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ORIGINAL ARTICLE

Dartintinnus alderae n. g., n. sp., a Brackish Water Tintinnid (Ciliophora, Spirotrichea) with Dual-ended Lorica Collapsibility

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ABSTRACT

A tintinnid ciliate isolated from waters of the Thames River (Connecticut, USA) is described through combined in vivo observation, protargol impregnation, and phylogenetic analysis. The novel genus *Dartintinnus* and its type species, *D. alderae* are distinct from established tintinnid taxa by a lorica that collapses on both anterior and posterior ends. *Dartintinnus* is placed in the family Eutintinnidae based on a hyaline, elongated lorica opened at both ends, a ciliary pattern including a ventral kinety, at least one dorsal kinety, and right, left and lateral fields, and a sister relationship with *Eutintinnus* in gene trees. Main differences between *D. alderae* and *Eutintinnus* species include a 5.5–6.5% divergence in the small subunit rRNA gene, the geometry of the lorica (resembling an isosceles tetrahedron when collapsed vs. a cylinder, respectively), the number of macronuclear nodules (two vs. four), and the number of dorsal kineties (one vs. usually two). Considering the features of the new genus, we improve the diagnosis of the family Eutintinnidae, including the presence of a lateral ciliary field that had been overlooked in some *Eutintinnus* species. This work exemplifies the potential for novel diversity, even in these relatively well-studied protists, and the importance of an integrated approach for the description of tintinnid taxa.

TINTINNID ciliates have a long taxonomic history, with the first description dating back to the 18th century (Müller 1779) and extensive documentation during classical oceanographic expeditions (Jörgensen 1924; Kofoid and Campbell 1929, 1939; Laackmann 1910) as well as coastal sampling sites (von Daday 1887; Fol 1881). Owing to their lorica, an external structure that is both resistant to sampling damage and easy to characterize by light microscopy, detailed inventories of tintinnid taxa exist around the world, although with disparities in geographical and temporal coverage (Alder 1999; Dolan 2017; Modigh and Castaldo 2002). Given that marine surveys commonly produce lists of known tintinnids (Dolan and Pierce 2013), the lineages included in this group are assumed to be known, at least based on lorica morphology.

Because tintinnids are mostly marine, they have been less frequently targeted in limnetic and brackish waters, where in turn the most recently discovered genera, *Membranicola* and *Nolaclusilis*, have been found (Foissner et al. 1999; Sniezek et al. 1991; Snyder and Brownlee 1991). Also isolated from brackish waters, the new genus *Dartintinnus* and its type species, *D. alderae*, are described here. Tintinnids presenting an elongated lorica with dual-ended collapsibility were first observed in the Black Sea, but remained undescribed (Gavrilova and Dolan 2007; Gavrilova and Dovgal 2016). The discovery of specimens with

similar features in the Thames River (Connecticut, USA) has allowed an integrated study of in vivo and preserved materials, including lorica and cell morphology, phylogenetic relationships, and some basic behavioral and ecological aspects as recommended for description of tintinnid taxa (Santoferrara et al. 2016). Such integrated approaches are key to improving tintinnid classification and evolutionary hypotheses, which have been hindered by the ambiguity of lorica features and the scarcity of information on more reliable characters, such as ciliary patterns and DNA sequences (Agatha and Strüder-Kypke 2013, 2014).

MATERIALS AND METHODS

Collection and cultivation

Surface waters along the Thames River (41.31–41.52°N, 72.07–72.09°W) were sampled in the summer and fall of 2016 and in the summer of 2017 (Table 1 and Fig. S1). GPS position, temperature and salinity were determined with a CastAway CTD (Xylem, Rye Brook, NY). Plankton samples were collected by towing a 20-μm-mesh net within the top half-meter of water. Approximately 200 liters of water were sampled at each station (based on the net radius and a tow rate of 2.5 m/s over a 5-min sampling period). From the resulting concentrate, a subsample of 50 ml was fixed with non-acid Lugol's solution (2% final concentration) and stored at 4 °C, while the remaining volume was kept unpreserved for cultivation and observation in vivo. Occurrence of *Dartintinnus alderae* was assessed from Lugol's samples observed with an inverted microscope (Olympus IX70; 400X). Living specimens were observed under a dissecting microscope (Olympus SZX16; 3.5–90X), then isolated with a drawn glass pipette and placed individually into 24-well culture plates. Clonal cultures were achieved in sample water filtered through a 3-μm membrane, and supplemented with *Rhodomonas lens* (strain CCMP739; 4×10^4 cells/ml) and *Isochrysis galbana* (strain T-iso; 1×10^4 cells/ml) as food. Weekly, cultures were refreshed with 1 ml of leftover filtered sample water. Cultures were maintained at 19 °C on a 12 h:12 h light:-dark cycle at ~50 μmol photons/m^2/s.

