The Journal of Eukaryotic Microbiology

ISOP Published by the International Society of Protistologists

Journal of Eukaryotic Microbiology ISSN 1066-5234

ORIGINAL ARTICLE

Labyrinthula diatomea n. sp.—A Labyrinthulid Associated with Marine Diatoms

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Keywords
ITS haplogroups; rDNA phylogeny; Stramenopila.

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Received: 5 July 2019; revised 19 December 2019; accepted January 19, 2020.
Early View publication February 23, 2020

doi:10.1111/jeu.12789

ABSTRACT

Labyrinthulomycetes are mostly fungus-like heterotrophic protists that absorb nutrients in an osmotrophic or phagotrophic manner. Members of order Labyrinthulida produce unique membrane-bound ectoplasmic networks for movement and feeding. Among the various types of labyrinthulids' food substrates, diatoms play an important role due to their ubiquitous distribution and abundant biomass. We isolated and cultivated new diatom consuming Labyrinthulida strains from shallow coastal marine sediments. We described *Labyrinthula diatomea* n. sp. that differs from all known labyrinthulids in both molecular and morphological features. We provided strain delimitation within the genus *Labyrinthula* based on ITS sequences via haplotype network construction and compared it with previous phylogenetic surveys.

LABYRINTHULIDA (Stramenopila, Labyrinthulomycetes) is a relatively understudied order of fungus-like aquatic protists (Leander et al. 2004). The distinctive feature of this group is the anastomosing membrane-bound ectoplasmic network secreted by a unique organelle, the bothrosome (Raghukumar and Damare 2011). The network serves as a track for individual labyrinthulid cells to glide through and absorb nutrients from the external environment. *Labyrinthula* can be found in a diverse range of habitats, including marine and freshwater, from the epipelagic surface to the deep sea (Raghukumar 2002). They have also been isolated from various substrates, including algal biofilms, mangrove leaves, seagrass, coral mucus, and mollusks (Raghukumar and Damare 2011). Most labyrinthulids are saprotrophic and often associated with detritus like fallen mangrove leaves, decomposing algae, and fecal pellets of marine invertebrates (Bremer 1995; Tsui et al. 2009). Moreover, *Labyrinthula* is endosymbiotic with the marine ameba *Thecamoeba hilla* (Dyková et al. 2008). *Labyrinthula magnifica* (Valkanov) L.S. Olive specializes in its nutrition on diatom microalgae (Valkanov 1969). Grell (1994) noted the *Labyrinthula* isolate as an effective decomposer of the diatom lawn. Several other *Labyrinthula* spp. are not able to feed on diatoms or any other kind of protists (Lindholm et al. 2016).

Taxonomy of Labyrinthulomycetes has undergone several rearrangements in past decades (Beakes et al. 2014; Gomaa et al. 2013; Honda et al. 1999; Leander et al. 2004; Leander and Porter 2001; Olive 1975; Porter 1989; Takahashi et al. 2014; Yokoyama and Honda 2007; Yokoyama et al. 2007). However, up-to-date higher level classification of Labyrinthulomycetes is largely unresolved (Pan et al. 2017; Tice et al. 2016). Labyrinthulomycetes appear to be composed of two main clades: the first one for holocarpic thraustochytrids and the second one for plasmodial labyrinthulids and aplanochytrids (Bennett et al. 2017).

According to the last revision, Labyrinthulomycetes contain five orders (Amphitremida, Amphifilida, Oblongichytrida, Labyrinthulida, and Thraustochytrida) and 21 genera (Adl et al. 2019). All recognized species of *Labyrinthula* are not well distinguished using morphological features (Dick 2001). Phylogenetic analyses of isolated strains and environmental DNA samples show that the real number of *Labyrinthula* species (Martin et al. 2016) and Labyrinthulomycetes species as a whole (Collado-Mercado et al. 2010; Pan et al. 2017) are underestimated.

Though most are consider saprophytes, *Labyrinthula* is also a well-known opportunistic protistan pathogen found in association with marine vegetation, including seagrasses, around the world (Vergeer and den Hartog 1994). In addition to seagrasses, *Labyrinthula* is also associated with infection of marine algae (Pokorny 1967; Raghukumar 1987), terrestrial plants (see Schwelm et al. 2018 for review), and molluscs (Collier et al. 2017).

Diatom algae are the most abundant and diverse group of phytoplankton eukaryote species (Simon et al. 2009). Marine diatoms contribute nearly 20% to the total primary production of the World Ocean. In coastal and other nutrient-rich zones, their contribution reaches 75% (Falkowski 2012; Field et al. 1998; Nelson et al. 1995). They also play a major role in marine biological pump and regulating global climate change (Young and Morel 2015). Given the important role of diatoms in marine food webs, nutrient cycling, and global climate, many studies are focused on protists that could influence the structure and function of this group of algae.

