consisting of isodiametric filaments with intercalary components, which become spherical in older material. Zoosporangia reduced, often producing a single zoospore; sexual reproduction unknown. In a clade with *Monoblepharidaceae* and *Gonapodyaceae*.
Type: *Telasphaerula* Longcore et T.Y. James.
Telasphaerula Longcore et T.Y. James **gen. nov**.
MycoBank MB 818659.
Etymology: *Tela* (Latin) refers to the web-like rhizomycelium, *sphaerula* (Latin) for small, spherical swellings.
Type species: *Telasphaerula gracilis* Longcore et T.Y. James sp. nov.
Growth indeterminate, consisting of swellings connected by isodiametric tubes. Swellings may be irregular but most become spherical in older growth. Zoospores ovate, slightly pointed at apex, released from terminal zoosporangia with delicate walls. Sexual reproduction not observed.
Telasphaerula gracilis Longcore et T.Y. James, **sp. nov**.
MycoBank MB 818660 Fig 1A–L.
Etymology: *Gracilis* (Latin) = slender, which refers to the intercalary growth of the rhizomycelium.
Growth indeterminate, consisting of swellings connected by fine isodiametric tubes (0.7–1.2 μm diam). Swellings become spherical in older growth (up to 13 μm, usually 9–10 μm diam). Zoospores ovate (~4.4 × 3.6 μm), released singly from terminal zoosporangia with delicate walls. Flagellum length 18–19 μm. 18S rDNA GenBank No. = KY130459; 28S rDNA GenBank No. = KY130460.
Type: UNITED STATES. MAINE; Pushaw Lake, Old Town (44°56′49.6″ N; 68°48′5.4″ W). MAINE-F-00007952 (the University of Maine Herbarium). Sample collected 31 August 2012; fungus found on isolation plate containing *Chara* from lake. Isotypes MAINE-F-00007953, MAINE-F-00007954, and MAINE-F-00007955 prepared from same pure culture as type. Ex type culture in JEL collection and CBS-KNAW.
Habitat: Associated with *Chara* from mesotrophic lake.



Topology	AU	KH	ELW
1	0.723	0.640	0.601
2	0.070	0.043	0.005
3	0.016	0.018	0.005
4	1e-004	0.013	<0.0001
5	3e-005	0.003	0.0004
6	0.036	0.050	0.013
7	0.001	0.010	<0.0001
8	2e-005	3e-004	<0.0001
9	2e-005	3e-004	<0.0001
10	0.029	0.024	0.014
11	0.035	0.036	0.024
12	0.029	0.024	0.015
13	5e-048	<0.0001	<0.0001

Fig 3 – Bayesian tree of *Monoblepharidomycetes* phylogeny based on SSU and LSU rDNA. Branches are labelled with posterior probability (the first number) and bootstrap support (the second number). Branches without labels have both parameters lower than 0.7/70, branches with '-' indicate parameters lower than 0.7 or 70; branches with both parameters higher than 0.95/95 are marked bold. Species without both SSU and LSU data are marked in accordance with available data. P-values of the approximately unbiased (AU) test, Kishino-Hasegawa test (KH), and confidence value (expected likelihood weight, ELW) of different alternative topologies are shown in the insert table. Alternative topology changes are shown by arrows with numbers. Changes in *Sanchytrium* clade position are marked orange, changes of *Telasphaerula gracilis* position are marked blue, changes of other *Monoblepharidomycetes* positions are marked green. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Telasphaerula gracilis was not obvious in the raw collection of algae and detritus but zoospores appeared on an isolation plate (Cd nutrient agar) and developed into a delicate rhizomycelium. The isolation plate, which was set up to isolate members of the *Cladochytriales*, contained pieces of *Chara*.

Sanchytriaceae Karpov et Aleoshin **fam. nov**.

MycoBank MB 818908

Thallus monocentric, epibiotic, penetrates host wall with rhizoid in parasitic species. Sporangia as in *Rhizophydiales* (*Chytridiomycetes*). Sexual reproduction not observed.

Type: *Sanchytrium* Karpov et Aleoshin gen. nov.

Sanchytrium Karpov et Aleoshin **gen. nov**.