Morphological investigations

Specimens were observed with a compound microscope (Olympus BX50; 400–1,000X) under bright-field and differential interference contrast. Cultured specimens were (i) examined in vivo in slide and coverslip preparations, (ii) fixed with Lugol's solution for lorica measurements, and (iii) fixed with Bouin's solution and impregnated with protargol (Wilbert 1975) to study the ciliary patterns. Morphometrics, micrographs, and digital videos were collected with NIS-Elements Advanced Research imaging software v. AR-3.00 (Nikon, Melville, NY). Lorica and cytological terminology follows Agatha and Riedel-Lorjè (2006), Agatha and Strüder-Kypke (2007), and Agatha and Simon (2012).

Table 1. Occurrence of *Dartintinnus alderae* along the salinity gradient in the Thames River, Connecticut, USA

Date	Number of stations	Temperature (°C)	Salinity		
			1.0–10.9	11.0–17.0	17.1–28.0
6/22/2016	6	24.0–25.0	n.s.	++	n.d.
6/29/2016	5	21.0–23.0	n.s.	++	n.d.
7/13/2016	6	24.0–26.0	+	++	n.d.
7/22/2016	5	24.0–26.0	+	++	n.d
9/8/2016	6	22.7–23.4	+	++	n.d.
11/17/2016	4	11.0–14.0	n.s.	n.d.	n.d.
6/15/2017	6	21.0–23.1	n.d.	n.d.	n.s.
7/10/2017	5	17.9–22.0	+	++	n.d.

Presence (indicated as + or ++ for densities of about < 100 or > 1,000 cells/liter, respectively; note that these are rough approximations limited by the sampling method), non-detection (n.d.) and non-sampling (n.s.) are indicated. See additional details in Table S1 and Fig. S1, S2.

DNA sequencing and phylogenetic inference

Single-cell sequencing of the small subunit ribosomal RNA gene (SSU rDNA) was done mostly as detailed before (Santoferrara et al. 2013). In brief, single living specimens were transferred into 20 μl of DNA buffer (1% SDS, 0.1 M EDTA at pH 8), incubated with 1 μl of Proteinase K (20 mg/ml) for 12 h at 55 °C, and subjected to DNA extraction with the Clean & Concentrator-25 kit (Zymo Research, Orange, CA). SSU rDNA was amplified with universal eukaryotic primers (Medlin et al. 1988) under the following conditions: 1 min at 95 °C, followed by 40 cycles at 94 °C for 15 s, 50 °C for 30 s, and 72 °C for 2 min, and a final extension for 10 min at 72 °C. Products were purified with the Zymoclean Gel DNA Recovery kit (Zymo Research). Because DNA yields were insufficient for sequencing, an additional cloning step was added: the purified products were cloned with the Takara DNA Ligation kit v. 2.1 and Takara pMD20 T-Vector (Takara Bio USA, Mountain View, CA), and plasmids were then purified with the GeneJET Plasmid Miniprep kit (Thermo Scientific, Waltham, MA). Sanger sequencing of three clones (all resulting in identical sequences) was done with a capillary Genetic Analyzer (Applied Biosystems, Foster City, CA) at the DNA Analysis Facility, Yale University. Sequences were quality-checked and assembled in MEGA v. 6 (Tamura et al. 2013), which was used also to estimate the p-distances between the new sequence and *Eutintinnus* spp.

