Tintinnophagus acutus n. g., n. sp. (Phylum Dinoflagellata), an Ectoparasite of the Ciliate Tintinnopsis cylindrica Dayad 1887, and Its Relationship to Dubosquodium collini Grassé 1952

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ABSTRACT. The dinoflagellate Tintinnophagus acutus n. g., n. sp., an ectoparasite of the ciliate Tintinnopsis cylindrica Dayad, superficially resembles Dubosquodium collini Grassé, a parasite of Eutintinnus fraknoii Dayad. Dinospores of T. acutus are small transparent cells having a sharply pointed epimere, conspicuous eyespot, posteriorly positioned nucleus with condensed chromosomes, and rigid form that may be supported by delicate thecal plates. Dinospores attach to the host via a feeding tube, losing their flagella, sulcus, and girdle to become spherical or ovoid cells. The trophont of T. acutus feeds on the host for several days, increasing dramatically in size before undergoing sporogenesis. Successive generations of daughter sporocytes are encompassed in an outer membrane or cyst wall, a feature not evident in trophonts. Tintinnophagus acutus differs from D. collini in host species, absence of a second membrane surrounding pre-sporogenic stages, and failure to differentiate into a gonocyte and a trophocyte at the first sporogenic division. Phylogenetic analyses based on short subunit (SSU) ribosomal DNA (rDNA) sequences placed T. acutus and D. collini in the class Dinophyceae, with T. acutus aligned loosely with Pfiesteria piscicida and related species, including Amyloocystis ocellatum, a parasite of fish, and Paulosrella vonstoschii, a parasite of diatoms. Dubosquodium collini nested in a clade composed of several Scripsiella species and Peridinium polonicum. Tree construction using longer rDNA sequences (i.e. SSU through partial large subunit) strengthened the placement of T. acutus and D. collini within the Dinophyceae.

Key Words. Ciliate, dinoflagellate, parasite, taxonomy, tintinnids.

FRESHWATER and marine ciliates host a diverse array of parasitic organisms including bacteria, fungi, flagellates, and even other ciliates (Ball 1969). Few of these parasites have been brought into culture, and, thus, little is known about their biology, mode of transmission, or influence on host organisms. Even less is known about their prevalence in natural systems and their impact on host populations.

Among the better studied pathogens of marine ciliates are the parasitic dinoflagellates (Cachon and Cachon 1987), of which three genera, Amoebophyra, Duboscquella, and Dubosquodium, include species categorized as intracellular parasites of tintinnids, aloricate chooretrichs, oligotrichs, prorodontids, and apostomes (Cachon 1964). Several other dinoflagellate genera act as extracellular parasites of protists, invertebrates, and vertebrates, but none of their species is known to infect ciliates. The three genera that infect ciliates are placed in the Syndinea, a subdivision of dinoflagellates whose members are endoparasites (i.e. intracellular, or in host body fluids) having a flagellate dinospore stage in their life cycle and possessing histones in their nucleus (Fensome et al. 1993). Small subunit (SSU) ribosomal DNA (rDNA) sequences place Amoebophyra, Duboscquella, and other syndinians with the Group I and Group II mastigophores, forming basal lineages to the Dinokaryota (Guillou et al. 2008; Harada, Ohtsuka, and Horiuchi 2007; Skovgaard, Menese, and Angélico 2009; Skovgaard et al. 2005).

The life cycles of dinoflagellates parasitic on ciliates encompass a bi-flagellated infective stage, the dinospore, that may actively penetrate the host cell membrane (e.g. Amoebophyra) or be ingested by the host (e.g. Duboscquella) (Cachon 1964). Once inside the host, the parasite grows into a trophont that occupies much of the host cytoplasm. In Amoebophyra, the trophont is multinucleate, ruptures through the host cell at maturity, and then completes cytokinesis to liberate numerous dinospores. Duboscquella, on the other hand, remains uninnucleate as a trophont, ruptures through the host cell membrane ingesting part, or all, of the remaining host cell, then undergoes rapid sequential nuclear and cytoplasmic divisions to produce dinospores. Far less is known about the life cycle of Dubosquodium, as species of that genus have not been studied since the very brief original descriptions provided by Grassé (1952 in Chatton 1952).

Dubosquodium collini Grassé, a parasite of Eutintinnus fraknoei, is the type species for the genus and was described along with Dubosquodium kofoidi Grassé, a parasite of Tintinnopsis campanula. Placement of D. kofoidi within the genus was provisional (designated as Dubosquodium (?) kofoidi; Fig. 297 in Chatton 1952), as it exhibited an unusual “rosace” pattern during sporogenesis. Neither species was reported inside a host cell, or in a lorica that also contained a host organism. Thus, description of the two parasites relied exclusively on attributes of their post-feeding stage (= toment) and sporogenesis, leaving uncertain whether the species are endoparasitic or ectoparasitic. Species of Dubosquodium differ from those of Duboscquella and Amoebophyra by possessing a typical dinokaryon with moniliform chromosomes and by producing “Gymnodinium-like,” rather than “Oxyrrhis-like” dinospores (Chatton 1952). In addition, D. collini was reported to possess a double outer membrane.

Here we describe Tintinnophagus acutus n. g., n. sp., an ectoparasitic dinoflagellate that infects the tintinnid ciliate Tintinnopsis cylindrica. We also provide phylogenies based on SSU only and longer SSU through partial large subunit (LSU) rDNA sequences that places T. acutus within the Dinophyceae and aligned with the Pfiesteriaceae. Finally, we consider the implications of new observations for D. collini from E. fraknoei and review past reports of dinoflagellate parasites of tintinnids, offering comment regarding ambiguities in parasite life cycle, dinospore morphology, and nomenclature.