In the present study, we isolated and cultured a novel *Labyrinthula* sp. strain associated with the marine diatoms *Cylindrotheca closterium* (Ehrenberg) Reimann & J.C. Lewin and *Micropodiscus weissflogii* Grunow from coastal marine sediment samples. We obtained both molecular and morphological data to define the position of our strain among other *Labyrinthula* strains.

MATERIALS AND METHODS

Environmental sample collection

Cultures of *Labyrinthula* were isolated from sea sediment samples collected on the south coasts of Sri Lanka (Tangalle town, 6°01′N 80°47′E) and Thailand (Pattaya city, 12°56′N 100°53′E) in February 2017, from a depth of about 50 cm, though not specifically from a diatom lawn. Samples were collected in sterile disposable 50-ml polypropylene centrifuge tubes and transported to the laboratory of Moscow State University within 48 h. We found the *Labyrinthula* strain associated with marine diatoms *C. closterium* while observing samples using light microscopy. We used a modified serum saline water agar (SSA) medium, containing agar, antibiotics, and artificial seawater (Yadagiri et al. 2012). We improved SSA by adding f/2 medium (1/10) for algal growth and adjusted artificial seawater salinity to ~30‰. Additionally, we used a liquid medium with algae growth supplements (f/2) and 30‰ salinity. We tested different algae cultures as potential hosts for the *Labyrinthula* strain: the dinoflagellates *Prorocentrum minimum* and *Amphidinium carterae*, the chlorophyte *Tetraselmis viridis*, and the diatom algae *M. weissflogii*. The *Labyrinthula* strain grows only in association with *M. weissflogii*. All tested algae cultures were algologically clean and cultivated on the same f/2 medium and under the same conditions. Since the differences in organic matter content between algae cultures were insignificant, the new *Labyrinthula* strain survived only on the diatom algae and was initially observed on diatoms, and we suggest that this strain is an obligate diatom eater. Unfortunately, after one-month of cultivation, we lost the original diatom host *C. closterium* and further co-cultured our isolate with the algae *M. weissflogii* at room temperature (23 °C) and 14-h light, 10-h dark cycle (for algae cultivation). Algae cultures were taken from the culture collection of marine microalgae housed at the Hydrobiology Department of Biological faculty, Lomonosov Moscow State University, Moscow, Russia.

Morphological observations

Light microscopy measurements of various features of the isolate *Labyrinthula* (length, width, thickness of ectoplasmic networks; $n = 50$ cells) were performed using ImageJ software. Small pieces of the growing culture were placed into a glass-bottom Petri dish with a few drops of autoclaved seawater. Images were taken using Carl Zeiss Axiovert 200M (Carl Zeiss Microscopy GmbH, Jena, Germany) inverted microscope equipped with a 100X NA 1.3 Plan-Neofluar phase-contrast lens 200M (Carl Zeiss Microscopy GmbH) and ORCAII-ERG digital camera (Hamamatsu Photonics, Hamamatsu, Japan).

DNA extraction, PCR, and sequencing

We isolated DNA from a binary culture when the predator almost completely ate the diatom prey. We used the DIAtom DNA Prep kit (Isogen, Moscow, Russia) following the protocol provided by the manufacturer. For phylogenetic analyses, we selected two common nuclear DNA markers, the ITS1-5.8S-ITS2 (ITS) and the small ribosomal RNA gene (SSU or 18S), which were previously applied for labyrinthulids. SSU and LSU rDNAs of *Labyrinthula diatomea* were amplified as overlapping fragments using Encyclo PCR kit (Evrogen, Moscow, Russia). We amplified DNA fragments with the set of previously designed primers (Medlin et al. 1988; Van der Auwera et al. 1994) and new labyrinthulid-specific primers lab_v7 (5′-TTAACGAACGAGACCTCAGCC-3′) and lab_28d23 (5′-TGC TTGCCTCGTCAGAGCTTT-3′) as an additional means of avoiding contamination by the diatom prey. PCR annealing temperatures and elongation times varied. The basic PCR cycling conditions include denaturation at 95 °C for 5 min, followed by 45 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 2 min, and final extension at 72 °C for 5 min. PCR products were separated with agarose gel electrophoresis and purified using Cleanup Mini kit (Evrogen). Amplicons were sequenced directly with an Applied Biosystems 3730 DNA Analyzer.