For phylogenetic analysis, the new sequence was added to a curated alignment of tintinnids (Santoferrara et al. 2017), with species of the order Choreotrichida (aloricate choreotrichs) as outgroup. Sequences were re-aligned with MAFFT v. 7 (Katoh and Standley 2013), and ambiguous positions were removed with the guidance of Gblocks v. 0.91b under default parameters (Castresana 2000). Maximum likelihood inference was done with RAxML v. 8.3.17 (Stamatakis 2014); the best-known tree was inferred out of 200 initial trees, and node support was estimated after 10,000 bootstraps. Bayesian inference

was done with MrBayes v. 3.2.1 (Ronquist et al. 2012). Five million generations were run and trees were sampled each 1,000 cycles. The initial 1,000 trees were discarded as burn-in, and the remaining 4,000 trees were used to estimate the Bayesian posterior probabilities. For each analysis, the GTR model with a Γ model of rate heterogeneity and a proportion of invariable sites was used, as previously identified with MrModeltest v. 2 (Nylander 2004) under the Akaike Information Criterion.

RESULTS

Isolation and cultivation

Dartintinnus alderae n. g., n. sp. (Fig. 1, 2), was detected along the Thames River at salinities and temperatures of 1–17 and 18–26 °C, respectively (Table 1). The species was relatively more abundant at salinities of 11–17 than 1–10. Cultures were more successful (i.e. lasted longer, up to 2 mo) if originated from samples at salinities of 15–16, and as long as they were refreshed weekly with filtered sample water, thus suggesting that *D. alderae* may benefit from picoplankton, nutrients or other unknown constituents that become depleted in culture. The swimming pattern of *D. alderae* in culture is usually continuous and spiraled (Fig. 1C), although reversals occur frequently around food. Cyst production was not observed in our cultures.

Lorica features

The lorica of *D. alderae* appears cylindrical in one plane (Fig. 1A, 2A), but conical upon rotation on the longest axis (Fig. 1B). The lorica flattens towards the anterior and posterior ends, and both ends are oriented on a different plane—if one end is oriented on the *x*-axis, the other one is oriented on the *y*-axis (i.e. at an angle approximately 90° from each other). This change in axis orientation is best approximated by the geometry of an isosceles tetrahedron (Fig. 1C).

Both lorica ends are open and collapsible, although the features of the anterior and posterior collapse differ. The anterior end folds upon retraction of the cell (Fig. 2B, C and Movie S1). The folding is flat (Fig. 1D), so that the anterior end appears truncated and slightly flared in lateral view (Fig. 1E), but narrowed (Fig. 1F) or pointed (Fig. 1G) in ventral and dorsal views. When the cell extends