MATERIALS AND METHODS

Sampling protocol. Tintinnopsis cylindrica parasitized by T. acutus n. g., n. sp. was collected from the Chesapeake Bay during cruises in fall to spring of 1989–1991 and from shore in January, February, and December of 2008 and 2009. Cruise stations yielding infected T. cylindrica were in the mesohaline portion of the Bay (Fig. 1): Stations 858 (38°58′N; 76°23′W), 845...
In vivo observation of parasite morphology and development. In vivo morphology and development of *T. acutus* n. g., n. sp. was examined using specimens obtained in winter and spring of 1991. To follow parasite development and assess generation time, *T. cylindrica* infected by *T. acutus* measuring 5–10 μm in diameter were isolated by micropipette, washed several times in filtered site water (0.45 μm filter, Millipore Corp., Billerica, MA), and incubated in humidity chambers at ambient water temperature (6°C and 8°C) following methods of Coats (1988). Specimens were examined (400–1,000X) at 6–12 h intervals using a Zeiss WL microscope or Zeiss Axioscope with epifluorescence capabilities (Zeiss 09 filter set: 450–490 nm excitation, 510 nm barrier, 520 nm emission) to determine parasite size, shape, and number of dinospores emerging from host loricae (Carl Zeiss Inc., Thornwood, NY). The time from isolation of specimens to the first indication of sporogenesis was taken as the duration of the vegetative growth phase, while the time from the first indication of cell division to emergence of one or more dinospores from the host lorica was taken as the duration of sporogenesis. Parasite generation time was calculated as the sum of the duration of the vegetative growth phase and sporogenesis.

Additional observations of trophonts, sporocytes, and dinospores of *T. acutus* were made using wet mount preparations of specimens collected in 2008 and 2009. To obtain dinospores, infected *T. cylindrica* were isolated from plankton samples using micropipettes, washed 6 times in 0.45 μm filtered site water, and transferred to 24-well Falcon plates (Becton Dickinson & Co., Lincoln Park, NJ). Wells receiving 2 ml of filtered site water and 10–100 infected host cells were incubated at ambient water temperature (~7°C) and allowed to produce dinospores over the following 2–5 d. Photographs and measurements of live specimens were obtained using a Zeiss Axioscam interfaced with a PC running Zeiss Axiovision software (Carl Zeiss Inc.).

Specimens of *D. collini* located in the lorica of *E. fraknoii* were viewed and photographed using an Olympus IX71 microscope equipped with DIC optics and a DP71 camera (Olympus France SAS, Rungis Cedex, France) or a Zeiss Axiosvert 25 with epifluorescence capabilities (Zeiss 09 filter set: 450–490 nm excitation, 510 nm barrier, 520 nm emission). The camera was interfaced with a PC running Olympus BioCell image analysis software with scale calibration (Olympus France SAS). Measurements for specimens were obtained from digital images using Axiovision software.

**Parasite cytology.** Organisms present in 4 L of each Niskin-bottle sample were concentrated to 20 ml using 20-μm Nitex screening (Sterling Net & Twine Co. Inc., Montclair, NJ). Resulting concentrates and a portion of each net tow were preserved with modified Bouin’s fixative (Coats and Heinbokel 1982) and processed by the Quantitative Protargol Staining (QPS) technique of Montagnes and Lynn (1993). For cytological observation of *T. acutus* dinospores, infected *T. cylindrica* were incubated in filtered site water as above, with emerging dinospores preserved in 2% (v/v) glutaraldehyde, 4% (v/v) formaldehyde, or 35% (v/v) methanol for direct examination and calcoflour staining (Dider et al. 1995; Eschbach et al. 2001; Fritz and Triemer 1985; Palacios and Marín 2008), or in modified Bouin’s for QPS staining. Preserved and stained specimens were examined, photographed, and measured using a Zeiss Axioscope, Axioscam, and Axiovision software as above.

**Scanning electron microscopy (SEM) of parasite dinospores.** Dinospores of *T. acutus* obtained as above were processed for SEM following numerous protocols used in recent years to visualize thecal plates of lightly armored dinoflagellates. The protocols included “membrane swelling” techniques (Glasgow et al. 2001; Parrow et al. 2006), “membrane stripping” techniques (Mason et al. 2003; Steidinger et al. 1996), and various formulations of osmium and mercurochloride (Hansen, Daughberg, and Henrikson 2007;
Moestrup, Hansen, and Daugbjerg 2008). Images provided in this manuscript are for specimens preserved in glutaraldehyde (2% [v/v] final concentration) and stored at 4 °C. Glutaraldehyde-fixed samples were concentrated onto 1 μm and the cell pellet stored at 4 °C.

The reactions were conducted in a Biometra T-gradient thermocycler (Biometra, Goettingen, Germany) using the following conditions: an initial denaturing step at 94 °C for 2 min, followed by 35 cycles of 94 °C for 15 s, 55 °C for 15 s, and 72 °C for 1.5 min, and a final extension at 72 °C for 5 min. Amplicons were visualized on 1% agarose gels stained with ethidium bromide, purified by polyethylene glycol precipitation (Morgan and Soltis 1995), washed with 70% ethanol, and resuspended in 10 μl pico-pure distilled water. Sequencing was done with 11 primers (EukA, SR3, SR4, SR5, SR8, SR9, EukB for the SSU; Dino1662 or the Amoebohrya variant as needed, 25F1, 25R1, LSUR2 for the ITS and LSU regions; Handy et al. 2009) using a Big-Dye Terminator v3.0 Cycle Sequencing kit (Applied Biosystems, Foster City, CA) and an ABI model 3730 sequencer (Applied Biosystems), according to the manufacturer’s protocols. The amplicons were sequenced until at least double-stranded coverage was reached. Sequencer 4.8 (Genecodes, Ann Arbor, MI) was used to remove low-quality regions and assemble the individual sequence reads. The sequences were deposited in GenBank, with accession numbers listed in figures and supplemental material.