Sequence alignment and molecular phylogenetic analysis

All available to date ITS and SSU sequences of *Labyrinthula* strains were downloaded from the nucleotide collection (nr) GenBank database. Then, all GenBank *Labyrinthula* sequences and *L. diatomea* sequence were used as query against the nr database for the searching of uncultured and unidentified *Labyrinthula* strains. Finally, all found sequences were used as query against the whole-genome shotgun contigs (WGS) GenBank database for the searching of metagenome assembly sequences. All sequences were aligned in MEGA 6.0 (Tamura et al. 2013) with MUSCLE and manually adjusted in BioEdit (Hall 1999). Initial sequence alignment lengths were 475 bp for ITS and 2,110 bp for SSU. After manually excluding ambiguously aligned regions, final sequence alignment lengths were 399 bp for ITS and 1,643 bp for SSU. Phylogenetic inference was performed by IQ-TREE (Nguyen et al. 2014) under ModelFinder method (-m MFP) and ultrafast bootstrap with 1,000 replicates (-bb 1,000). The best-fit model according to ModelFinder was TIM + R3. Phylogenetic trees were visualized with MEGA 6.0. The initial phylogenetic trees were constructed using all *Labyrinthula* ITS and SSU sequences downloaded from GenBank. For final phylogenetic tree construction, the majority of redundant sequences with over 98% similarity were removed from alignments.

Strain demarcation

Aligned ITS sequences of *Labyrinthula* spp. were used to determine the relationship between strains. The software Popart 1.7 (Leigh and Bryant 2015) was then used for comparative analysis and identification of differences between populations as well as for construction of a TCS haplotype network (Clement et al. 2002).

RESULTS

Morphology

Colonies, trophic cells, and clumps (sori) are colorless. In the liquid medium, colonies form dense clumps of cells without walls and measure up to 60.0 μm in diameter (Fig. 1A). Trophic cell shapes vary from oblong to fusiform. Cells are very mobile, and their size ranges from 3.5 to 5.0 μm (avg 4.3 ± 0.5 μm) in width and from 10.0 to 12.5 μm (avg 10.5 ± 0.7 μm) in length (Fig. 1B and Movie S1). Nuclei are located in the middle of the cells (Fig. 1B). The ectoplasmic network consists of long, thin ramifying filaments.

In the absence of the prey, separate cells of *L. diatomea* are evenly distributed over the ectoplasmic network and move randomly (Fig. 1B). In the presence of prey cells, *L. diatomea* slide along ectoplasmic network filaments, toward the prey (Fig. 1C). Free-swimming zoospores were not observed.

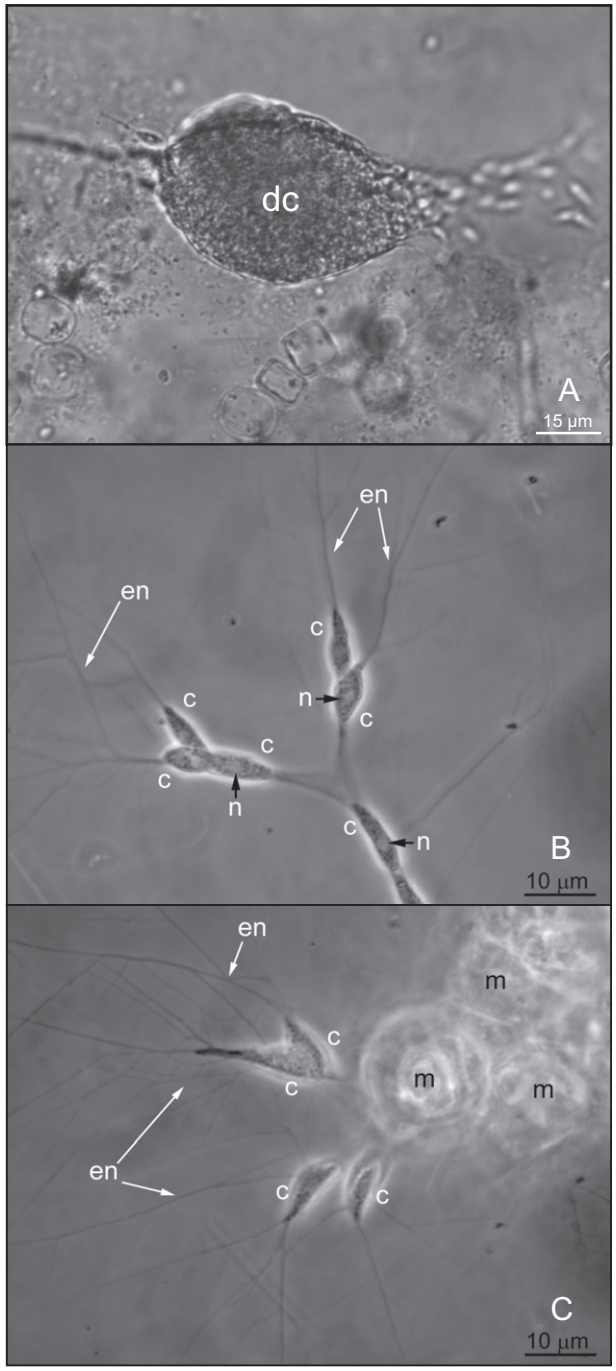

Figure 1 Light morphology of *Labyrinthula diatomea*. **A.** Colony morphology with a dense clump. **B.** Trophic cells with centrally located nuclei. **C.** Trophic cells with *Micropodiscus weissflogii* cells. C = trophic cell; en = ectoplasmic network; dc = dense clump; m = cell of *M. weissflogii*; n = nuclei.