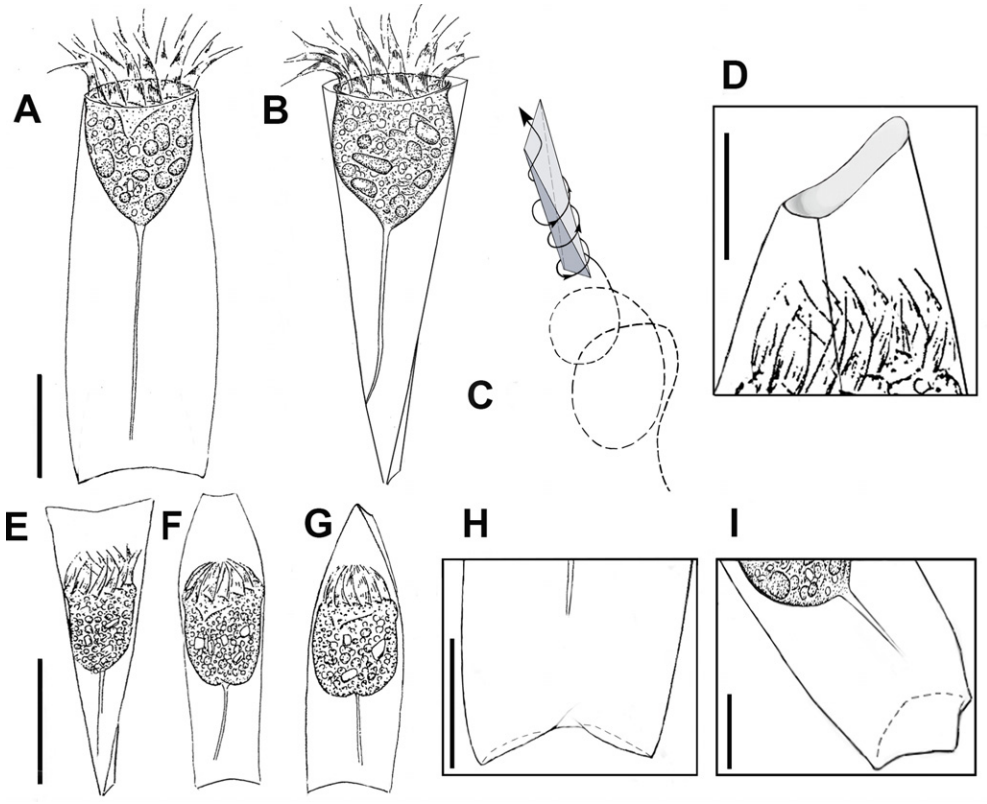

Figure 1 *Dartintinnus alderae* in vivo. (**A** and **B**) Same specimen in ventral (A) and lateral (B) views. (**C**) Swimming pattern in culture. The lorica geometry resembles an isosceles tetrahedron, thus differing in appearance depending on the angle observed. Note, however, that during swimming the cell extends beyond the lorica, and thus the lorica shape changes accordingly. (**D**) Collapse of the lorica anterior end. (**E**–**G**) Same specimen showing variations in lorica shape when the cell has retracted and the anterior end is collapsed; lateral (E) and ventral (F, G) views. (**H**, **I**) Detail of the lorica posterior end, which is usually collapsed (H), but triangular when expanded (I). Scale = 10 μm (A, B, D, H, I), 20 μm (E, F, G).

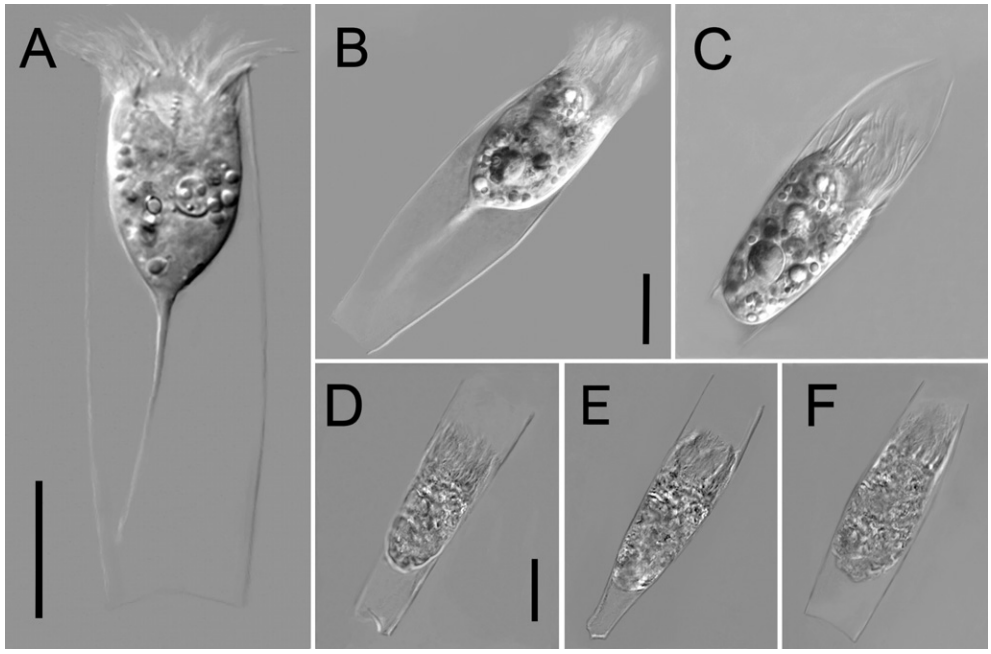

Figure 2 In vivo (**A**–**C**) and Lugol's-preserved specimens (**D**–**F**). (A) Ventral view. (B, C) Same specimen in ventral view with non-collapsed (B) and collapsed lorica anterior end (C); when the cell retracts, it no longer supports the anterior end of the lorica, resulting in collapse. (D–F) Same specimen showing the lorica posterior end at different focal planes and upon rotation (lateral view, D and E; dorsal view, F). Scale = 10 μm.

outward, the lorica expands to fit the diameter of the cell, and the anterior rim becomes elliptical (Fig. 1A, B). The anterior collapse occurs repetitively at the same line of fold.