**Alignments.** To place the novel *T. acutus* n. g., n. sp., *D. collini*, and *S. trochoidea* sequences into context, an alignment of 88 SSU rDNA sequences (hereafter, SSU alignment) was compiled to encompass 86 dinoflagellates as in-group taxa with 63 identified to species, 18 to genus, and five to class, and two *Perkinsus* species as an outgroup. The alignment included sequences from all known genera of parasitic dinoflagellates for which data were available in GenBank (20 sequences from 12 genera). An additional 23 sequences were selected for inclusion in the alignment based on BLASTN searches using the novel sequences from this study as queries. The sequences were selected based on BLASTN identity (> 95% to *T. acutus*; > 98% to *D. collini* or *S. trochoidea*), where bitscores and query coverage were high (> 2,875 and > 96% to *T. acutus*; 3,074 and > 97% to *D. collini* or *S. trochoidea*). The BLAST tree widget was used to screen for identical sequences, with redundant sequences eliminated. When different length redundant sequences were available, the longer sequence was selected. Sequences of another 39 dinoflagellates not identified in the queries above were included to broaden taxonomic coverage and provide intensive sampling of genera most closely related to *T. acutus* (i.e. 13 sequences representing *Pfiesteria, Pseudopfiesteria*, and *Luciella* and 8 sequences representing *Scrippsiella* and *Peridinium*). Finally, environmental clone sequences were excluded from the alignment. Sequences were aligned using CLUSTALX 1.83 (Thompson et al. 1997) and adjusted manually using MacClade 4.08 (Maddison and Maddison 2002). Highly variable regions of the alignment were removed using GBLOCKS (Castresana 2000) with default parameter settings, except that minimum length of a block was set to five bases and the gap parameter was set to half positions.

A longer rDNA sequence alignment, hereafter termed the SSU–LSU alignment, included the SSU, ITS1, 5.8S, ITS2, and LSU regions of *T. acutus, D. collini*, and *S. trochoidea*, the two strains of *Amoebohrya* as an outgroup, and 29 other taxa in the SSU alignment for which sufficient data were available in GenBank.

**Phylogenetic analysis.** Phylogenetic trees were inferred with maximum likelihood (ML), ML distances with minimum evolution, and Bayesian inference. Modeltest v.3.7 (Posada and Crandall 1998) was used to select the most appropriate model of substitution for the ML and ML-distance methods. The GTR+I+Γ (i.e. general time reversible with invariant sites and gamma rate correction) model was identified as the best-fit model for both the SSU and the SSU–LSU datasets.
Maximum likelihood analyses were performed using RAXML with the rapid bootstrapping option and 1,000 replicates (Stamatakis 2006). Trees were visualized and graphic versions exported using FigTree v1.2.2. ML-distance analyses were performed using PAUP* 4b10 (Swofford et al. 2002), with the parameters obtained from the best-fit model (GTR+I+Γ) of nucleotide substitution. Heuristic tree searches were started with a stepwise random addition of taxa with 10 replicates, followed by a tree-bisection-reconnection (TBR) branch-swapping algorithm. Maximum likelihood-distance bootstrapping was executed with 1,000 replicates starting from a neighbor joining tree and followed by TBR branch swapping.

Bayesian analysis used MrBayes 3.1.1 (Huelsenbeck and Ronquist 2001) running four simultaneous Monte Carlo Markov Chains for 2,000,000 generations and sampling every 100 generations, following a burn in of 100,000 generations.

Taxa and GenBank accession numbers used to infer phylogenetic trees are provided in supplemental material and are indicated in the figures.

RESULTS

Description of *Tintinnophagus acutus* Coats n. g., n. sp (Table 1–3; Fig. 2–27). All life-history stages of *T. acutus* lacked chloroplasts, as indicated by the absence of chlorophyll a autofluorescence under blue–violet excitation. Early in the infection cycle, *T. acutus* measured 13–18 × 14–20 μm in vivo (Table 1) and appeared as a hyaline orb typically attached to the host stalk (Fig. 2). These colorless cells lacked flagella, were connected to the host by a thin feeding tube, and contained an acrunchy, roughly spherical nucleus with a large central nucleolus (Fig. 2 inset). Growth of the parasite was accompanied by the formation of a conspicuous food vacuole (Fig. 3 inset) located in the upper half of the cell (i.e. toward the oral opening of the host lorica) and near the origin of the feeding tube. With continued feeding and growth, the cytoplasm of the parasite trophont became opaque due to the presence of numerous translucent granules, while the food vacuole became red to dark brown in color (Fig. 3–7). Numerous short rod-like chromosomes were evident in the nucleus of small to medium-size trophonts (Fig. 5), but were less conspicuous as the parasite matured (Fig. 6).

At the end of the growth phase, the mature parasite measured 36–48 × 72–108 μm (Table 1) before undergoing a series of sporogenic divisions to produce 18–46 dinospores (29.8 ± 3.7; n = 8). The first division was marked by migration of the translucent granules toward the equator of the cell, as the nucleus divided transversely (Fig. 7). The second fission was also transverse in most instances, producing four equal-sized sporocytes arranged sequentially within the host lorica (Fig. 8). The food vacuole did not divide and usually passed to the most anterior of the first four sporocytes. The third sporogenic division typically occurred perpendicular to the first and second fissions, with orientation of subsequent divisions difficult to assess (Fig. 9). The sporocyte receiving the residual food vacuole often divided more slowly than the others and sometimes failed to fully differentiate into dinospores. Sporocytes eventually appeared as a compacted mass in the posterior half of the host lorica (Fig. 10). Condensed chromosomes were clearly visible in the nuclei of sporocytes (Fig. 11), and some of the cells had two short flagella. The host often survived infection, remaining within the lorica even though its peduncle was usually ruptured during growth and sporogenesis of the parasite. On some occasions, however, the host abandoned its lorica before the parasite completed sporogenesis. In such specimens, the mass of sporocytes eventually disaggregated as the individual cells began to move in brief, short jumps. In several

Table 1. Morphological attributes for *Tintinnophagus acutus* n. g., n. sp. trophonts in vivo.

<table>
<thead>
<tr>
<th>Trophonts</th>
<th>Range</th>
<th>Mean ± SE</th>
<th>Sample size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell width</td>
<td>12.8–47.6</td>
<td>28.6 ± 1.76</td>
<td>31</td>
</tr>
<tr>
<td>Cell length</td>
<td>13.7–108.2</td>
<td>42.9 ± 4.42</td>
<td>31</td>
</tr>
<tr>
<td>Early infection¹</td>
<td>Cell width</td>
<td>12.8–18.4</td>
<td>15.0 ± 0.88</td>
</tr>
<tr>
<td></td>
<td>Cell length</td>
<td>13.7–19.8</td>
<td>17.4 ± 0.80</td>
</tr>
<tr>
<td>Mature parasite²</td>
<td>Cell width</td>
<td>35.5–47.6</td>
<td>40.2 ± 2.62</td>
</tr>
<tr>
<td></td>
<td>Cell length</td>
<td>71.5–108.2</td>
<td>89.2 ± 7.53</td>
</tr>
</tbody>
</table>

Measurements are in μm, with mean given ± standard error (SE) to the mean.

