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Genetic Identities of Cryptic Species in the *Strombidium stylifer/apolatum/oculatum* Cluster, Including a Description of *Strombidium rassoulzadegani* n. sp.

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ABSTRACT. To assess diversity among cryptic species of the ciliate genus *Strombidium*, we characterized the small subunit ribosomal DNA gene (SSU-rDNA) from several lineages that had been identified previously as distinct based on the internal transcribed spacer regions of the rDNA locus. We sequenced SSU-rDNA from four members of a cryptic species cluster of ciliates from tidepools in the North Atlantic Ocean. One of the sequences was very similar (>99% similarity) to that of *Strombidium apolatum*. The other three sequences differed from each other and from the closest named species on GenBank by 4–10%. We were able to cultivate only one of these three species. Here we name it *Strombidium rassoulzadegani* n. sp. and describe its morphology, behavior, and feeding. The history of observations of tidepool Strombidiidae is discussed along with hypotheses about how they may partition the tidepool niche for coexistence. Given the apparent high degree of cryptic diversity of ribotypes in the Strombidiidae, we recommend that no new species descriptions be made without accompanying genetic information.

Key Words. Biogeography, ciliate, mixotrophy, new species, oligotrich, tidepool.

THE Strombidiidae are a family of spirotrich ciliates that are among the most abundant marine planktonic forms (Montagnes, Poulton, and Shammon 1999; Montagnes et al. 1988). As an important component of the microbial food web, they comprise an intermediate step in the transfer of material and energy from smaller protists and bacteria to higher trophic levels, including metazoa (Calbet and Saiz 2005; Gifford and Dagg 1988, 1991; Stoecker and Capuzzo 1990; Stoecker and Egloff 1987; Stoecker, Guillard, and Kavee 1981). Many strombidiids are ''mixotrophs'' able both to ingest other organisms for nutrition and also photosynthesize. In particular, some species of *Strombidium*, *Tontonia*, and *Laboea* have the habit of retaining the chloroplasts of ingested algae and holding them for at least several days, during which time they are functional (Stoecker, Michaels, and Davis 1987; Stoecker and Silver 1990). *Laboea strobila*, for example, harbors chloroplasts from ingested cryptophyte algae and is an obligate mixotroph, requiring both light and food for growth (McManus and Fuhrman 1986; Stoecker et al. 1988).

One remarkable mixotrophic member of this family is the morphospecies *Strombidium oculatum*, which harbors green chloroplasts derived from the zoospores of green macroalgae (Ulvophyceae; McManus, Zhang, and Lin 2004). It lives in tidepools, encysting and excysting on a circatidal rhythm to avoid being flushed out of the pools during the high-tide period. In a series of elegant experiments and observations, Fauré-Fremiet (1948), Jonsson (1994), and Montagnes et al. (2002a, b) showed that individuals have a 6 h:18 h cycle of excysted:encysted condition, and that they have strong phototactic behavior (i.e. positive early post-excystment and negative pre-encystment). Because individuals are generally only active on alternate low tides, any given tidepool may contain two somewhat isolated populations. However, variation in encystment cycles among individuals can lead to some transfer of individuals between the two populations in a pool (Montagnes et al. 2002b).

When Katz et al. (2005) sequenced the internal transcribed spacer (ITS) regions of the ribosomal locus from wild populations of the morphospecies *S. oculatum*, they found evidence for cryptic species. Although all of the members of this species complex are of similar size, with green chloroplasts and a prominent red eyespot, analysis of ITS sequences indicated four common distinct haplotypes, designated by them as Clades ii, iv, vii, and viii, which differed by up to 15%. Based on an ITS mutation rate of 0.5–1% per 10^6 yr (Lajeunesse 2005), the clades diverged on the order of 10 Mya. Here we report the genetic identities of the four clades through analyses of the small subunit ribosomal DNA gene (SSU-rDNA). We also report on the behavior, physiology, and morphology of Clade vii, which we have been able to cultivate, and formally describe this form as *Strombidium rassoulzadegani* n. sp.

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MATERIALS AND METHODS

Collections. In earlier studies, we collected one or more members of the cryptic species cluster from various sites in the British Isles, including Scotland, Ireland, and the Isle of Man, as well as coastal New England (Katz et al. 2005). For the DNA sequences reported here, we used samples collected in Ireland from Galway Bay (53°14′N, 9°22′W) and the Irish Sea (53°17′N, 6°07′W), and in the United States from Long Island Sound (41°16′N, 72°36′W). Experimental results are reported from cultures of Clade vii that were established from samples collected in Dunbar Scotland (56°00′N, 2°31′W), Long Island Sound, and São Sebãstiao, Brazil (23°50′S, 45°27′W).

Cultures. We initially cultivated Clade vii by picking individuals with a drawn pipette and placing them in six-well plates with pieces of the thallus of the green macroalga *Ulva* sp. The *Ulva* had been held in the dark overnight in moist tissue paper at 4 °C to induce zoospore formation. Subsequently, when the ciliates were not abundant enough in the samples for direct isolation, we obtained cultures numerous times (>10) by enriching samples with the unicellular prasinophyte microalga *Tetraselmis chui* (strain PLY429) at approximately 10^4 cells/ml. None of the other green algae—*Nannochloropsis* sp. (UTEX2341), *Stichococcus bacillaris*, *Chlorella autotrophica*, and *Dunaliella tertiolecta*—proved suitable for cultivating Clade vii. Cultures were routinely grown on *Tetraselmis* in seawater at 18 °C on a 12 h:12 h light:-dark cycle. Inorganic nutrients, including vitamins and trace metals (F/2 formulation; Guillard and Ryther 1962), were added to the medium. Salinity of the medium was 33 (practical salinity scale), but positive growth was achieved at salinities as low as 10 and as high as 40 (data not shown). Despite our best efforts, only Clade vii has proven amenable to cultivation. We tried a variety of microalgae as food, both individually and in combination, in particular *T. chui*, *Prorocentrum minimum*, *Isochrysis galbana*, and

Rhodomonas salina but were not able to cultivate any of the other clades. Even when DNA sequencing indicated the presence of multiple clades in a field sample, only Clade vii could be grown from enrichments of that sample.