Molecular phylogeny

Ribosomal rRNA and ITS sequences of the *L. diatomea* strains from Sri Lanka and Thailand are identical. Phylogeny for genus *Labyrinthula* was reconstructed

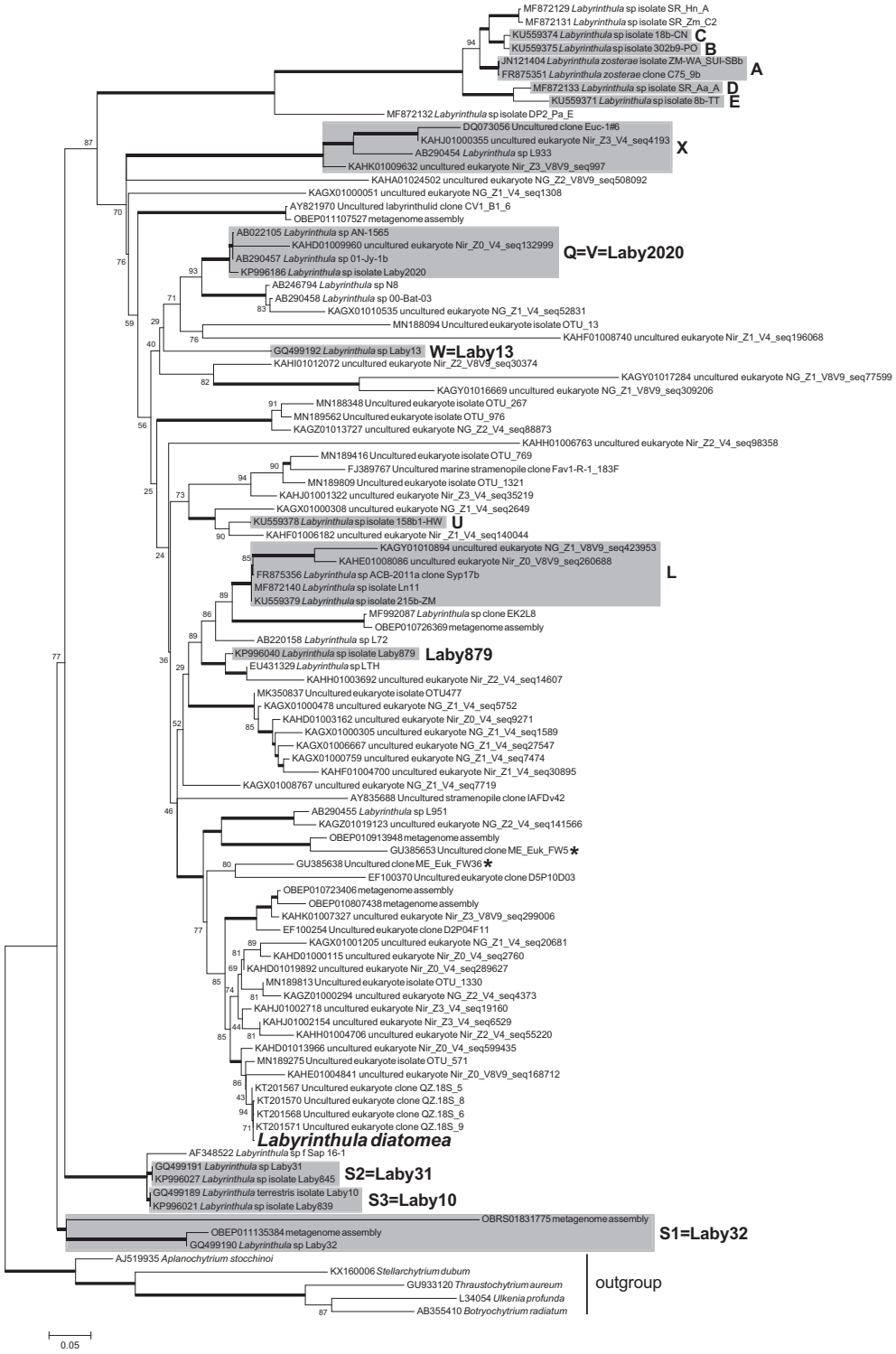

Figure 2 The maximum likelihood tree of *Labyrinthula* phylogeny based on 18S sequences. Branches are labeled with bootstrap support. Branches with bootstrap support higher than 95 are marked bold. Haplogroups named according to Martin et al. (2016), Douhan et al. (2009), and Chitrampalam et al. (2015). Haplogroups are squared in gray. *Only the first 800 bp of the sequence.

separately for the SSU and the ITS regions. Putative species letter groups are defined according to Martin et al. (2016), Douhan et al. (2009), and Chitrampalam et al. (2015). Originally, most of these groups were defined after ITS haplotypes; therefore, all *Labyrinthula* spp. ITS, but only few SSU sequences, belong to any lettered group.