¹Trophonts that lacked food vacuoles and associated pigmentation.

²Specimens in first nuclear division, but lacking a distinct fission furrow.

Table 2. Morphological attributes for living, preserved, and stained dinospores of *Tintinnophagus acutus* n. g., n. sp.

<table>
<thead>
<tr>
<th>Specimens in vivo</th>
<th>Range</th>
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<tbody>
<tr>
<td>Cell width</td>
<td>8.7–11.6</td>
<td>10.5 ± 0.42</td>
<td>7</td>
</tr>
<tr>
<td>Cell length</td>
<td>12.7–15.7</td>
<td>14.7 ± 0.39</td>
<td>7</td>
</tr>
<tr>
<td>Episome length</td>
<td>8.4–9.6</td>
<td>9.0 ± 0.18</td>
<td>7</td>
</tr>
<tr>
<td>Hyposome length</td>
<td>4.2–5.6</td>
<td>5.7 ± 0.29</td>
<td>7</td>
</tr>
<tr>
<td>Angle at cell apex</td>
<td>70.0–76.6</td>
<td>73.0 ± 0.80</td>
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Maximum likelihood analyses were performed using RAXML with the rapid bootstrapping option and 1,000 replicates (Stamatakis 2006). Trees were visualized and graphic versions exported using FigTree v1.2.2. ML-distance analyses were performed using PAUP* 4b10 (Swofford et al. 2002), with the parameters obtained from the best-fit model (GTR+I+Γ) of nucleotide substitution. Heuristic tree searches were started with a stepwise random addition of taxa with 10 replicates, followed by a tree-bisection-reconnection (TBR) branch-swapping algorithm. Maximum likelihood-distance bootstrapping was executed with 1,000 replicates starting from a neighbor joining tree and followed by TBR branch swapping.

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instances, the sporocytes moved out of the host lorica before fully differentiating into dinospores.

Mature dinospores were small, dinokont cells with a sharply pointed episphere, rounded hyposphere, clearly defined chromosomes, and a conspicuous yellow to orange, crescent or rod-shaped eyespot located near the origin of the flagella (Fig. 12, 13). Dinospores measured 13–16 × 9–12 µm in vivo and 11–17 × 8–12 µm after glutaraldehyde fixation (Table 2). The episphere was slightly longer than the hyposphere, appeared rigid, formed an apical angle of 70–80°, and had a noticeable anterior constriction that set the apex apart as a short cylinder (Fig. 12, 14). The sulcus was broad posteriorly, narrowed toward the girdle, and lacked a conspicuous anterior extension. The proximal and distal ends of the girdle were offset by about half the width of the girdle (Fig. 14). The trailing flagellum was 20–30 µm long. Dinospores readily attached to the body of host cells that had abandoned their lorica.
Protargol-stained dinospores measured 11–14 × 8–9 μm (Table 2) and had a large ovoid to reniform nucleus (5–7 μm maximum dimension) positioned posteriorly in the cell (Fig. 15). The nucleus contained two to five small nucleoli and was tightly packed with short, rod-like chromosomes (Fig. 15). Scanning electron microscopy revealed the presence of mastigonemes on the transverse flagellum, a thin, pointed peduncle at the base of the trailing flagellum, and a “donut-shaped” apical “knob” (Fig. 16,
17). Attempts to visualize thecal plates of dinospores using calcicolour staining and SEM were unsuccessful. However, some specimens showed partially stripped membranes that appeared to delineate thecal sutures (Fig. 16).

Once attached to its host, T. acutus had a spherical to ovoid nucleus that increased in size with cell growth (Fig. 18–20, 23, 28). Young trophonts had conspicuous, densely staining chromosomes (Fig. 19). Nucleolar number ranged from 1 to 5, with mean number of nucleoli in 5–7 μm trophonts being about half that of dinospores (Table 3; Fig. 29). The number of nucleoli per cell decreased during early growth of the parasite, stabilizing at about 1.5 in trophonts >10 μm in length. The multiple small nucleoli of early infections were usually clustered near the center of the nucleus (Fig. 18), while slightly larger trophonts (Fig. 19) generally had a single large nucleolus (Fig. 19, 23), suggesting coalescence of the multiple small nucleoli into a single nucleolus. The “coalesced” nucleolus increased in size as the nucleus became larger (Fig. 30).

Early in the infection process, protargol-stained host cells had macronuclei typical in appearance to those of uninfected cells (i.e. ovoid with a lightly staining nucleoplasm, numerous dark granules, and in some instances a replication band; Fig. 19). By the middle of the infection cycle, however, host nuclei were highly elongated or broken into globular fragments (Fig. 20). The feeding tube was clearly visible in some specimens and had two argenticophilic granules at its proximal end (Fig. 21). These granules, presumably basal bodies, did not have associated flagella. On rare occasions, a small food particle was present in the feeding tube. Food particles appeared to coalesce into a single large food vacuole that often contained densely staining material similar to that of fragmented host macronuclei (Fig. 23).

The nucleus of mature parasites was granular in appearance due to the presence of numerous small chromosomes and usually contained a single large nucleolus (Fig. 23). With the onset of sporogenesis, the nucleolus separated into numerous fragments that were distributed to daughter nuclei along with the chromosomes (Fig. 24–27). Each cell division occurred within a thin, seemingly rigid outer membrane or cyst wall (Fig. 25–27).

Tintinnophagus acutus n. g., n. sp. development time and effect on host cells. When incubated at 6–8 °C in filtered estuarine water, early infections by trophonts 5–10 μm in diameter required approximately 4 d to mature and another 2 d to complete sporogenesis (Table 4). Ignoring the time required for dinospores to encounter new host cells, generation time of T. acutus was about 6 d.