The ability of Clade vii to grow on non-green algae was evaluated quantitatively by measuring growth rates on the dinoflagellate *P. minimum*, the prymnesiophyte *I. galbana*, and the cryptophyte *R. salina* Unialgal cultures were diluted into a range of concentrations from 10^2 to 10^5 cells/ml. Clade vii cells were acclimated to each food concentration overnight, added to wells at the target food concentrations (i.e. 10 ciliates per well, in 10-ml wells), and grown for 3 d. Growth rates were calculated assuming exponential growth, and parameters of the numerical response equation (see Montagnes, Berger, and Taylor 1996) were estimated iteratively using the SigmaPlot computer program. A growth experiment with *T. chui* as food was conducted using the same design, except that an additional dark treatment was added at each food concentration to compare survivorship and growth in light vs. dark conditions.

Live observations. Documentation of phototaxis and morphology of live ciliates from our cultures of Clade vii were performed using an Olympus SZX stereomicroscope, an Olympus BX50 compound microscope, and an Olympus IX70 inverted microscope (Olympus, Center Valley, PA). Bright field, phase contrast,and differential interference contrast optics were used. To measure live dimensions we used a homemade Roto-Kompressor (Heunert and Uhlig 1966; Uhlig and Heimberg 1981), which allowed the ciliates to be gently compressed into a single plane. They were then photographed at 100X while actively swimming and measured on the photographs using the program Image J (http://rsbweb.nih.gov/ij/). To characterize swimming patterns, ciliates were observed with the inverted microscope while swimming freely in 10-ml wells. Digital videos were made at 10 frames/s and swimming motions were subsequently traced while stepping through the recordings frame by frame using NIS Elements imaging software (version AR-3.00; http://www.lim.cz).

Electron microscopy. Individual ciliates were picked from cultures using a drawn capillary pipette. They were fixed in ice-cold 2.5% (v/v) glutaraldehyde for 15 min, rinsed once in filtered seawater, and transferred through a graded ethanol series (i.e. 50%, 70%, 95%, 100%, 100% aqueous ethanol, for 10 min each step). During these transfers, the ciliates were held in 6.5-mm-well inserts (made for 12-well tissue culture plates; Falcon product # 35-3181; Fisher Scientific, Pittsburgh, PA) containing 3-μm pore size polyethylene terephthalate filters glued onto the bottom. Before each transfer, the ethanol solution was wicked away by placing the well insert in contact with a piece of tissue paper. After the second 100% ethanol rinse, a few drops of trimethylsilane were added and the ciliates were air-dried at room temperature. Subsequently, the filter was cut from the bottom of the well insert and the ciliates were swept onto a stub covered with double-sticky tape using a fine needle with a human eyebrow hair glued to it. The stubs were then sputter-coated with gold and observed in a LEO/Zeiss DSM 982 digital field emission scanning electron microscope (Carl Zeiss, Thornwood, NY).

Protargol staining. Membranellar patterns and somatic kineties were made visible by staining with silver proteinate (protargol). We used the protocol of Wilbert (1975), except that the staining step was carried out at higher temperature (~45 °C), as recommended by Skibbe (1994).

DNA sequencing and analysis. DNA was extracted with phenol/chloroform and amplified using the polymerase chain reaction (PCR). The clades were originally discriminated by differences in the ITS region. To link these sequences with SSU, we sequenced a portion of the ribosomal genes approximately 2.1 kbp long, comprising the SSU-ITS1–5.8S-ITS2 region. This segment was amplified using SSU 5′← and LSU start⁻ primers (Table 1), gel-isolated, and sequenced using the Big Dye terminator kit (Applied Biosystems, Foster City, CA). The sequencing PCR was done using two additional internal primers (Table 1). Sequence reads were assembled and proofread manually, using the SeqMan program (DNASTAR Inc., Madison, WI), and aligned in McClade (Sinauer Inc., Sunderland, MA). A phylogenetic tree was constructed with the SSU-rDNA portion of the sequence using MrBayes (version 3.1.2), and included sequences from other spirotrich ciliates available on GenBank. In constructing the tree, we used a General Time Reversible model of nucleotide substitutions, estimating a proportion of invariable sites and a γ-shaped distribution of rates across sites. This model was determined to be appropriate using the online server Datamonkey (http://www.datamonkey.org/).

Table 1. Primers used for amplifying and sequencing the SSU-rDNA and 5.8S ribosomal genes, including internal transcribed spacer regions.

Primer name	Sequence
LSU start	TAKTRAYATGCTTAAGTYCAGCG
SSU-rDNA 5′←	ACCTGGTTGATCCTGCCAGT
SSU-rDNA INT+1	YGGAGARDSRGCYTGAKARAYGGC
SSU-rDNA INT − 1	GACCTGKTATTGCCTYAMRTCTCC

RESULTS

All four lineages of *Strombidium* fall within the oligotrich clade on the SSU-rDNA tree (Fig. 1). Clade vii is nearly identical (>99% similarity) to an unidentified environmental sequence retrieved from a New England Salt Marsh (Stoeck and Epstein 2003). Clade ii is >99% identical in SSU-rDNA to *Strombidium apolatum* (Wilbert and Song 2005; Xu, Song, and Hu 2005); the two sequences differ by only five single nucleotide polymorphisms, all transitions. Clades ii, iv, and viii cluster together with *S. apolatum* with a posterior probability of 0.82. Clades iv and viii do not appear to have been sequenced before, because they are no closer than 96% identical to any sequences published on GenBank.

Available data on morphological differences among the species in this group reveal varying levels of similarity (Table 2). For example, the ventral membranelles of *S. apolatum* and *S. oculatum* are distinctly shorter at their bases than the anterior membranelles, whereas the two fields of membranelles grade into one another more gradually in *Strombidium stylifer* and *S. rassoulzadegani*. Also, the macronucleus in *S. apolatum* is more fusiform or spindle-like than those of the other species, and its ventral kinety is much longer, extending up to the girdle kinety. *Strombidium stylifer* and *S. rassoulzadegani* have the distinctive tail or ''stachel'' originally described by Levander (1894), while the others do not. It is not clear if all of these species contain a pigmented eyespot, because some observers have noted this feature and the others may have ignored it (Table 3). Thus, while there is some overlap among species in morphometrics and measurements associated with ciliature, the differences are consistent with our observation of genetic heterogeneity, and suggest at least four distinct species.