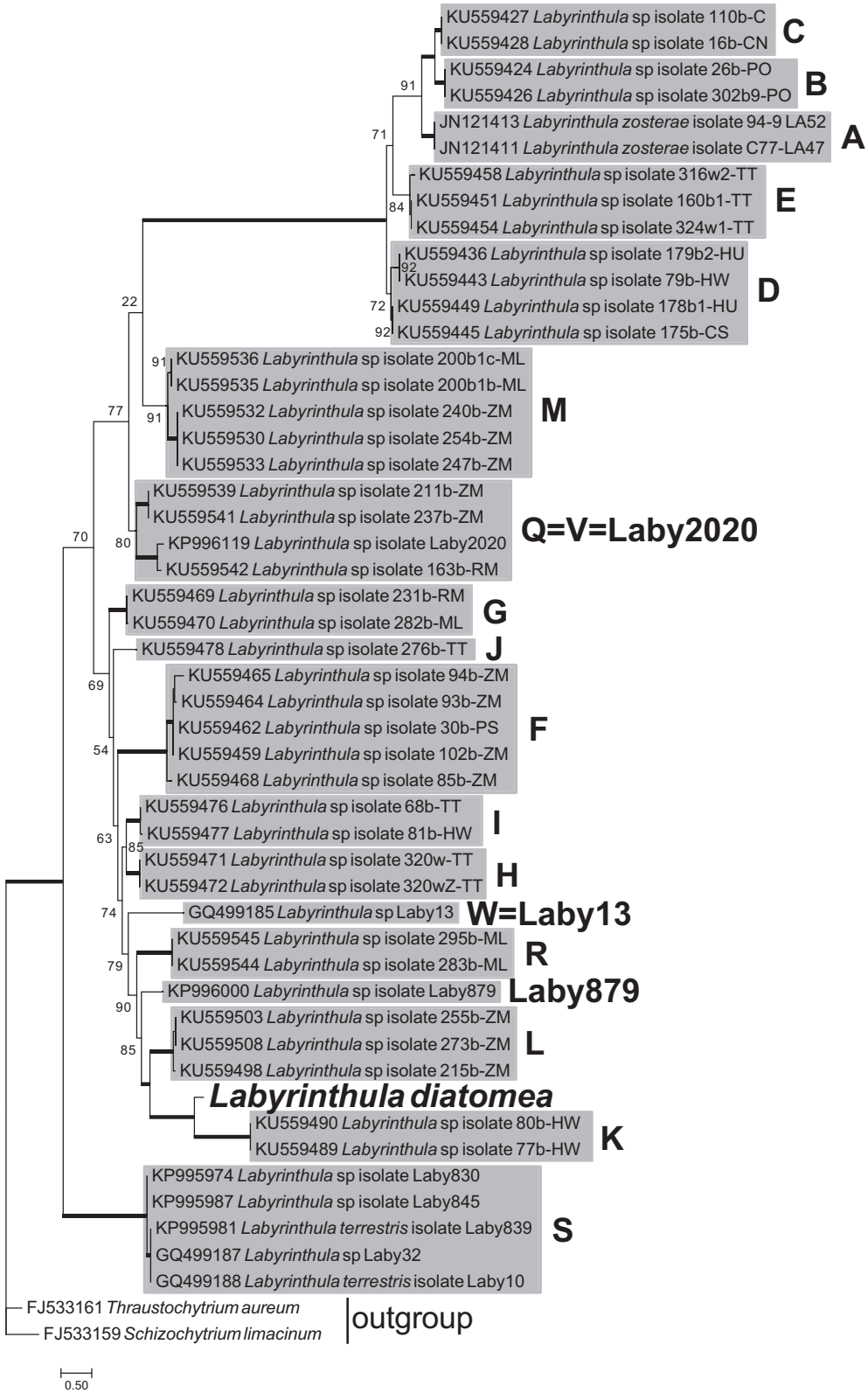

Figure 3 The maximum likelihood tree of *Labyrinthula* phylogeny based on ITS sequences. Branches are labeled with bootstrap support. Branches with bootstrap support higher than 95 are marked bold. Haplogroups named according to Martin et al. (2016), Douhan et al. (2009), and Chitrampalam et al. (2015). Haplogroups are squared in gray.