Host cells supporting mature T. acutus were visibly smaller than uninfected organisms, indicating that utilization of host biomass by the parasite exceeded host growth rate. In protargol-stained material, parasite biomass was roughly 3X that of the host and represented 71 ± 4.4% of the total biomass of host+ parasite. In addition to being smaller in size and having disrupted macronuclei, infected hosts appeared to lose the ability to reproduce, as indicated by the proportion of cells undergoing stomatogenesis (Fig. 31, 32). While ~50% of uninfected T. cylindrica had developing oral structures for their posterior daughter cell, only 18% of infected host cells showed signs of stomatogenesis. Furthermore, uninfected hosts were equally partitioned as early and late stages of stomatogenesis, while very few infected hosts (~1%) had progressed to late stomatogenesis.

Dubrosquodinium collini from Eutintinnus fraknoii. Dubrosquodinium collini measured 55 ± 2.9 × 29 ± 0.8 μm, with individuals being tightly lodged in the lorica of their host (Fig. 33). None of the loricae was occupied by living host cells, but some showed cellular debris apparently derived from recently dead host organisms. In those instances, the parasite was located above the remains of the host (i.e. toward the oral opening of the lorica). Dubrosquodinium collini lacked obvious pigmentation and chlorophyll autofluorescence, but possessed a large food vacuole, a small spherical nucleus with condensed chromosomes, and a thin outer membrane or cyst wall that had an acute peak directed towards the remains of the host (Fig. 33).
The sequence of the single specimen from January had four nucleotides in length and was successfully amplified (Fig. 36, 37).

Fig. 29. Mean nucleolar number for dinospores (DS), trophonts partitioned by nuclear length (T-1 = 5–7 μm; T-2 = 8–10 μm; T-3 = 11–13 μm; T-4 = 14–16 μm; T-5 = 17–19 μm; T-6 = 19 μm), and specimens undergoing the first sporogenic division (ED) of Tintinnophagus acutus n. g., n. sp. Standard error of the mean and sample size is indicated for each category. Data are for protargol-stained specimens.

toward the oral opening of the host lorica (Fig. 33, 34). After fixation, the outer membrane was distended away from the cell forming a smooth covering with little or no indication of the peak evident in living specimens (Fig. 35). Protargol-stained specimens showed food vacuoles in various states of digestion, nuclei with condensed chromosomes, and karyokinesis typical of dinoflagellates (Fig. 36, 37).

rDNA sequences. The rDNA region of T. acutus n. g., n. sp. was 3,619 nucleotides in length and was successfully amplified from nine individuals in late sporogenesis. All specimens were collected from the Rhode River, MD, USA with one obtained in January 2008 and eight in December 2008. Sequences for specimens isolated in December were fully resolved and were identical. The sequence of the single specimen from January had four ambiguous base positions in the ITS regions, but was otherwise identical to the other eight sequences. BLASTN searches of GenBank provided a 96% maximum match of the T. acutus SSU rDNA sequence to representatives of other dinoflagellate genera, including Paulsenella (AJ968729), Pentapharsodinium (AF022201; AF274270), Peridinium (AY443018), Prorocentrum (EU780638; Y16232), Scrippsiella (AM494499; AB183677; AF274276), and Stoeckeria (FN557541).

Two D. collini individually isolated from a single sample collected from the Bay of Villefranche-sur-Mer, France in September 2009 yielded rDNA sequences of 3,009 and 3,620 nucleotides, with discrepancy in length due to incomplete sequencing of the LSU rDNA. The sequences differed by one base position in the SSU and five in the ITS through the LSU regions (0.06% difference in the SSU rDNA; 0.2% overall). A BLASTN query matched the SSU rDNA of D. collini to Scrippsiella spp. (AB183677, AF274277, AJ145515, AM494499, AY743960) with 99% identity. A BLASTN query using the SSU sequence of Scrippsiella trochoidea (CCMP 2771; total sequence length = 3,530 nucleotides) gave identical results to those obtained for queries using D. collini.

Comparison of the SSU rDNA sequences showed marked difference between T. acutus and D. collini (63 bases; 3.5%). The SSU rDNA of D. collini and S. trochoidea, however, differed by only four bases (0.2%). Sequence divergence was greater when compared across the entire rDNA region, with T. acutus and D. collini differing by 331 bases (9.1%), while D. collini and S. trochoidea differed by 84 bases (2.4%).

Phylogenetic analyses. Total length of the SSU alignment for 86 in-group taxa (i.e. seven syndinians and 79 dinophyceans) and two outgroup taxa (Perkinsozoa) was 1,807 bases, with sequence length ranging from 1,206 to 1,754 bases. Trimming the alignment using Gblocks left 1,706 positions for phylogenetic analyses.

The ML tree inferred from the SSU alignment had a ln value of −17486.960781 and is presented with ML bootstrap values, Bayesian posterior probability, and ML-distance bootstrap values (Fig. 38). The three analyses produced trees of comparable topology, showing a moderately supported split (ML and ML-distance bootstrap values >70%; posterior probability ≥0.90) between the Dinophyceae and Syndiniophyceae, with T. acutus n. g., n. sp. and D. collini nested among thecate dinoflagellates deep within the Dinophyceae. Clades representing the Suessiales, including the fish parasite Piscinooodinium sp., Prorocentrum, Heterocapsa, and a prominent “Pfiesteria group” containing Luciella masanensis, Cryptoperidiniopsis species, Pseudopfiesteria shumwayae, and Pfiesteria piscicida were well supported (ML and ML-distance bootstrap values >85%; posterior probability ≥0.95) and consistent across the phylogenies. A “Scrippsiella group,” including Peridinium polonicum, D. collini from E. fraknoii, and all but one of the Scrippsiella species (i.e. S. hangoei) was well supported by Bayesian and ML-distance analyses, but poorly supported by ML bootstrapping (<65%). Small
subunit rDNA sequences clustered in the *Scrippsiella* group differed by a maximum of 1.6%, with the two sequences for *D. collini* differing from that of *S. sweeneyae*, type species for the genus, by only 0.3–0.4%. The genera *Haplozoan* and *Chytridiunium* each formed a monophyletic lineage of parasites; however, their placement within the Dinophyceae was not well resolved. *Blastodinium* species from copepods consistently failed to group together and were polyphyletic. *Paulsenella vonstoschii*, a parasite of diatoms, branched adjacent to the *Pfiesteria* group with moderate support (i.e. bootstrap values of 65–80%, or posterior probabilities of 0.90–0.95). The positions of *Amyloodinium ocellatum*, a parasite of fish, and *T. acutus* from *T. cylindrica* were not well resolved, but each consistently branched outside the *Pfiesteria* group.