Description of *Strombidium rassoulzadegani* sp. n. The ciliate's size in vivo is 49–82 μm long × 35–50 μm wide, average 66 × 41 μm. Body shape is only slightly variable among individuals, generally broadly obconical and circular in cross-section, widest at the shoulder region. The anterior is domed with a buccal lip on the right of the oral groove; the lip leads to a central apical bump (about 5 μm high), which is prominent in vivo but may disappear or be undetectable after protargol impregnation (Fig. 2, 9,

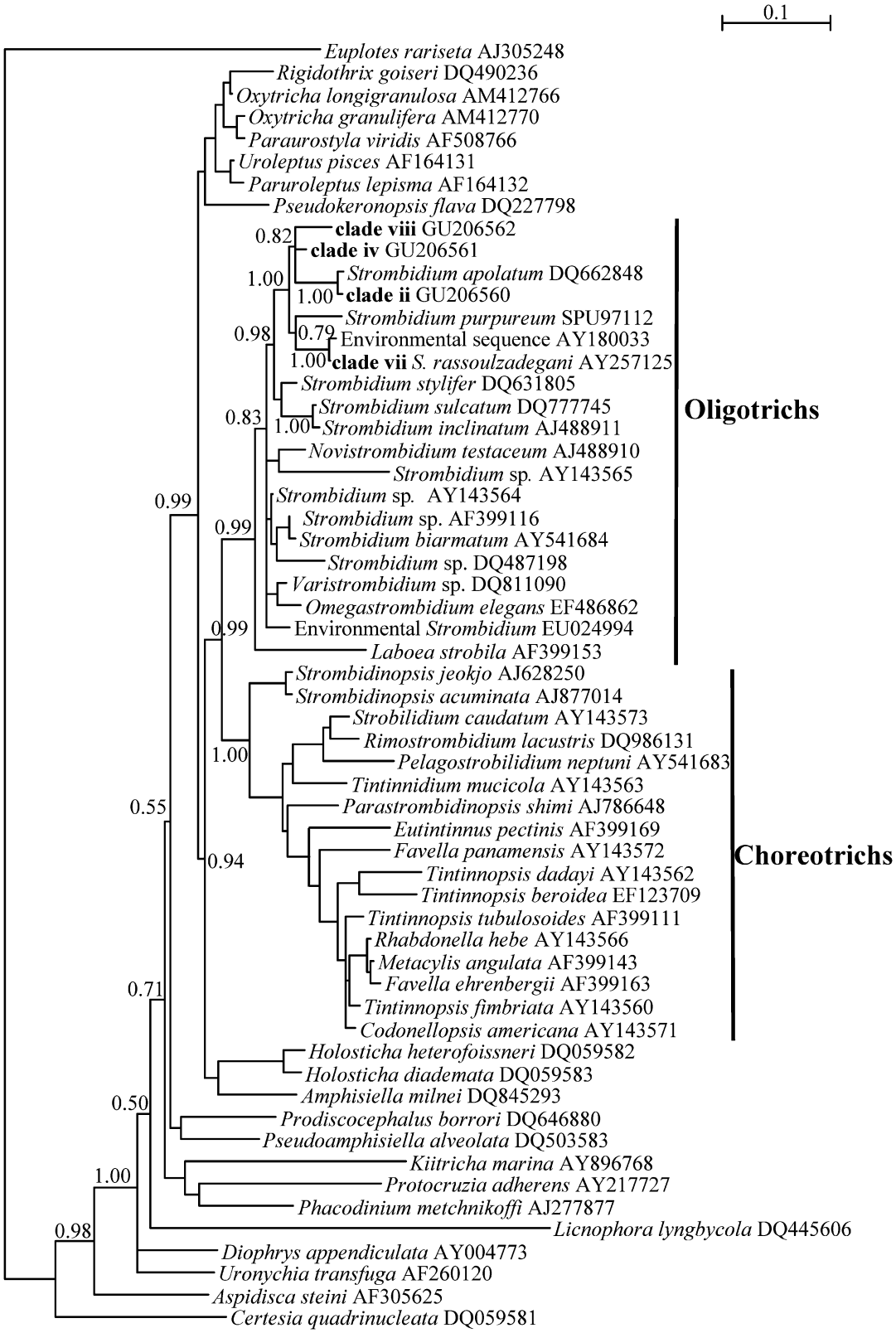

Fig. **1.** Small subunit rDNA tree of spirotrichs and related groups, showing the cryptic rockpool species cluster (i.e. Clades ii, iv, vii, and viii) within the Oligotrichs, which, along with the Choreotrichs, comprise the two dominant clades of planktonic marine ciliates. The tree was produced using Bayesian inference with the program MrBayes. Bayesian posterior probabilities are indicated at nodes relevant to higher-order taxonomy and to the specific clades sequenced for this report.

Table **2.** Morphological distinctions among four related species, including two separate populations each of *Strombidium styliferum (= stylifer)* and *Strombidium apolatum*.