MycoBank MB 818909

Etymology: *San* (Greek) – as, like, *chytrium* – chytridiomycete. In general, like a chytridiomycete.

Type species: *Sanchytrium tribonematis* Karpov et Aleoshin **sp. nov**.

Parasite of algae. Sporangia epibiotic, spherical to ovate with one or more discharge papillae. Zoospores attach to algal cell wall, encyst, and penetrate host wall with a short, branched rhizoid.

Sanchytrium tribonematis Karpov et Aleoshin **sp. nov**.

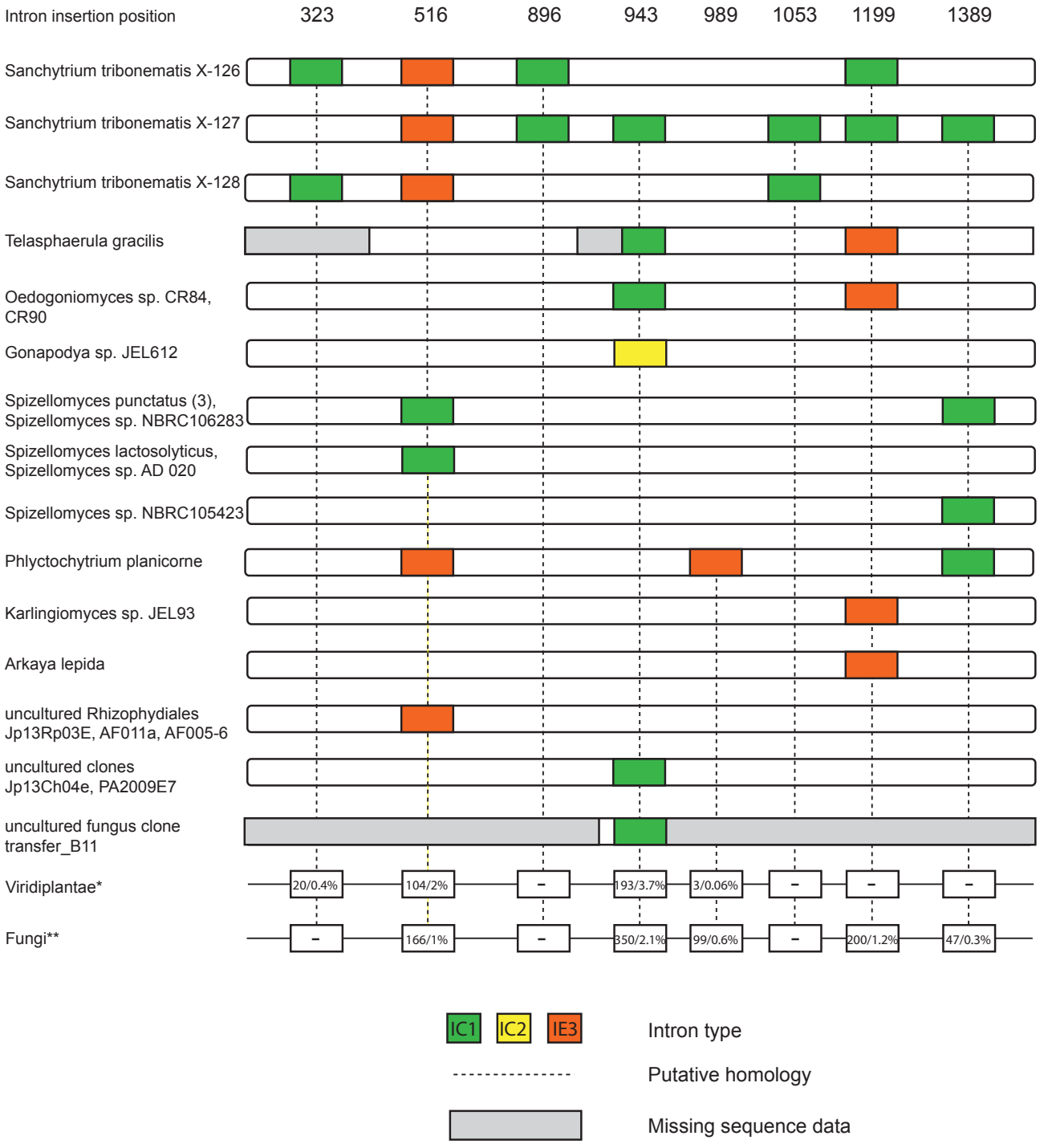


Fig 4 – Map of intron location in SSU gene within *Chytridiomycota*. First number in the square is total number of species with this intron, the second number is the ratio of first number, and total number of species within this group. Insertion positions specified in accordance with the 16S gene reference of *Escherichia coli* (Cannone *et al.* 2002). * without *Embryophyta* ** without *Chytridiomycetes*.