The SSU–LSU alignment of 29 in-group taxa and two Amoebophrya spp. as outgroup taxa was 4,188 nucleotides long, with individual sequence length ranging from 3,046 to 6,332 bases. After trimming with Gblocks, 2,853 positions remained for use in phylogenetic analyses. The selected positions represented 99% of the SSU rDNA, 16% of ITS1, 99% of the 5.8S, 11% of ITS2, and 58% of the LSU rDNA in the aligned dataset. The ML tree inferred from the SSU–LSU alignment had a ln value of \(-16599.185661\) (Fig. 39). The SSU–LSU trees were similar to the SSU trees in topology and were largely congruent across methods; however, disagreements were found using the ML-distance analysis (cf., Fig. 38, 39). The ‘‘Pfiesteria group’’ was a strongly supported clade in all three analyses with bootstrap values of 100% and posterior probability of 1.00 (Fig. 39). *Stoeckeria* sp. and the dinophycean ‘‘Shepherd’s crook’’ formed a well-supported sister lineage to the ‘‘Pfiesteria group,’’ to which *T. acutus* and *D. collini* were consistently basal. *Dubosquodinium collini* and *S. trochoidea* grouped together with strong support in all three analyses (Fig. 39). Placement of *T. acutus* was less well resolved, as it fell between the *Stoeckeria*–‘‘Shepherd’s crook’’ clade and the *Scrippsiella–Dubosquodinium* clade in ML and Bayesian analyses, but clustered with *S. trochoidea* and *D. collini* by the ML-distance method.

**DISCUSSION**

Comparison of *Tintinnophagus acutus* n. g., n. sp. with other dinoflagellate parasites of tintinnids. Haeckel (1873) noted the presence of 10–20 small, spherical cells in the cytoplasm of two
tintinnid species, *Cyttarocylis cassis* (reported as *Dictyocysta cassis*) and *T. campanula* (reported as *Codonella campanella*), interpreting them to be ciliate reproductive propagules or spores. When removed from the ciliate, the “spores” lacked cilia or flagella and contained a spherical nucleus. In one *T. campanula*, Haecckel (1873) also reported larger (30 × 20 μm), uniformly ciliated cells that he interpreted to be ciliate embryos. Haecckel’s spherical spores are now considered to be the first report of dinoflagellate parasitism in ciliates, being placed by Chatton (1920) in the genus *Duboscquella*, while the nature of the enigmatic ciliated “embryos” remains uncertain.

Several years later, Laackmann (1906, 1908) mistook parasitism of *T. campanula* and *Cyttarocylis helix* (basionym: *Tintinnus helix* Claparède & Lachmann, 1858; used hereafter) as sexual reproduction. His 1906 article provided an account, but no illustrations, of parasites, called sporocysts by Laackmann, which pinched off the posterior end of *T. campanula* and divided to produce either microspores or macrospores. Microspores and macrospores formed in separate host loricae and were, respectively, characterized as ~5 μm spherical cells that numbered well over 100 per tintinnid and much larger gymnodinoid cells (17–20 μm long by 10–12 μm wide) that numbered 12–24. Arrangement and number of flagella were not determined, but spores were reported to move rapidly in a sinusoidal fashion while spinning around their longitudinal axis. Laackmann (1908) expanded his observations for *T. campanula*, noting that many specimens contained a darkly staining mass positioned posteriorly in the cell, but were otherwise normal in appearance. He interpreted these bodies as developing “sporocysts” (i.e. parasites) and provided an illustration (his Fig. 33) that conforms nicely in position, shape, and nuclear morphology to parasites now placed in the genus *Duboscquella* (Cachon 1964; Coats 1988; Coats et al. 1994). Laackmann (1908) also described parasite development in *T. helix*, including several illustrations, but never mentioning “sporocysts” in the cytoplasm of the host. Rather, host loricae contained a large cytoplasmic mass, presumably the tintinnid zooid, to which was attached a small “sporocyst.” In vivo, these small “sporocytes” gradually increased in size, while the cytoplasmic mass of the host cell diminished. Large “sporocysts” in *T. helix* eventually divided to produce gymnodinoid macrospores (his Fig. 21) that were similar in appearance and behavior to those observed in *T. campanula* (Laackmann 1906), but no microspores were reported. The gradual growth of parasites attached to the outside of *T. helix* and the lack of microspore formation, strongly suggest that Laackmann was dealing with two different dinoflagellates, an intracellular parasite in *T. campanula*, resembling a species of *Duboscquella*, and an extracellular parasite in *T. helix*, perhaps related to *T. acutus* n. g., n. sp.

Lohmann (1908) described colorless, parasitic gymnodinoid macrospores that emerged from the lorica of *Tintinnopsis nucula*
(now considered a species of *Stenosemella*, possibly *S. nivalis*, Agatha, pers. comm.) and had a distinct girdle and sulcus with associated transverse and longitudinal flagella. Lohmann (1908) clearly illustrated macrospores of the parasite (his Fig. 6) and, in his figure legend, provided for them the new species name *Gymnodinium tintinnicola*. He believed *G. tintinnicola* to be an intracellular parasite,
although he did not report seeing the parasite in the cytoplasm of the host cell. Rather, his conclusion appears to be based on the account of "sporocyst" development provided by Laackmann (1908). In light of the discussion above, Lohmann’s *G. tintinnicola* could be either an intracellular or an extracellular parasite.