The posterior end of the lorica is usually collapsed, but not completely sealed (Fig. 1H). It appears truncated in ventral and dorsal views, but pointed in lateral view (Fig. 1A, B, 2A, D–F and Movie S2). Because the posterior end is partially sealed, its appearance changes from bi-cylindrical (Fig. 2D) to sharpened (Fig. 2E) when examining different focal planes and angles of the lorica. Infrequently, the opening of the posterior end is slightly expanded, showing a triangular outline (Fig. 1I).

Neither the anterior nor posterior collapse appears to occur actively (the cell lacks distinctive structures for lorica collapse), but instead as a result of lorica flimsiness. The lorica wall is extremely thin and hyaline (as compared to other hyaline tintinnids, e.g. *Eutintinnus* spp.), nearly imperceptible if not for the cell. The lorica has an average length of 48 μm, but its width varies depending on orientation and collapse (Table 2). On the truncated side, the maximum widths of the anterior and posterior ends average 13 μm and 11 μm, respectively.

Cell features

The goblet-shaped cell proper has an average length and width of 22 and 12 μm in vivo (23 and 13 μm after protargol impregnation), respectively (Table 2). There are two ovoidal macronuclear nodules, and each of them is usually accompanied by a micronucleus located posteriorly (Fig. 3A, 4A). The cell attaches sub-terminally to the lorica wall by a contractile stalk, which is up to 20 μm long when fully extended (Fig. 2A). Upon contraction of the stalk, an invagination at the posterior cell end is sometimes visible, especially after fixation.

The somatic ciliature includes a ventral and a dorsal kinety, and right, left and lateral ciliary fields (Fig. 3, 4 and Table 2). Based on protargol impregnation, the ventral and lateral kineties are entirely monokinetidal; the right and left ciliary fields are monokinetidal except for an anteriormost dikinetid; the dorsal kinety appears monokinetidal, but we cannot rule out that it is composed of dikinetids in which one unciliated basal body did not impregnate (transmission electron microscopy is needed for verification). The ventral kinety (Fig. 4B) is 4–6 μm long and consists of 4–6 monokinetids, which are 0.85–1 μm apart. The ventral kinety commences 2.2–2.5 μm posteriorly to the collar membranelles and displays a slight clockwise curvature as it advances to the posterior part of the cell, increasing in distance from 1 to 2.25 μm from the right field (kinety 2). The right field (Fig. 4C) includes 4–6 kineties that are 5–7 μm long (5–7 kinetids), with length generally increasing towards the dorsal kinety. The kineties of the right field commence 2 μm posteriorly to the collar membranelles. These kineties are parallel and 2.5 μm apart, with a distance of 1 μm between kinetids. The dorsal kinety (Fig. 4D) is 5–7 μm long and comprises 5–7 kinetids, which are spaced 0.95–1 μm apart. It commences at a distance of 2.5–2.75 μm from the collar membranelles, and maintains a 15° angle from the main cell axis, decreasing in distance