Characteristic	Minimum	Maximum	Mean	*n*
Cell length				
S. rassoulzadegani n. sp.	50	74	60.5	15
S. styliferum (Song & Packroff, 1997)	38	61	46.9	16
S. styliferum (Lei et al., 1999, Qingdao pop.)	34	59	—	—
S. apolatum (Wilbert & Song, 2005, Antarctic pop.)	31	49	42.1	11
S. oculatum (Montagnes et al. 2002a, b)	48	86	64.0	—
Cell width				
S. rassoulzadegani	34	50	43.0	15
S. styliferum (Song & Packroff, 1997)	29	46	34.4	16
S. styliferum (Lei et al., 1999, Qingdao pop.)	25	42	—	—
S. apolatum (Wilbert & Song, 2005, Antarctic pop.)	25	38	33.1	11
S. oculatum (Montagnes et al. 2002a, b)	23	52	37.0	—
Apex to cytostome, distance				
S. rassoulzadegani	20	28	24.0	15
Apex to girdle kinety, distance				
S. rassoulzadegani	23	33	28.2	15
Apex to the beginning of ventral kinety, distance				
S. rassoulzadegani	33	55	40.5	15
Macronucleus length				
S. rassoulzadegani	21	30	25.2	15
S. styliferum (Song & Packroff, 1997)	12	30	20.2	16
S. styliferum (Lei et al., 1999, Qingdao pop.)	ca. 20			
S. apolatum (Wilbert & Song, 2005, Antarctic pop.)	23	32	28.6	11
S. oculatum (Montagnes et al. 2002a, b)	21	48	32.5	—
Macronucleus width				
S. rassoulzadegani	12	16	13.7	15
S. styliferum (Song & Packroff, 1997)	9	27	18.4	16
S. styliferum (Lei et al., 1999, Qingdao pop.)	ca. 19			
S. apolatum (Wilbert & Song, 2005, Antarctic pop.)	7	10	8.8	11
S. oculatum (Montagnes et al. 2002a, b)	7	18	12	—
Anterior membranelles, number				
S. rassoulzadegani	15	18	16	15
S. styliferum (Song & Packroff, 1997)	13	16	14.1	16
S. styliferum (Lei et al., 1999, Qingdao pop.)	14	17	—	21
S. apolatum (Wilbert & Song, 2005, Antarctic pop.)	12	14	12.6	7
S. oculatum (Montagnes et al. 2002a, b)	10	16	15	—
Ventral membranelles, number				
S. rassoulzadegani	7	11	8	15
S. styliferum (Song & Packroff, 1997)	9	11	9.8	16
S. styliferum (Lei et al., 1999, Qingdao pop.)	11	13	—	22
S. apolatum (Wilbert & Song, 2005, Antarctic pop.)	6	8	7.3	6
S. oculatum (Montagnes et al. 2002a, b)	10	18	14	—
Dikinetids in girdle kinety, number				
S. rassoulzadegani	50	72	62	15
S. styliferum (Song & Packroff, 1997)	ca. 50[a]			
S. styliferum (Lei et al., 1999, Qingdao pop.)	44	52	—	8
S. apolatum (Wilbert & Song, 2005, Antarctic pop.)	ca. 60			
S. oculatum (Montagnes et al. 2002a, b)	34	63	54	—
Dikinetids in ventral kinety, number				
S. rassoulzadegani	12	18	15	15
S. styliferum (Song & Packroff, 1997)	ca. 20[a]			
S. styliferum (Lei et al., 1999, Qingdao pop.)	9	14	—	21
S. apolatum (Wilbert & Song, 2005, Antarctic pop.)	ca. 45			
S. oculatum (Montagnes et al. 2002a, b)	9	23	15	—

All linear dimensions are given in μm.
[a]Estimated from published figure.

10). The cell tapers posteriorly and terminates in an elongated spine, which is about 10–15 μm long, thin, fragile but not contractile, and often not detectable after fixation (Fig. 3). The pellicle is thin, with polygonal plates on the hemitheca, as in other Strombidiidae (Fig. 2, 9). The cytoplasm often contains green chloroplasts, giving the cell an opaque and dark appearance at low magnification. An orange-red eyespot 5 μm in diameter is found in or just below the apical bump (Fig. 2). Extrusomes are

Table **3.** Presence or absence of eyespot and posterior stylet, color, and ingested food composition from observations of coastal or tidepool Strombidiidae similar to *Strombidium rassoulzadegani*.

Species	Eyespot	Stachel	Color	Food reported
S. oculatum Faure-Fremiet	Y	N	Green	Diatoms, flagellates
S. oculatum Montagnes	Y	N	Green	Diatoms, flagellates
S. apolatum Xu et al, Wilbert & Song	Y[a]	N	Brown/gray	Diatoms, yellowish algae
S. stylifer Levander	N	Y	Greenish yellow	Diatoms, no zoochlorellae
S. styliferum Kahl/Calkins	Y	Y	N	''saprobic''—decaying vegetable matter
S. stylifer Faure-Fremiet	N	Y	Yellowish green	Diatoms, dinos, no zoochlorellae
S. styliferum Song & Packroff				Flagellates and diatoms
S. styliferum Borror	Y	Y	Bluish green and yellow green bodies	Diatoms; ''cytoplasmic inclusions''
S. longipes Meunier 1910	N	Y		
S. rassoulzadegani (clade vii)	Y	Y	Green	Can eat a variety of algae, green zoospores

[a]From published figure.

14 ± 1.3 μm (SD) long, extending posteriorly and inward from the girdle and ending about halfway to the posterior end of the cell. The single macronucleus lies left of the mid-line, is broadly ellipsoidal, and contains some small heterochromatin bodies ca. 2–4 μm across (Fig. 8, 10, 11). A micronucleus was not observed. Neither a contractile vacuole nor a cytopyge was recognized. These cells are fragile, and sensitive to the pressure of a coverslip as they easily burst.

Somatic cilia are 1.5 μm long, arranged in a girdle and a ventral kinety (Fig. 5, 8). The girdle kinety is pre-equatorial, on average 33% posterior to anterior cell end, continuous, and composed of ca. 62 horizontally oriented dikinetids, each having a cilium associated only with the left basal body (Fig. 10). The ventral kinety extends meridionally on the ventral side in a furrow between the girdle kinety and the posterior end of the cell, terminates at the beginning of the posterior spine, and comprises ca. 15 dikinetids, each having a cilium associated only with the anterior basal body (Fig. 10).

The oral apparatus is typical of the genus, consisting of an endoral membrane on the inner wall of the buccal cavity and a conspicuous adoral zone of membranelles that surrounds the apical bump (Fig. 8–10). The endoral membrane is composed of a single row of monokinetids. The adoral zone of membranelles contains a distinct ventral opening and is divided into two parts that are continuous with each other: an anterior portion with 15–18 membranelles and a ventral portion with 7–11 membranelles. The anterior membranelles are 10 μm long at the base. The bases of the ventral membranelles gradually shorten from anterior to posterior; their cilia are about half as long as those on the anterior membranelles. Both the anterior and ventral membranelles are comprised of three ciliary rows.