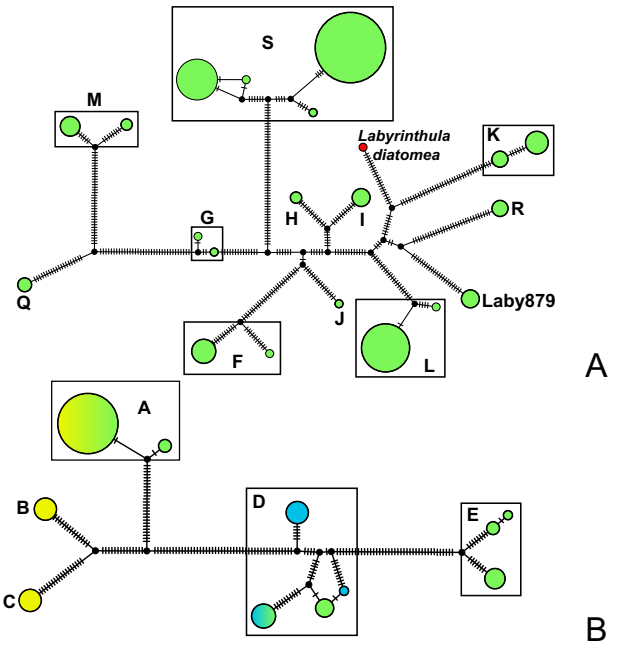

Figure 4 TCS network of *Labyrinthula* strains. **A.** The *L. terrestris* alignment group. **B.** The *L. zosterae* alignment group. Each circle is separate ITS haplotype. A haplotype is colored according to the location where it was collected. Haplotypes found in different locations are colored in the gradients between appropriate location colors. Asia is red, Europe is yellow, America is green, and Australia is blue. Circle sizes indicate the number of strains with the same ITS haplotype. Each dash is a one nucleotide substitution. Haplogroups named according to Martin et al. (2016) and Chitrampalam et al. (2015) with changes. Haplogroups with several different haplotypes are squared.

Therefore, due to the presence of ITS and SSU sequences from the same isolates we defined the identity of some groups: Q, V, and Laby2020; W and Laby13; S1 and Laby32; S2 and Laby31; and S3 and Laby10. Thus, some groups are described for both ITS and SSU data (A, B, C, D, E, L, Laby879, Q, S, and W), while others are available only for ITS (F, G, H, I, J, K, M, and R) or SSU (U and X) data. Both obtained trees (Fig. 2, 3) demonstrate the similar topologies with three well-supported clades of seagrass pathogenic *Labyrinthula* isolates (species letter groups A, B, C, D, and E), terrestrial *Labyrinthula* isolates (S2 and S3 haplogroups in the SSU tree and all isolates in the ITS tree), and a group of *Labyrinthula* isolates from various aquatic substrates (F, G, H, I, J, K, L, Laby879, M, R, Q, U, W, and X). The branching of seagrass pathogenic putative species (A, B, C, D, and E) is similar in both trees. Terrestrial *Labyrinthula* isolates are monophyletic in the ITS tree and paraphyletic in the SSU tree forming two branches. The rest of the sequences form the weakly supported (60% bootstrap support) third group in the SSU tree. If the pathogenic status is available, the majority of the third clade isolates are nonpathogenic, with the exception of *L. diatomea*. In the SSU tree, *L. diatomea* groups with four environmental DNA sequences isolated from benthic diatom film in the southeast coast of China. The identity between these sequences and *L. diatomea* is higher than 99%. In the ITS tree, *L. diatomea* groups with two *Labyrinthula* sp. strains isolated from seagrass tissues in Florida, USA, with the identity of 87%. In general, clade compositions and tree topologies are consistent with previous *Labyrinthula* SSU rDNA studies (Martin et al. 2016; Trevathan-Tackett et al. 2018). Considering the presence of metagenome data, the total amount of available *Labyrinthula* SSU sequences is significantly large than ITS sequences.