Entz (1909) mentioned gymnodinoid spores produced by a parasite of *Favella ehrenbergii* (reported as *Cyttarocylis ehrenbergii*), but did not provide illustrations. A year later, Duboscq and Collin (1910) described what they interpreted as sexual spores of *F. ehrenbergii* and *T. campanula*, but, as mentioned above, the status of those "embryos" is very uncertain. Chatton (1920) created the genus *Duboscquella* to include parasites of *T. campanula* (Laackmann 1906), *Stenosemella* cf. *nivalis* (Lohmann 1908), and *F. ehrenbergii* (Duboscq and Collin 1910; Entz 1909). He also included Haeckel’s "ciliate embryos" from *T. campanula*, mistakenly referring to the host as *Codonella galea*, but, as mentioned above, the status of those "embryos" is very uncertain. Chatton (1920) viewed infections of the various hosts as representing a single species of intracellular parasite and included all under the type species *Duboscquella tintinnicola* (Lohmann 1908). Chatton 1920. In making that decision, Chatton (1920) accepted the report of Duboscq and Collin (1910) as a redescription of *G. tintinnicola*, even though dinospore morphology was not consistent with that reported by Lohmann (1908). Seven additional species of *Duboscquella* have been subsequently described (Cachon 1964; Chatton 1952; Coats 1988), none of which produces dinospores having a clearly defined girdle and sulcus. Macrosperes of *Duboscquella* species described in these more recent manuscripts have a rounded episphere, cylindrical to conical hyposome, a trailing flagellum, and a transverse flagellum loosely wrapped around the cell. In many respects, they resemble an inverted form of the spores described by Duboscq and Collin (1910). The resemblance was sufficiently striking for
Cachon (1964) to suggest that Duboscq and Collin (1910) were mistaken about the polarity of their specimens. Since Lohmann’s (1908) account of *G. tintinnicola*, the only dinoflagellate parasites of tintinnids reported to have gymnodinoid spores belong to the genus *Tintinnaphagus* (this manuscript) and *Duboscquodinium* (Grassé 1952 in Chatton 1952), the latter said to produce gymnospores, but no illustrations of the spore were provided. The descriptions provided by Grassé (1952 in Chatton 1952) for *D. collini*, the type species infecting *E. fraknoi*, and *D. kofoidi*, a parasite of *T. campanula*, are the only accounts of these parasites in the published literature. Cachon (1964), who worked extensively on parasites of protozoans in the Mediterranean Sea, did not encounter tintinnids infected by *Duboscquodinium*. Our specimens, obtained from a single, fortuitous sample taken from the Bay of Villefranche-sur-Mer, did not permit examination of the parasite’s complete life cycle, but were sufficient to identify the organism as *D. collini* based on tomont morphology and host species. Aside from a distinctive peak in the outer membrane or cyst wall at one end of the parasite, our specimens conformed in all respects to the tomont of *D. collini*. Importantly, the peak was not present in all specimens, nor did it persist following fixation, suggesting that it is a transient feature, which could have been easily overlooked by Grassé (1952 in Chatton 1952).

Grassé (1952 in Chatton 1952) believed *D. collini* and *D. kofoidi* to be intracellular parasites, but did not report seeing infected host cells, as his observations were based on pre-sporogenic and sporogenic stages of the parasite in loricae devoid of host organisms. Our observations were likewise hampered by the absence of living hosts in loricae containing *D. collini*. Thus, we cannot comment on the nature of the parasitic relationship of *D. collini* with its host, or compare life styles (i.e. intra- vs. extracellular parasitism) of *Tintinnaphagus* and *Duboscquodinium*. *Tintinnaphagus acutus* resembles *D. collini* by its dinokaryotic nucleus with condensed chromosomes, conspicuous food vacuole in the trophocyte, and palintomic sporogenesis leading to gymnodinoid dinosavors. However, *T. acutus* differs from *D. collini* by host species, the absence of an outer membrane or cyst wall surrounding pre-sporogenic stages, failure of the first sporogenic division to differentiate a trophocyte (i.e. non-dividing daughter cell that presumably continues to feed) and a gonocyte (i.e. daughter cell that continues to divide; = gametocyte of Grassé), and rDNA sequence (see below for further molecular comparison). Late in the sporogenesis of some specimens, however, *T. acutus* did produce a sporocyte that failed to divide further and eventually degenerated, as Grassé (1952 in Chatton 1952) reported for the trophocyte of *D. collini*. *Tintinnaphagus acutus* differs from *D. kofoidi* by the absence of a ‘rosace’ stage during sporogenesis and by host species. Were it not for this ‘rosace’ stage, Grassé’s description of *D. kofoidi* would correspond nicely with Lachmann’s (1906, 1908) account of macrospore formation in *T. campanula*.

The striking difference in spore morphology between Lohmann’s *G. tintinnicola* from *S. cf. nivalis* and the account provided by Duboscq and Collin (1910) for parasitism of *F. ehrenbergii* raises the possibility that the two studies may have been dealing with different parasitic organisms. Indeed, based on macrospore morphology, Lohmann’s *G. tintinnicola* from *S. cf. nivalis* would seem more closely aligned with *Duboscquodinium* and *Tintinnaphagus* than with other species currently included in the genus *Duboscquella*. Should future investigation of parasitism in *S. cf. nivalis* support that conclusion, then major restructuring of the genus *Duboscquella* would be indicated. In recognizing the possibility that *T. acutus* may be related to *D. tintinnicola* of Lohmann (1908), it is necessary to emphasize that the two organisms show important differences. While their dinosavors are similar in size, those of *T. acutus* have a sharply pointed episome and a conspicuous eyespot at the base of the flagella. By contrast, the macrospores described by Lohmann (1908) had a smooth, rounded episome and lacked an eyespot.

**Prior reports of parasitism in *Tintinnopsis cylindrica***. Parasites of *T. cylindrica* (≡ *kofoidi*) have been reported on three prior occasions, once without providing a taxonomic identification (Hada 1932) and twice as *Duboscquella* sp. (Agatha and Riedel-Lorjé 2006; Akelman and Santinelli 1989). Hada (1932) appears to have seen an intracellular parasite, with his illustrations being more or less consistent with a species of *Duboscquella*. Akelman and Santinelli (1989) provided a single illustration of parasite sporogenesis and insufficient description of the parasite to determine if they were working with a species of *Duboscquella*, *T. acutus*, or some other organism. The specimens of Agatha and Riedel-Lorjé (2006), however, were certainly parasitized by *T. acutus*, as their illustrations clearly show growth and sporogenesis of the extracellular parasite. They also report changes in the host macronucleus as the parasite ‘sucks out the tintinnid cell.’