MycoBank MB 818910 Fig 2A–D.

Etymology: after *Tribonema*, the genus name of the first observed host.

Sporangium round to ovate, smooth, ~10 μm diam with 1–3 discharge papillae; sessile on algal surface. Rhizoid almost invisible inside host. Zoospores 3 × 2.5 μm with posterior flagellum 5 μm in length. GenBank Accession Numbers KY652373–KY652378.

Type: Fig 2A–D Karpov *et al.* this publication. RUSSIA, Novgorod region, pond in the village Zaytsevo (58°22′06″ N; 32°04′00″ W). Sample collected by Maria Mamkaeva in April 2014. Ex type culture deposited in Culture Collection of

Parasitic Protists of Zoological Institute of Russian Academy of Science (CCPP ZIN RAS) (Malysheva *et al.* 2016) under No: X-128.

Other collections: No: X-126 (Fig 2E and F) FINLAND, vicinity of Kotka (60°28′00″ N; 26°55′00″ W). Sample collected by Maria Mamkaeva in 2014. Culture deposited in ZIN collection (CCPP ZIN RAS) as X-126. No. X-127 (Fig 2G and H) RUSSIA, Novgorod region, roadside ditch in the village Zaborka (58°22′06″ N; 32°04′00″ W). Sample collected by Maria Mamkaeva in 2014. Culture deposited in ZIN collection (CCPP ZIN RAS) as No: X-127.

Comments: Strains X-126 and X-127 are morphologically similar to *Sanchytrium tribonematis*. All three strains infect and reproduce on the alga *Tribonema gayanum* (Xanthophyceae); however, we did not see branched rhizoids of X-126 and X-127 inside the infected alga (Fig 2E and G). The sporangium adheres to the infected algal cells, which are totally destroyed after sporangium maturation. None of the green algae, *Cosmarium* (five strains), *Closterium* (five strains) or *Chlorococcum oleofaciens*, became infected when inoculated with *S. tribonematis*.

Discussion

Higher level taxonomy

The addition of two new families to the class is cause for reevaluation of upper level taxonomy of the Monoblepharidomycetes. We accept six families (Table 3) in the *Monoblepharidomycetes* and, until description of additional genera and species make this arrangement unwieldy, we maintain these families in a single order, the *Monoblepharidales*. Each of these families is supported by morphological differences and by our analysis based on SSU plus LSU rDNA sequences. Because *Harpochytrium* and *Oedogoniomyces* are in the same poorly resolved clade, we reject the family *Oedogoniomycetaceae* (Barr 1990) and retain both genera in the *Harpochytriaceae* (Wille) Emerson & Whisler (1968).