Unlike wild populations of *S. oculatum*, *S. rassoulzadegani* n. sp. cultures do not encyst on a circatidal rhythm, but we cannot rule out that their failure to encyst rhythmically in vitro may reflect plasticity in this trait. We have not observed natural populations at high enough abundances to discern whether *S. rassoulzadegani* has rhythmic encystment in situ. Among the many isolates of this species we have observed in culture, some have produced cysts (Fig. 4) and some have not. The cysts appear typical for strombidiids, a rounded vessel capped by a frothy-appearing plug. Excystment has been observed, but we have not elucidated the cues that initiate either encystment or excystment.

Cells swim by rotation about the main cell axis, interrupted by sudden changes in direction. *Strombidium rassoulzadegani* is positively phototactic. In a shallow Petri dish, the ciliate swims in tight helices in the direction of the light source, resulting in its encounter with the bottom when viewed with a stereomicroscope, and subsequent swimming in tight counterclockwise circles (viewed from the posterior) just above the bottom (Fig. 12). Unlike some other Strombidiidae and many Choreotrichia, it does not appear to make long straight-line jumps and is relatively easy to capture with a drawn pipette.

Strombidium rassoulzadegani n. sp. is incapable of sustained growth in darkness, even at saturating food concentrations (Fig. 13). It also does not show sustained growth if starved in the light. It thus appears to be an obligate mixotroph. When feeding on the prasinophyte *T. chui*, growth was saturated at light levels above 30 μmol photons/m^2/s (data not shown) and food concentrations above 2,000 cells/ml (Fig. 13). Although it can grow on other classes of algae (Fig. 14), to date, we have observed highest maximum growth rates on prasinophytes or zoospores of green macroalgae (McManus et al. 2004). We have so far not observed whether its photosynthetic metabolism is different when it feeds on non-green algae, but the cells appear to still contain an eyespot and exhibit positive phototaxis on other diets. Parameters of the numerical response curve (μ_{max}, k, and t; Montagnes et al. 1996) can vary widely among different foods and even with the same food at different times irrespective of culture age, suggesting that the metabolic link between enslaved chloroplasts and ciliate growth is not constant.

DISCUSSION

In the past 135 years, a number of Strombidiidae bearing some of the characteristics of Clades ii, iv, vii, and viii have been described from tidepools or other inshore waters, and these reports may provide insight into the identities of the clades and, in particular, how they coexist in tidepools (Table 3).

Fromentel (1874–1876) described *Strombidium caudatum* from freshwater. This species had a tail or stalk, and when Calkins (1901) observed a similar species in New England coastal water he identified it as *S. caudatum*. Calkins mentioned a ''heap of pigment granules'' located in an anterior bump, but did not mention anything about green chloroplasts. Levander (1894) had meanwhile described *S. stylifer*, with a distinct thorn-like tail, and said that the ciliate was full of greenish-yellow colored bodies, but did not mention an eyespot. Likewise, Fauré-Fremiet (1912, 1924) described *S. stylifer* as being full of yellowish-green remains of diatoms and dinoflagellates, but specifically states that green chloroplasts (''zoochlorelles'') were not seen. This keen observer also did not mention any eyespot or reddish pigment content. Kahl (1932) described two related species, one with and one without algal pigments. He referred to these as *Strombidium styliferum* and *S. styliferum* var minor, respectively, and renamed *S. styliferum* var minor as *Strombidium calkinsi*. In a later volume, Kahl (1932, 1935, p. 840) renamed it as *S. styliferum* var minor