Strain delimitation via ITS analysis

Strain delimitation was conducted with all available to date *Labyrinthula* ITS sequences from GenBank and new *L. diatomea* strain. All sequences were aligned and derived into two large alignment groups, poorly aligned between each other. The first alignment group named after *L. terrestris* contains 195 sequences including *L. terrestris*, *L. diatomea*, and *Labyrinthula* spp. isolates. The second alignment group named after *L. zosterae* contains 84 sequences including *L. zosterae* and *Labyrinthula* spp. isolates. All ITS sequences from the *L. terrestris* alignment group, except *L. diatomea*, were found in North America. Sequences from the *L. zosterae* alignment group were found in North America, Europe, and Australia. Each alignment was used for the construction of a TCS haplotype network (Fig. 4). Both haplotype networks contain several haplogroups with one or a few similar haplotypes. Formally, some haplotypes were integrated into one haplogroup if the total amount of nucleotide substitutions between all these haplotypes did not exceed 20. With the exception of two haplogroups, all haplogroups coincide with the ITS putative species letter groups that were defined in previous species delimiting within genus *Labyrinthula* (Martin et al. 2016), so we used this lettered system for our haplogroups naming. The *L. terrestris* alignment group (Fig. 4A) contains 13 haplogroups, and the *L. zosterae* alignment group (Fig. 4B) contains five haplogroups (Table 1). We identified one new haplogroup within the *L. terrestris* alignment group—*L. diatomea*, with a single sequence. Within the *L. terrestris* alignment group, there are six haplogroups with several haplotypes (F, G, K, L, M, and S) and seven haplogroups with one haplotype (H, I, J, R, Q, Laby879, and *L. diatomea*). Within the *L. zosterae* alignment group, there are three haplogroups with several haplotypes (A, D, and E) and two haplogroups with one haplotype (B and C). The number of sequences in one haplogroup varies from one (J and *L. diatomea*) to 105 (S).

TAXONOMY

Class Labyrinthulomycetes Arx, 1970; Dick 2001.
Order Labyrinthulida Doflein 1901.
Family Labyrinthulidae Cienkowski 1867.
Genus *Labyrinthula* Cienkowski 1864.

Table 1. *Labyrinthula* ITS haplogroups

Alignment group	Haplogroup[a]	Number of sequences	GenBank accession numbers	References
Labyrinthula zosterae	A	46	JN121409–JN121413, KU559380–KU559420	Bergmann et al. (2011) and Martin et al. (2016)
	B	6	KU559421–KU559426	Martin et al. (2016)
	C	6	KU559427–KU559432	
	D	18	KU559433–KU559450	
	E	8	KU559451–KU559458	
Labyrinthula terrestris	F	10	KU559459–KU559468	
	G	2	KU559469, KU559470	
	H	2	KU559471, KU559472	
	I	5	KU559473–KU559477	
	J	1	KU559478	
	K	12	KU559479, KU559481–KU559491	
	L	37	KU559492–KU559528	
	M	8	KU559529–KU559536	
	R	5	KU559542–KU559546	
	Q	3	KU559539–KU559541	
	S	105	GQ499186–GQ499188, KP995973–KP995999, KP996002–KP996008, KP996012, KP996053–KP996118, KU559547	Chitrampalam et al. (2015), Douhan et al. (2009), and Martin et al. (2016)
	Laby879	5	KP996000, KP996001, KP996009–KP996011	Chitrampalam et al. (2015)
	Labyrinthula diatomea[b]	1	MN101174	This study

[a]Haplogroups are coded according to Martin et al. (2016) and Chitrampalam et al. (2015).
[b]Haplogroup described in this study.

Table 2. Morphological features of different diatom eater *Labyrinthula* species

Species	*Labyrinthula diatomea*	*Labyrinthula magnifica*	*Labyrinthula* sp.
Color	No	No	No
Plasmodium size	n/d	Up to 50 cm	n/d
Sori size	60 μm	n/d	n/d
Sori wall thickness	No	20–30 μm	n/d
Trophic cell shape	Oblong to fusiform	Ellipsoidal	Fusiform
Trophic cell width, μm	3.5–5	n/d	4–6
Trophic cell length, μm	10–12.5	15–18	11–14
Sea aquatory	South China Sea; Laccadive Sea	Black Sea	Mediterranean Sea; Atlantic Ocean
References	This study	Valkanov (1969)	Grell (1994)

Labyrinthula diatomea Popova & Belevich, n. sp.

Etymology: after diatoms, the class name of the first observed hosts.

Colonies and trophic cells are colorless. In liquid culture, colonies form dense clumps (sori) of cells without wall about 60.0 μm in diameter. Trophic cells shape varies from oblong to fusiform. Cells are very mobile and ranged 3.5–5.0 μm wide and 10.0–12.5 μm long. The ectoplasmic network consists of long thin filaments.

MycoBank 831573.

GenBank Accession number MN101174.

Type: Fig. 1. Popova et al. this publication. Thailand, south coast. Sample collected in February 2017. Culture deposited in the culture collection in Hydrobiology department of Biological faculty, MSU.

Other collection: Sri Lanka, coast near Tangalle town. Sample collected in February 2017. Culture deposited in the culture collection in Hydrobiology department of Biological faculty, MSU.

Comments: Both cultures are morphologically identical. Both strains were initially associated with diatom *Cylindrotheca closterium* and then were cultivated on diatom *Micropodiscus weissflogii*.