Telasphaerula gracilis

The position of *Telasphaerula gracilis* as sister to mycelial species raises the questions of as to what is the difference between a rhizomycelium and mycelium. Neither of these terms indicates a phylogenetic relationship. As shown by Dee *et al.* (2015), fungal hyphae, which make up a mycelium and are defined as 'tubular filaments' (Alexopoulos *et al.* 1996), have arisen independently at least twice within the Fungi and the same is probably true of the rhizomycelial growth form. By referring to the thallus of *T. gracilis* as a rhizomycelium, we mean that the branching, filamentous growth is indeterminate but not evenly tubular. Instead the thallus consists of swellings that are connected by narrower filaments. The indeterminate, polycentric growth of representatives of various chytridiomycete orders is also described as rhizomycelial. The difference among polycentric, rhizomycelial thalli lies in the morphology of the swollen areas and in the diameter and uniformity of the intercalary filaments. The rhizomycelial habit is present in genera in five orders of the *Chytridiomycota*: *Cladochytrium* and *Nowakowskiella* in the *Cladochytriales* (Mozley-Standridge *et al.* 2009), *Physocladia obscura* in the *Chytridiales* (James *et al.* 2006), *Polychytrium aggregatum*, and *Lacustromyces hiemalis* in the *Polychytriales* (Longcore & Simmons 2012) and *Catenomyces persicinus* in the *Rhizophlyctidales* (James *et al.* 2006). The finding of *Telasphaerula* with rhizomycelial growth adds the *Monoblepharidales* to this list. The rhizomycelium of *T. gracilis* is recognizable because the connecting filaments between swellings are uniformly narrow (~1 μm) and the swollen areas become spherical. In isolates of *Cladochytrium*, the genus with the most similar non-reproductive morphology, the intercalary swellings become elongate and septate. The connections between swellings in *Nowakowskiella* and the other chytridiomycete genera can be quite broad and uneven in diameter (e.g., Longcore 1993; Mozely-Standridge *et al.* 2009). Although *Cladochytrium* species occasionally are recovered from pollen bait, species with indeterminate, rhizomycelial morphology most frequently are found in or on larger substrates such as moribund plant

Table 3 – Families of the *Monoblepharidales* based on analysis of combined 18S and 28S rDNA, morphological characters, and included genera.

Families	MycoBank#	Morphology	Genera
Monoblepharidaceae A. Fisch. 1892	MB#81021	Hyphal; oogamous sexual reproduction common.	*Monoblepharis*, *Monoblepharella*
Harpochytriaceae Wille 1900	MB#82102	Rod-shaped with basal holdfast; sexual reproduction not reported.	*Harpochytrium*, *Oedogoniomyces*
Gonapodyaceae H.E. Petersen 1909	MB#82121	Hyphae with pseudosepta and often with swellings in hyphae; oogamous sexual reproduction common.	*Gonapodya*
Hyaloraphidiaceae Doweld 2001	MB#585033	Planktonic with non-flagellate spores; sexual reproduction not reported.	*Hyaloraphidium*
Telasphaerulaceae Longcore and T.Y. James 2017	MB#818658	Rhizomycelial; zoospores produced in reduced sporangia; sexual reproduction not seen.	*Telasphaerula*
Sanchytriaceae Karpov & Aleoshin 2017	MB#818908	Monocentric, epibiotic sporangium with endobiotic, reduced rhizoids inside algal host; sexual reproduction not seen.	*Sanchytrium*