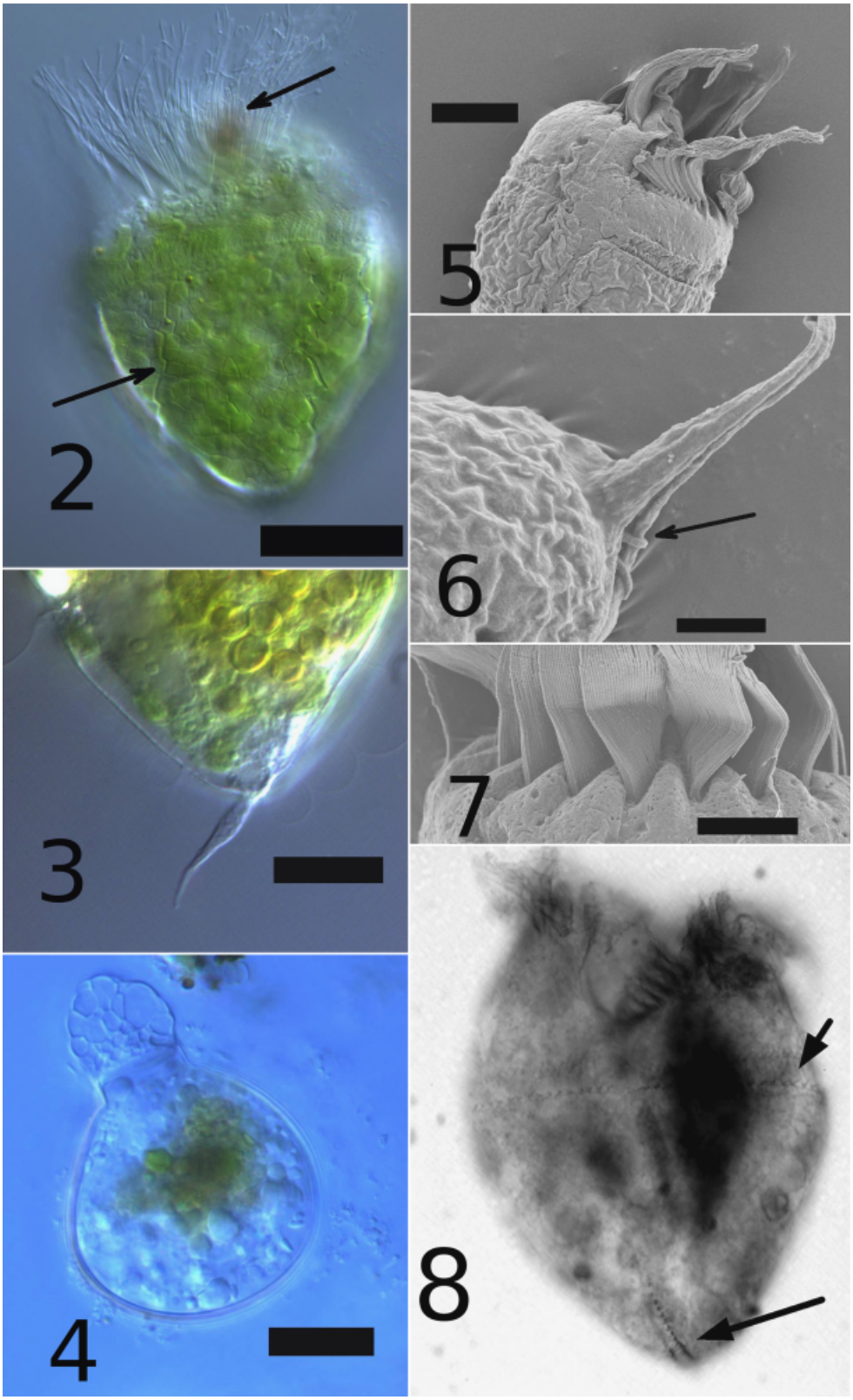

Fig. **2–8.** Images of Clade vii *Strombidium rassoulzadegani* n. sp., from cultures. **2.** Live cell, showing anterior reddish eyespot (upper arrow) and cortical plates on the posterior half of the cell (lower arrow), scale bar = 20 μm. **3.** Formaldehyde-preserved cell, showing the posterior stylet or ''stachel,'' which is not always present in cultivated cells, scale bar = 10 μm. **4.** Live cyst, showing characteristic frothy plug at the anterior, scale bar = 20 μm. **5.** Scanning electron microscopy (SEM) of ventral side, showing slight offset of ventral polykinetid zone of membranelles, scale bar = 10 μm. **6.** Obliquely ventral view, showing cilia of ventral kinety in a groove that extends to the base of the stylet (arrow), scale bar = 2 μm. **7.** Higher magnification SEM indicating insertion of oral membranelles, which are composed of three ciliary rows, scale bar = 5 μm. **8.** Micrograph of protargol-stained specimen, indicating position of girdle (upper arrow) and ventral (lower arrow) kineties.

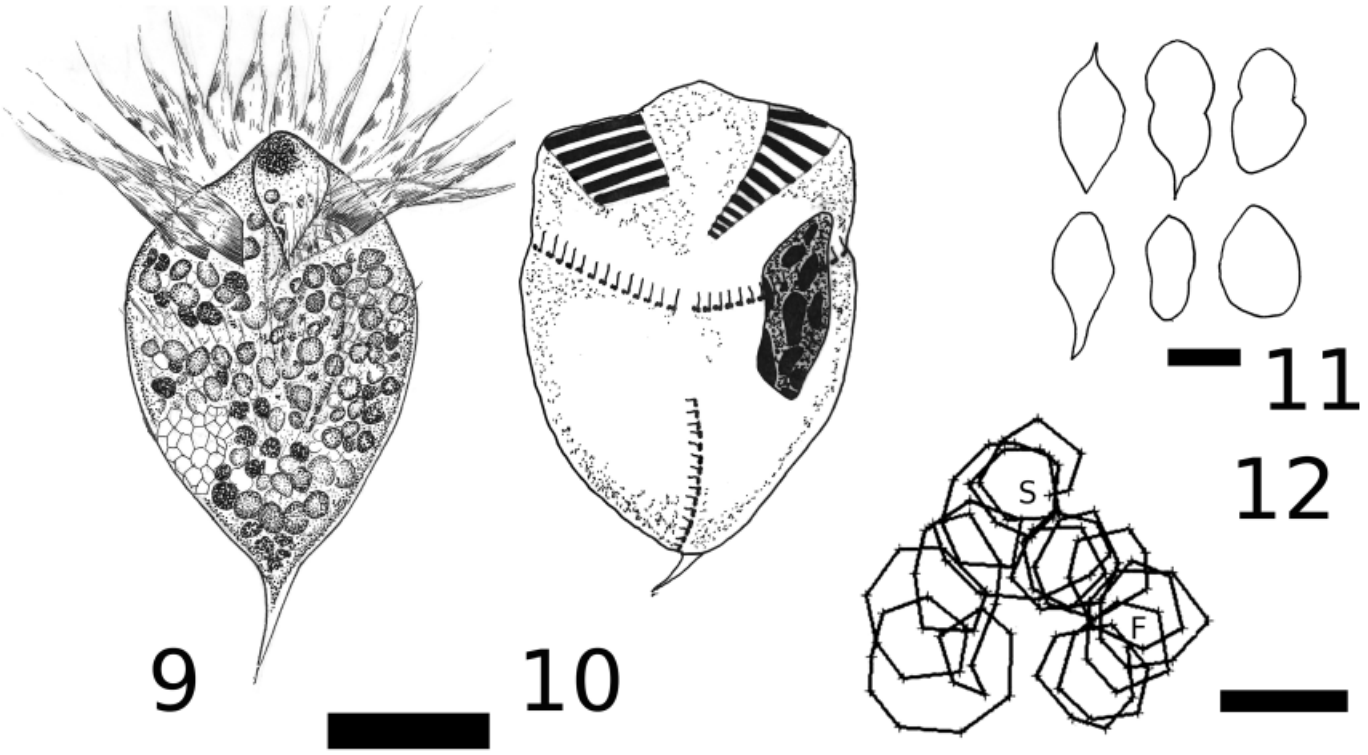

Fig. **9–12.** Line drawings of Clade vii *Strombidium rassoulzadegani* n. sp. from cultures. **9.** Drawing of live cell. **10.** Schematic of protargol-stained specimen, indicating positions of anterior membranelles, girdle and ventral kineties, and macronucleus, based on camera lucida drawings of several cells, scale bar 20 μm. **11.** Outlines of macronuclei from six individuals traced with a camera lucida from protargol-stained cells, scale bar 15 μm. **12.** Ten-second trace of typical swimming motion of *S. rassoulzadegani* n. sp. S, where the cell started and F, where it finished; cells swim in a helical motion in the direction of light until they encounter a surface, such as the bottom of a Petri dish, then they swim in tight circles; the scale bar = 150 μm, indicating that this cell translated its position only a few body lengths over the interval.

because the name *calkinsi* had been taken. Maeda and Carey (1985) later synonymized Calkins's *S. caudatum* and Kahl's *S. styliferum* var minor as *Strombidium minor*.