Table 2. Lorica and cell morphometrics

Characteristics	Mean	M	SD	SE	CV	Min	Max	*N*
Lorica								
Length	47.8	48.2	3.4	0.7	1.4	38.4	55.1	40
Anterior aperture diameter (truncated view)	12.6	12.3	1.9	0.4	3.0	9.3	18.5	40
Posterior aperture diameter (truncated view)	11.0	11.6	2.6	0.2	2.2	4.2	17.1	24
Length*	47.6	48.1	2.8	0.8	1.8	40.1	50.3	11
Anterior aperture diameter (truncated view)*	13.4	13.5	2.5	0.8	5.2	10.9	19.0	11
Posterior aperture diameter (truncated view)*	10.6	11.3	2.7	0.8	7.6	5.9	15.5	11
Length:anterior aperture ratio	3.9	3.9	0.7	0.1	3.2	2.5	5.0	40
Length:anterior aperture ratio*	3.4	3.5	0.6	0.2	5.6	2.1	4.2	11
Cell								
Length of cell proper*	22.3	22.3	1.9	0.6	2.6	18.5	25.6	17
Width of cell proper*	12.2	12.2	1.2	0.4	3.0	10.8	15.1	17
Length:width ratio*	1.8	1.9	0.2	0.1	3.9	1.5	2.3	17
Cell stalk length*	17.0	1.8	1.7	0.7	0.0	14.9	20.1	6
Length of cell proper	23.1	22.0	2.6	0.6	2.6	20.0	30.0	19
Width of cell proper	12.8	13.0	1.0	0.2	1.7	11.0	15.0	20
Length:width ratio	1.8	1.7	0.2	0.0	2.5	1.5	2.3	19
Macronuclear nodules, number	2.0	2.0	0.0	0.0	0.0	2.0	2.0	22
Anterior macronuclear nodule, length	5.9	6.0	1.2	0.2	4.2	4.0	9.0	22
Anterior macronuclear nodule, width	4.1	4.0	1.0	0.2	5.3	3.0	6.0	22
Posterior macronuclear nodule, length	7.3	7.0	1.9	0.4	5.4	3.0	10.0	22
Posterior macronuclear nodule, width	3.6	3.0	1.4	0.3	8.4	2.0	9.0	22
Anterior cell end to anterior macronuclear nodule, distance	4.3	4.0	0.9	0.2	4.4	2.0	6.0	22
Anterior cell end to posterior macronuclear nodule, distance	10.6	11.0	2.1	0.4	4.2	7.0	15.0	22
Micronuclei, number	2.0	2.0	0.0	0.0	0.0	2.0	2.0	10
Anterior micronucleus, length	0.8	0.8	0.1	0.1	7.1	0.6	1.0	7
Anterior micronucleus, width	0.7	0.0	0.2	0.1	7.8	0.5	0.9	7
Posterior micronucleus, length	1.2	1.1	0.4	0.2	12.2	0.8	1.9	7
Posterior micronucleus, width	0.9	0.9	0.2	0.1	7.8	0.7	1.3	7
Collar membranelles, number	14.2	14.0	0.8	0.4	2.6	13.0	15.0	5
Buccal membranelles, number	1.0	1.0	0.0	0.0	0.0	1.0	1.0	4
Number of kineties								
Total	16.9	17.0	0.7	0.3	1.5	16.0	18.0	7
Ventral kinety	1.0	1.0	0.0	0.0	0.0	1.0	1.0	10
Right field	5.0	5.0	0.8	0.3	6.2	4.0	6.0	7
Dorsal kinety	1.0	1.0	0.0	0.0	0.0	1.0	1.0	9
Left field	7.0	7.0	0.6	0.2	3.1	6.0	8.0	7
Lateral field	2.8	3.0	0.5	0.2	6.0	2.0	3.0	8
Length and structure of kineties								
Ventral kinety (1), length	5.4	5.6	0.7	0.3	5.5	4.2	5.8	5
Ventral kinety (1), number of kinetids	5.4	5.5	0.7	0.2	4.6	4.0	6.0	8
Right field, kinety 2, length	5.9	5.9	0.2	0.1	1.2	5.7	6.1	5
Right field, kinety 2, number of kinetids	5.6	5.0	0.8	0.4	6.4	5.0	7.0	5
Right field, kinety 3, length	6.2	6.1	0.3	0.1	2.4	5.9	6.6	5
Right field, kinety 3, number of kinetids	6.1	6.0	0.6	0.2	3.9	5.0	7.0	7
Right field, kinety 4, length	6.8	6.8	0.4	0.2	2.8	6.1	7.2	5
Right field, kinety 4, number of kinetids	6.7	7.0	0.5	0.2	2.5	6.0	7.0	7
Right field, kinety 5, length	6.2	6.0	0.6	0.3	4.4	5.5	7.1	5
Right field, kinety 5, number of kinetids	6.0	6.0	0.0	0.0	0.0	6.0	6.0	7
Right field, kinety 6, length	6.3	6.3	0.5	0.2	3.4	5.6	6.7	5
Right field, kinety 6, number of kinetids	6.0	6.0	0.5	0.2	3.4	5.0	7.0	7
Dorsal kinety (7), length	5.8	5.8	0.4	0.2	3.6	4.8	6.8	5
Dorsal kinety (7), number of kinetids	5.5	5.9	0.5	0.2	3.1	5.0	7.0	8
Left field, kinety 8, length	9.2	9.0	0.4	0.2	2.1	8.8	9.9	5