**Phylogenetic placement of *Tintinnophagus acutus* n. g., n. sp. and *Duboscquodinium collini***. Historically, most species of parasitic dinoflagellates have been classified as members of the Blastodiniphyceae or Syndiniophyceae, with only a few genera placed among the Dinophyceae (see Coats 1999 for review). Recent work, however, has demonstrated that the Blastodiniphyceae is an artificial assemblage, with several of its members now distributed among the Dinophyceae (Gómez, Moreira, and López-García 2009; Landsberg et al. 1994; Levy et al. 2007; Litaker et al. 1999; Saldarriaga et al. 2001; Skovgaard and Salomonsen 2009; Skovgaard, Massana, and Saiz 2007). As in these reports, our phylogenies placed *Amphyooidinium*, * Blastodinium*, *Chrytidinium*, *Dissodinium*, and *Piscinoodinium* within the Dinophyceae, well removed from the syndinian genera *Syndinium*, *Hematodinium*, *Amoebophrya*, *Duboscquella*, and *Ichthyodinium*. Our results also placed *T. acutus* and *D. collini* (previously classified as a syndinane) deep within the Dinophyceae and nested among thecate dinoflagellates, establishing that neither species belongs to the Syndiniophyceae.

The rDNA sequences of *T. acutus* and *D. collini* differed by 3.5% in the SSU rDNA and by 9.1% across the SSU, ITS1, 5.8S region, ITS2, and partial LSU rDNA region, supporting morphological criteria for separation at the species level. Phylogenetic analyses using SSU rDNA sequences linked *T. acutus* to a well-supported clade containing genera typically assigned to the *Pfiesteriaceae* (Mason et al. 2007; Steidinger et al. 2006) and closely associated with *collini* with *Scrisspistella*, including the type species *S. sweeneyae*.

Expanding our phylogenies to consider a longer region of the rDNA region (i.e. SSU through partial LSU rDNA region) provided strong support for an affiliation between *D. collini* and *S. trochoidea*, suggesting that the two species may be congeneric. That notion was also supported by the gross similarity in rDNA sequences of *D. collini* and *S. trochoidea*, as the two differed by <1% in the SSU rDNA and by only 2.4% across the SSU through partial LSU rDNA region. Certainly, additional study of *D. collini* life-history stages, including assessment of plate tabulation in the dinospare stage, is needed before its taxonomic status can be adequately assessed.

Even our SSU-LSU phylogenies failed to clearly define associations between *T. acutus* and closely related Dinophyceae, although two of our three tree building methods (i.e. ML and Bayesian inference) placed it as a sister lineage to the *D. collini*–*S. trochoidea* clade and basal to clades predominantly composed of lightly thecate Dinophyceae. Thus, we refrain from assigning *T. acutus* to a family of dinoflagellates, opting instead to include it in the Dinophyceae and provisionally within the subclass Peridiniphyceae, based on phylogenetic analyses and
morphological observations suggesting the presence of delicate thecal plates.

TAXONOMIC SUMMARY

Phylum Dinoflagellata Batschli, 1885, emend. Fensome et al. 1993
Subphylum Dinokarya Fensome et al., 1983
Class Dinophyceae Pascher, 1914
Subclass Peridiniphyceidae Fensome et al., 1983 (incipitae sedis)

Tintinnophagus Coats n. g.

Diagnosis. Aplasticid ectoparasites feeding on tintinnid ciliates. Trophont lacks flagella, cingulum, and girdle, feeds myzocyotically on host, and possesses a dinokaryon with chromosomes visible in vivo or after staining. Reproduces by palmyzocytotically on host, and possesses a dinokaryon with chromosomes visible in vivo or after staining. Reproduces by palmyzocytotically on host, and possesses a dinokaryon with chromosomes visible in vivo or after staining. Reproduces by palmyzocytotically on host, and possesses a dinokaryon with chromosomes visible in vivo or after staining. Reproduces by palmyzocytotically on host, and possesses a dinokaryon with chromosomes visible in vivo or after staining.

Type species. Tintinnophagus acutus Coats, 2010

Type host. Tintinnopsis cylindrica Dayad, 1887

Epithet. Genus name is derived from type host genus, Tintinnopsis, and the Greek phaein (-phagos) meaning eat (-eating) and is Latinized in the masculine to imply tintinnid eater.

Tintinnophagus acutus Coats n. sp.

Diagnosis. Dinospore 13–16 by 9–12 µm with rigid form, sharply pointed epimere terminating in a cylindrical “knob,” and a conspicuous yellow to orange cylindrical or crescent-shaped eyespot at base of flagella. Large, ovoid to reniform nucleus positioned posteriorly in cell. Cingulum offset by one-half its width at union with sulcus. Trophont variable in dimension depending on age, with nucleus and nucleolus increasing in size with cell growth.

Type host. Tintinnopsis cylindrica Dayad, 1887

Type habitat. The mesohaline portion of Chesapeake Bay, a moderately stratified estuary bordered by the states of Maryland and Virginia, USA.

Type locality. Rhode River, MD, USA (38°53.14’N; 76°32.50’W).

Type material. Hapantotypes, slides with protargol-impregnated T. cylindrica infected by T. acutus, have been deposited in the International Protozoan Type Slide Collection, National Museum of Natural History, Washington, DC, USA and given the following registration numbers: 1142429 [IZ] and 1142430 [IZ].

Epithet. Species name is the Latin acutus meaning pointed and is used to reflect the sharp convergence of the epimere at the cell apex.

Gene sequence. The SSU–LSU rDNA sequence is deposited as GenBank Accession No. HM483397.

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LITERATURE CITED


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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. TAXA AND GENBANK ACCESSION NUMBERS USED TO INFER PHYLOGENETIC TREES.

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