Kahl's error (1932, 1935, p. 840) of referring to *S. stylifer* as ''styliferum'' has persisted in some more modern reports. For example, Borror (1972) found *S. styliferum* in salt marsh pools in New England, United States, and reported the presence of an eyespot, ingested diatoms, and bluish-green and yellow-green cytoplasmic inclusions. Wilbert (1986) reported a *S. styliferum* from a saline lake in North America. Song and Packroff (1997) redescribed *S. styliferum* from Chinese coastal waters. Their isolate was colorless and their illustrations do not show an eyespot. In April 2006, McManus and colleagues published an SSU-rDNA sequence for Clade vii as *S. styliferum* (GenBank accession number AY257125); simultaneously (June 2006), Gao and colleagues published an SSU-rDNA sequence for their isolate of *S. stylifer* from Chinese coastal waters (DQ631805). These two sequences differ by more than 4%, a level beyond intraspecific variation within the genus (Fig. 1; see also Gao et al. 2009). Because the Chinese isolate has been well described morphologically (Lei, Xu, and Song 1999; Song and Packroff 1997), and displays some morphological differences in addition to the genetic ones (e.g. it is smaller and has fewer girdle dikinetids), we defer to their identification of the species, and hence erect Clade vii as a new species, *S. rassoulzadegani* n. sp. In size and ciliature our Clade vii more closely resembles the *S. stylifer* described in Song and Packroff (1997) than the *S. stylifer* population described in Lei et al. (1999), supporting the idea that there are at least two morphologically and genetically distinct strombidiids containing a posterior spine.

Strombidium oculatum is another tidepool strombidiid with green chloroplasts and a prominent eyespot. Its ecology has been well studied, and its distinctive habit of encysting on a circatidal rhythm is clearly a defining characteristic. Although its morphology has been well documented (Montagnes et al. 2002a), there are no positive identifications of it that are linked to DNA sequences. In fact, when we collected green eyespot-containing strombidiids from tidepools known to contain *S. oculatum* populations, we found evidence for multiple ribotypes, the most common of which were Clades ii, iv, vii, and viii (Katz et al. 2005). Because Clade ii is $>99\%$ identical in SSU-rDNA to the morphospecies *S. apolatum* and Clade vii contains the diagnostic tail feature it shares with *S. stylifer*, we believe that either Clade iv or Clade viii (or both) will correspond in morphology to *S. oculatum*. To confirm this, one would need to capture circatidal cysts from a wild population, hatch them to confirm morphological identity with *S. oculatum*, and sequence them for comparison with Clades iv and vii. While this may seem to be a tall order, the highly amplified nature of the ciliate macronuclear genome makes it possible to obtain sequences from individual ciliates, so it is not far-fetched to suppose that this approach is feasible (Duff, Ball, and Lavrentyev 2008; Lynn and Pinheiro 2009).

Our analysis of the SSU-rDNA sequences supports earlier results based on the ITS region, which indicated the presence of cryptic species among green tidepool Strombidiidae (Katz et al. 2005). There appear to be at least three morphologically similar species (i.e. *S. oculatum*, *S. apolatum*, and *S. rassoulzadegani*), and possibly a fourth, either Clade iv or viii, one of which may be *S. oculatum*. A morphologically similar fifth species, not observed by us from tidepools, would be *S. stylifer* Song & Packroff.

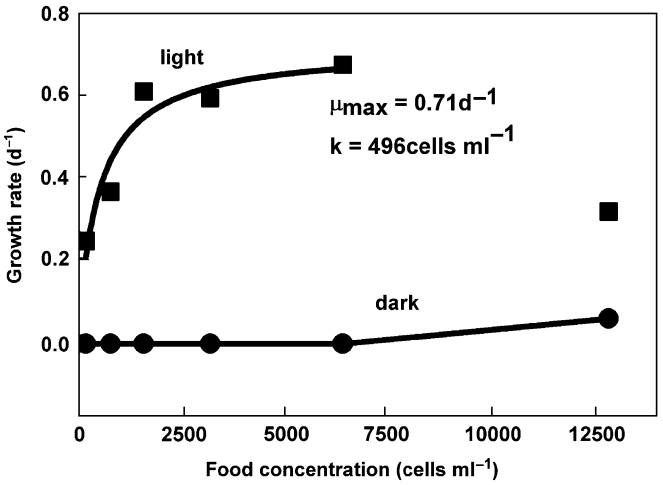

Fig. **13.** Growth rates of Clade vii *Strombidium rassoulzadegani* n. sp. fed the prasinophyte *Tetraselmis chui* in darkness or light. A value of one was added to all dark treatments to avoid ln(0); thus a growth rate of zero indicates 100% mortality. In this experiment, growth was reduced in the light at the highest food concentration (point at 12,500 cells/ml not included in curve fitting), but this was not consistently observed in other experiments (data not shown).

The species we collected have all been observed in the field as being ''grass-green,'' suggesting the use of chlorophyte or prasinophyte chloroplasts, and all contain a prominent red eyespot. The presence of several closely related and morphologically similar species in the restricted tidepool habitat raises the question as to how they can all be sustained in the same environment without competitive exclusion. We have shown that one of the species, *S. rassoulzadegani* n. sp., is somewhat plastic in the food it can eat; and its isolation and culturability at different temperatures and salinities suggest that it is quite adaptable. What is needed now is to get more members of this species cluster into culture so that aspects of ecophysiology can be measured to determine how they partition the tidepool habitat into separate niches for coexistence.