DISCUSSION

In this study, we isolated and cultured two genetically related strains of *Labyrinthula* from the surface marine sediments of coastal sites of the Indian Ocean and the Pacific Ocean. These strains utilize living cells of marine diatoms

C. closterium (in natura) and *M. weissflogii* (in vitro) as prey for their growth. We described a new species *L. diatomea* and established its phylogenetic position.

Besides *L. diatomea,* there are two other studied diatom consuming *Labyrinthula* strains: *L. magnifica* (A. Valkanov) L.S. Olive and *Labyrinthula* sp. (Grell 1994). Their known morphological features are summarized in Table 2. All three strains are colorless and associated with diatoms. If the comparison is applicable, all other features differ. *L. magnifica* has thick sori wall, while *L. diatomea* sori do not have walls. The shape of trophic cells is fusiform in *L. diatomea* and *Labyrinthula* sp. instead of an ellipsoidal shape in *L. magnifica*. *L. magnifica* has the largest trophic cell size among these *Labyrinthula* (up to 18 μm in length) while trophic cells of *Labyrinthula* sp. (11–14 μm in length) are slightly larger than *L. diatomea* cells (10–12 μm in length). Moreover, all three strains were found in different geographic regions: *L. diatomea* in the Indian Ocean and the Pacific Ocean, *L. magnifica* in the Black Sea, and *Labyrinthula* sp. in the Mediterranean Sea and the Atlantic Ocean. We consider the morphological distinctions between diatom consuming *Labyrinthula* strains as contributing evidence that they are separate species.

Among *Labyrinthula,* only two described species (*L. zosterae* and *L. terrestris*) have sequences associated with them in GenBank. Other *Labyrinthula* sequences are not identified to species; over half of them are associated with a diverse range of hosts, including invertebrates, corals, seagrass, and algae. Moreover, there are many unidentified *Labyrinthula* sequences in the WGS GenBank database received from metagenomic researches (Karst et al. 2018; Kim et al. 2016). Based on current knowledge, the genus *Labyrinthula* is primarily associated with coastal environments. Diatoms are the major primary producers in coastal areas, so the *Labyrinthula*–diatom interaction may play a great role in marine detritus decomposition, especially as diatoms are large contributors of benthic fouling. At the least, *L. diatomea* inhabits the southern coasts of China, Thailand, and Sri Lanka (Fig. S1). Despite the global distribution of *Labyrinthula* spp., the majority of known sequences are from North America. Consequently, *Labyrinthula* diversity is still underestimated.

Recent SSU rDNA analyses and ITS strain demarcation indicate a huge variety and a large number of undescribed species in *Labyrinthula* (Sullivan et al. 2017). Haplogroup compositions based on TCS haplotype networks from our study generally coincide with haplogroups previously delimited by the methods of neighbor joining and the maximum likelihood (Martin et al. 2016). As well as in Martin et al. 2016, all *Labyrinthula* sequences in our study are distributed into three clades. The terrestrial clade matches with Martin's T clade (terrestrial); the seagrass pathogenic clade matches with Martin's P clade (pathogenic); and all other sequences including *L. diatomea* matches with Martin's N clade (nonpathogenic). The term "pathogenic" is applicable to seagrass, so the presence of diatom pathogen *L. diatomea* in the nonpathogenic clade does not contradict with Martin's clade system of *Labyrinthula*. Moreover, N clade was described as "occurring on various aquatic vegetation" which may include diatom lawn. Haplogroup compositions in both studies are the same; however, we described one new haplogroup—*Labyrinthula diatomea*. However, the haplogroups' relative positions are more different: G + H + I + J do not form a monophyletic group, G is an ancestor of M and Q, J groups with F, and K locates much further from M and Q.

The lack of the morphological and molecular gap between strains from geographically remote areas of Thailand and Sri Lanka points on the presence of a large *L. diatomea* population in the northern Indian Ocean and in the western Pacific Ocean. Environmental sequences of the same species from the south coast of China expand the territory of this population. The wide occurrence of this single predator species indicates that it may have a large ecological role. This species likely consumes different types of substrates or distributes together with diatoms. Future research of labirinthulids and diatoms will help construct a clearer understanding of the relationships within the tropical marine biocenoses.

ACKNOWLEDGMENTS

We thank A. V. Tchesunov for kindly providing sea water samples that carried living labirinthulid cells. We are deeply grateful to the reviewers for their great efforts and productive dialogue that refined the paper. The reported study was funded by RFBR according to the research projects no. 18-04-01210 and 19-15-00273.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. *Labyrinthula diatomea* collection locations.
Movie S1. *Labyrinthula diatomea* cells glide through the ectoplasmic network in real time.