(continued)

Table 2. (continued)

Characteristics	Mean	M	SD	SE	CV	Min	Max	*N*
Left field, kinety 8, number of kinetids	9.4	9.5	0.7	0.2	2.6	8.0	10.0	8
Left field, kinety 9, length	9.9	9.9	0.3	0.1	1.3	9.6	10.3	5
Left field, kinety 9, number of kinetids	10.9	10.5	0.9	0.3	3.0	10.0	12.0	8
Left field, kinety 10, length	9.3	9.1	0.7	0.3	3.3	8.6	10.3	5
Left field, kinety 10, number of kinetids	9.6	10.0	1.4	0.5	5.2	7.0	11.0	8
Left field, kinety 11, length	6.0	6.0	0.4	0.2	3.4	5.5	6.5	4
Left field, kinety 11, number of kinetids	7.0	7.0	0.9	0.4	5.7	6.0	8.0	5
Left field, kinety 12, length	6.3	6.2	0.5	0.2	3.9	5.9	6.9	4
Left field, kinety 12, number of kinetids	5.7	6.0	0.5	0.2	2.9	5.0	6.0	7
Left field, kinety 13, length	6.9	7.1	0.5	0.2	3.3	6.1	7.4	5
Left field, kinety 13, number of kinetids	5.6	6.0	0.5	0.2	3.4	6.0	6.0	7
Left field, kinety 14, length	6.5	6.5	0.6	0.3	3.9	5.6	7.0	5
Left field, kinety 14, number of kinetids	5.3	5.0	0.7	0.3	5.0	4.0	6.0	7
Lateral field, kinety 15, length	5.2	5.5	0.6	0.3	5.3	4.3	5.8	5
Lateral field, kinety 15, number of kinetids	5.2	5.0	0.4	0.2	2.9	5.0	6.0	6
Lateral field, kinety 16, length	5.4	5.5	0.3	0.1	2.6	5.0	5.8	5
Lateral field, kinety 16, number of kinetids	5.6	6.0	0.5	0.2	3.5	5.0	6.0	6
Lateral field, kinety 17, length	5.3	5.7	0.9	0.4	7.3	4.3	6.1	5
Lateral field, kinety 17, number of kinetids	5.3	5.0	0.7	0.2	4.5	4.0	6.0	8

M = median; SD = standard deviation; SE = standard error of mean; CV = coefficient of variation (%); *N* = number of individuals examined. Data are the result of randomly selected morphostatic specimens from clonal cultures established on 7/22/2016 and 7/10/2017. Loricae and cells were examined after Lugol's fixation or protargol impregnation, respectively, except if in vivo (*). Measurements are in μm.

to the right field from 4 to 3.5 μm as it progresses posteriorly. The left field (Fig. 4D–F) includes 6–8 kineties. These kineties become shorter towards the lateral field (kineties 8 and 14 are 9–10 μm and 6–7 μm long, and include 8–10 and 4–6 kinetids, respectively). The kineties of the left field begin 2 μm posteriorly to the collar membranelles. These kineties are spaced 2 μm apart, with kinetids spaced 1 μm apart. The lateral field (Fig. 4F) consists of 2 or 3 kineties, each of them 4–6 μm long and including 4–6 monokinetids (0.75–0.95 μm apart). The kineties of the lateral field commence at a distance of 2.1–2.5 μm from the collar membranelles. These kineties are nearly parallel to each other, and follow a curvature similar to that of the ventral kinety (distanced 0.5–1.25 μm from kinety 17), although they terminate more anteriorly. The distances separating the lateral field kineties are smaller (0.5–1.25 μm) compared to those of the right and left fields. In about 30% of cells, one to three kinetal fragments with 2–4 kinetids are observed to the right of the posterior part of the dorsal kinety (Fig. 4D). These kinetal fragments appear similar in structure and kinetid spacing to the dorsal kinety. In dividers, the oral primordium is located to the left of the ventral kinety, posterior to the lateral ciliary field (Fig. 4A, F).