Using clone libraries created from field-collected samples, Doherty et al. (2007) showed that the number of ribotypes among oligotrichs is much greater than the number of distinct morphological types either in the same sample or in extensive morphological surveys from the same region (e.g. Doherty et al. 2010; Montagnes et al. 1988). This pattern did not hold for the choreotrichs, a sister group that contains the well-studied tintinnid ciliates. It thus appears that there is a high degree of cryptic diversity in the oligotrichs, among which the Strombidiidae are the largest family. Because we do not currently know how this cryptic genetic diversity corresponds to subtle morphological or ecological differences, it probably is best to stop naming new species in this group without accompanying genetic data (Lynn and Simpson 2009). Without such information, inferences cannot be made about biogeography, ecology, and evolution of individual species.

TAXONOMIC SUMMARY

Class Spirotrichea Bütschli, 1889
Order Oligotrichida Bütschli, 1889
Family Strombidiidae Fauré-Fremiet, 1970
Genus *Strombidium* Claparède and Lachmann, 1859
Strombidium rassoulzadegani n. sp.

Diagnosis. Size in vivo about 65 × 40 μm; generally broadly obconical, tapering posteriorly with a fragile but not contractile spine. An orange-red eyespot located in or just below an apical bump. About 16 anterior and 8 ventral membranelles. Girdle and ventral kineties consisting of 60 and 15 dikinetids, respectively. The GenBank accession number for its small subunit sequence is AY257125.

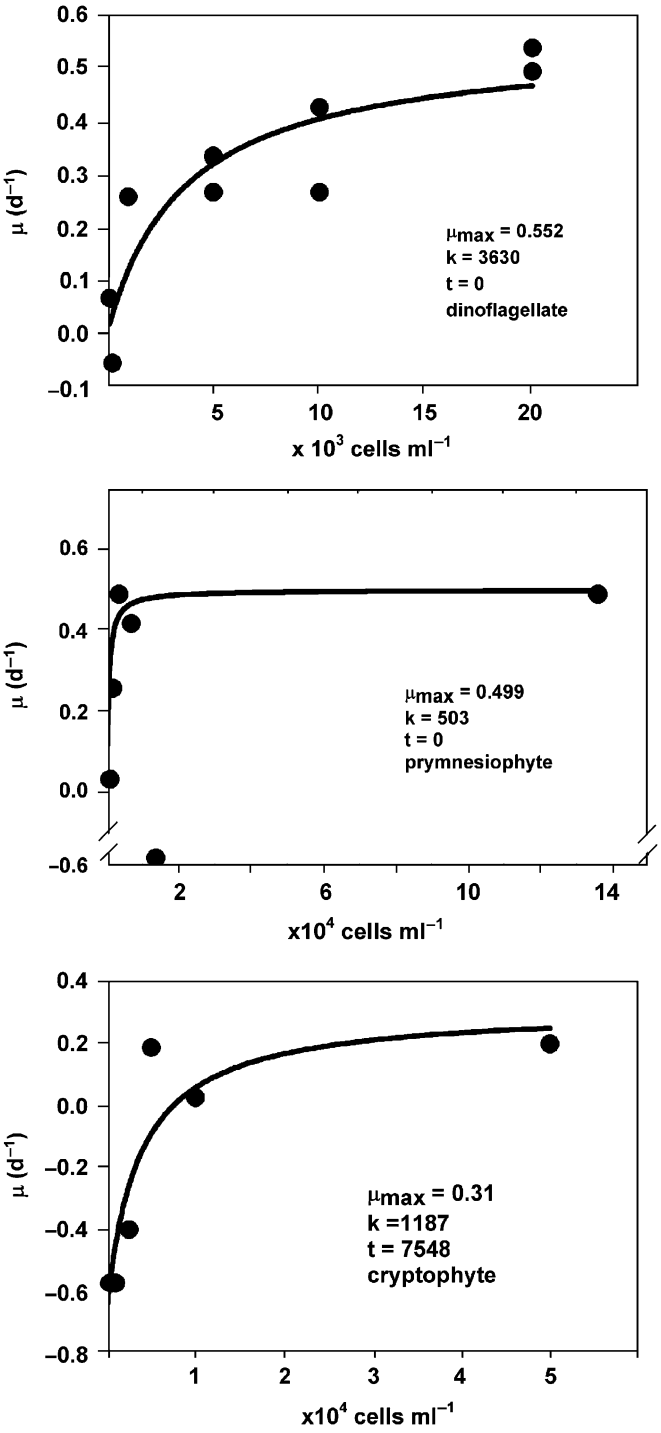

Fig. **14.** Numerical response curves for Clade vii *Strombidium rassoulzadegani* n. sp. feeding on the dinoflagellate *Prorocentrum minimum* (top), the prymnesiophyte *Isochrysis galbana* (middle), and the cryptophyte *Rhodomonas salina* (bottom). Data point showing negative growth on the prymnesiophyte at 15,000 cells/ml was omitted from the curve fitting.

Remarks. Because the new species is morphologically similar to its congeners *S. oculatum*, *S. stylifer*, and *S. apolatum*, the combination of morphological as well as molecular characteristic features is necessary for species identification.

Type locality. Type locality is Long Island Sound, NW Atlantic Ocean (41°16′N, 72°36′W).

Type deposition. A slide of protargol-stained specimens has been deposited as holotype at the National Museum of Natural History, Smithsonian Institution, USA (catalog number 1137682).

Dedication. This species is named in honor of Dr. Fereidoun Rassoulzadegan of the Laboratoire d'Oceanographie de Villefranche, who has had a long and distinguished career studying the ecology of oligotrich ciliates in the sea.

Habitat. This species has been collected from the littoral zone on rocky shores in both temperate (New England, British Isles) and tropical (Sao Sebastiao, Brazil) regions. We have isolated it from Scotland, Brazil, and the Northeast coast of the United States. In culture, it is both eurythermal and euryhaline.

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