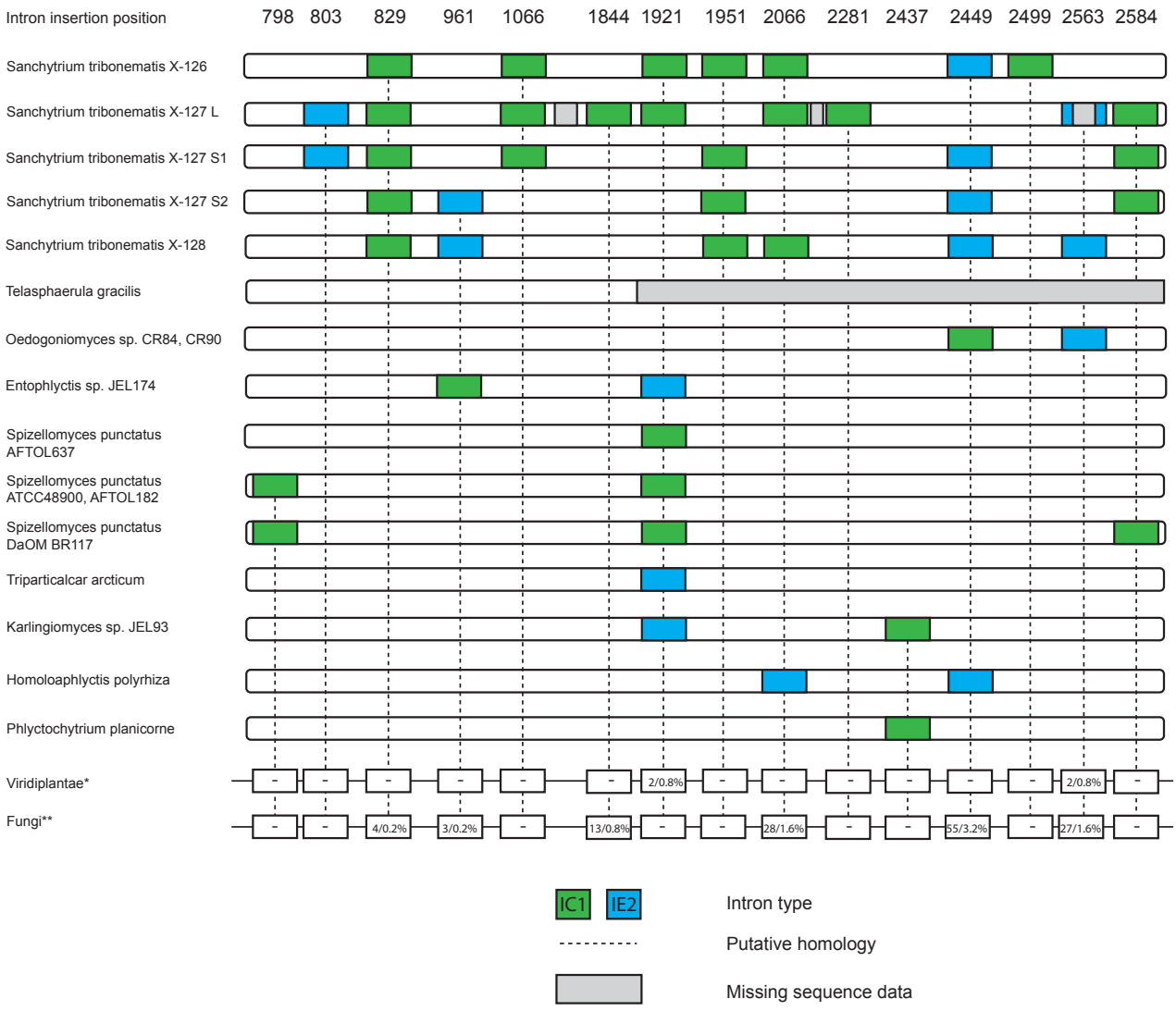


Fig 5 – Map of intron location in LSU gene within *Chytridiomycota*. First number in the square is total number of species with this intron, the second number is the ratio of first number, and total number of species within this group. Intron insertion positions specified in accordance with the 23S gene reference of *Escherichia coli* (Cannone *et al.* 2002). * without *Embryophyta* ** without *Chytridiomycetes*.

material (for most), and insect exuviae or other chitinous material (for members of the Polychytriales). We found *T. gracilis* separate from its substrate but hypothesize that its natural substrate is probably algal filaments, such as the *Chara* that we plated, or moribund aquatic plant material.

Telasphaerula gracilis also differs in the production of its zoospores. We repeatedly saw zoospores associated with pieces of rhizomycelium placed in distilled water, but the only structures that appeared to be zoosporangia were empty, thin-walled, sack-like structures that were large enough to accommodate one or, at most, two zoospores. This type of zoosporangium differs from that of other genera of the *Monoblepharidomycetes* and also from those of other polycentric *Chytridiomycota*, all of which form zoosporangia that are recognizable before zoospore discharge and contain multiple zoospores.

Sanchytrium tribonematis

The discovery of *Sanchytrium tribonematis* provides the first evidence of parasitism within the *Monoblepharidomycetes* (Powell & Letcher 2014; Dee *et al.* 2015). *S. tribonematis* zoospores encyst on living algal cells and form an intracellular, short, branched rhizoid inside of the cell; by the time that the fungus is mature the algal cell contents are disrupted. Its ability to parasitize algae and its morphology make *S. tribonematis* indistinguishable from members of the *Chytridiomycetes*. Thus, gene sequences are necessary to distinguish members of these two sister classes of fungi. Among the parasitic *Chytridiomycetes* the species *Phlyctidium laterale* (Braun) Sorokin has similar sporangial characteristics (sessile, spherical, ovoid 10–16 μm in diameter with one to three papillae; wall smooth, colourless), but the endobiotic part of *P. laterale* consists of

a peglike projection, which barely penetrates the host wall (i.e., rhizoid is absent). Further, the zoospores (oblong, 2 μm diam) have a much longer flagellum (10–12 μm) (Sparrow 1960). Because *S. tribonematis* has a branched rhizoid and different zoospore dimensions, we infer that it is not the same species as *P. laterale*. As further evidence, the *Phlyctidium* species has been reported from filamentous chlorophyte algae, most commonly *Ulothrix* (Sparrow 1960), rather than from the xanthophyte alga *Tribonema*.

Zoospore release was observed in a few sporangia; zoosporangial shape, from spherical to ellipsoid, seems to be a variable trait but the dimension of mature sporangia is approximately the same in all three strains.

The SSU and LSU gene sequences in all three *Sanchytrium* isolates are similar to each other, differing in just a few nucleotides. At least two strains (X-127 and X-128) live in a single geographic region and in the same habitat, but each strain has a unique intron pattern. If strains interbred with each other we would expect to see intron patterns of both parents or, if the crossing occurred long enough ago for unification of the ribosomal repeat, we would have seen a uniform pattern. The unique pattern for each strain (Figs 4 and 5) strongly suggests that no crossing takes place between them. A similar case has been described recently in *Myxomycetes* where some strains with different intron patterns do not interbreed and belong to separate biological species (Feng & Schnittler 2015). Intraspecific polymorphism in intron patterns have been described in rDNA of many parasitic, lichenized, and lichen-associated fungi (An *et al.* 2012; DePriest & Been 1992; DePriest 1993; Hafez *et al.* 2012; Nyati *et al.* 2013), but no clear evidence indicates that these discrete forms with alternative intron patterns hybridize with each other in the wild. Because *Sanchytrium* strains are genetically isolated from each other, they could be considered separate, morphologically identical (sibling) species. However, isolation from each other seems to have occurred just recently because: 1) the coding regions and even related introns in all three strains differ by only a few substitutions, and 2) the ITS2 of the strains have no compensatory base changes (Fig S4) that correlate with time divergence and interbreeding capability (Coleman 2000, 2009; Müller *et al.* 2007; Wolf *et al.* 2013). Moreover, the polymorphic intron pattern in LSU rRNA gene of X-128 indicates an even earlier stage of speciation. Although the three *Sanchytrium* strains appear not to interbreed and seem to be on their way to becoming separate species, for now, we maintain them in a single species. We suggest, however, that intron content and position is an additional area for research in determining species status and interbreeding in this group.

The clustering of environmental sequences from GenBank with *Sanchytrium* suggests that the genetic diversity in the *Sanchytriaceae* may be comparable with that in other monobleph families. We expect that as more chytrid-like organisms are isolated and studied with molecular tools, additional members of the *Monoblepharidomycetes* will be found, some new to science and some newly recognized as members of the class, and, perhaps, some narrowing the border between monoblephs and chytrids.

Intergeneric relationships within Monoblepharidomycetes *based on phylogenetic analysis*

Our phylogenetic analysis based on concatenated SSU and LSU rDNA data confirmed previously reported relationships of main monobleph genera (Dee *et al.* 2015). An earlier study based only on partial 28S rDNA sequences suggested that *Gonapodya* was not monophyletic (Chambers 2003) and placed *Gonapodya polymorpha*, and *Gonapodya prolifera* on two independent branches. We found the same positioning based on SSU data, but analysis of concatenated SSU and LSU rDNA sequences showed the monophyly of *Gonapodya*, as found by Dee *et al.* (2015). Analyses of alternative topologies also favoured the monophyly of *Gonapodya*. Polyphyly of *Harpochytrium* relative to *Oedogoniomyces* with weak (≤50 %) bootstrap support in the ML analysis was described earlier (Dee *et al.* 2015) and our Bayesian analysis also indicated polyphyly of *Harpochytrium*. We tested two alternative topologies with SSU rDNA data: 1) the monophyly of both genera and 2) the monophyly of *Oedogoniomyces* outside *Harpochytrium* and partial polyphyly of *Harpochytrium*. Support for both alternative topologies was significantly less than for the Bayesian combined SSU – LSU rDNA tree. So we conclude the monophyly of the combined *Oedogoniomyces* plus *Harpochytrium* clade, monophyly of *Oedogoniomyces*, and polyphyly of *Harpochytrium* relative to *Oedogoniomyces*.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.funbio.2017.05.002.

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