The oral ciliature includes 13–15 collar membranelles surrounding the peristomial field, accompanied by a single buccal membranelle. The cilia of the collar membranelles are up to 10 μm long and stretch outwards when swimming. The polykinetids of the collar membranelles are 4 μm long, except for three elongated polykinetids of 5–6 μm length that extend into the buccal cavity.

Sequence analysis

Sequencing of SSU rDNA yielded a product 1,672 bp in length with a 47.1% GC content (GenBank accession number MF039886). *Dartintinnus alderae* has a sister relationship with *Eutintinnus*, with full support in both Maximum Likelihood and Bayesian inferences (Fig. 5). On average, p-distance between *D. alderae* and the *Eutintinnus* sequences available in GenBank is 6.1% (range = 5.5–6.5%). In contrast, all the *Eutintinnus* sequences are, on average, 3.1% dissimilar (range = 0.2–4.8%) among them.

GenBank sequence KU715759 was excluded from our analyses because its genus affiliation cannot be confirmed. This sequence has been affiliated to *Eutintinnus* based on a micrograph, instead of direct microscopy observation (Zhang et al. 2016). However, it is genetically inconsistent with all the other sequences obtained for *Eutintinnus* so far (see details in Santoferrara et al. 2017). This sequence has a p-distance of 1.4% to *D. alderae*, but the available information prevents us from suggesting a common generic affiliation.

DISCUSSION

Dartintinnus is a new genus of the family Eutintinnidae

Dartintinnus is different from any other described tintinnid in that its lorica collapses on both anterior and posterior ends (Fig. 1, 2). The affiliation of *Dartintinnus* within Eutintinnidae is based on the features shared

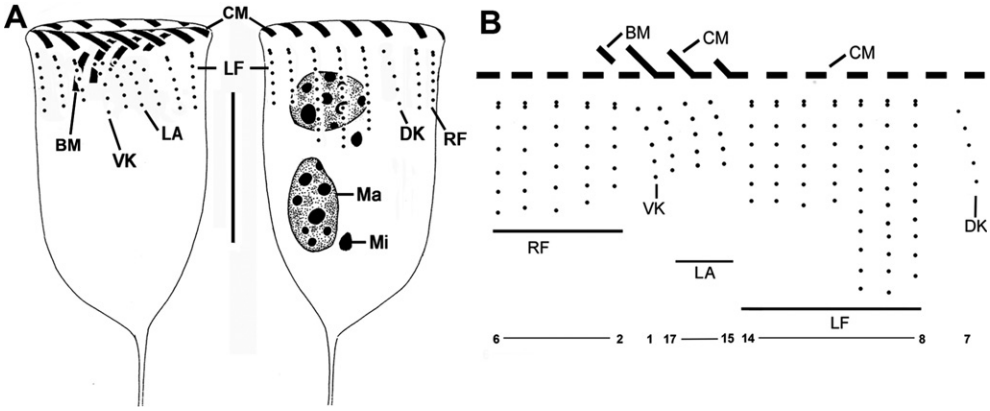

Figure 3 *Dartintinnus alderae* after protargol impregnation. (**A**) Ventrolateral (right) and dorsolateral (left) views of a representative specimen. (**B**) Ciliary map. The numeral identification at map bottom corresponds to kinety number, initiating with the ventral kinety and continuing in a clockwise direction via top view. BM = buccal membranelle; CM = collar membranelle; DK = dorsal kinety; LA = lateral field; LF = left field; Ma = macronuclear nodule; Mi = micronucleus; RF = right field; VK = ventral kinety. Scale = 10 μm.

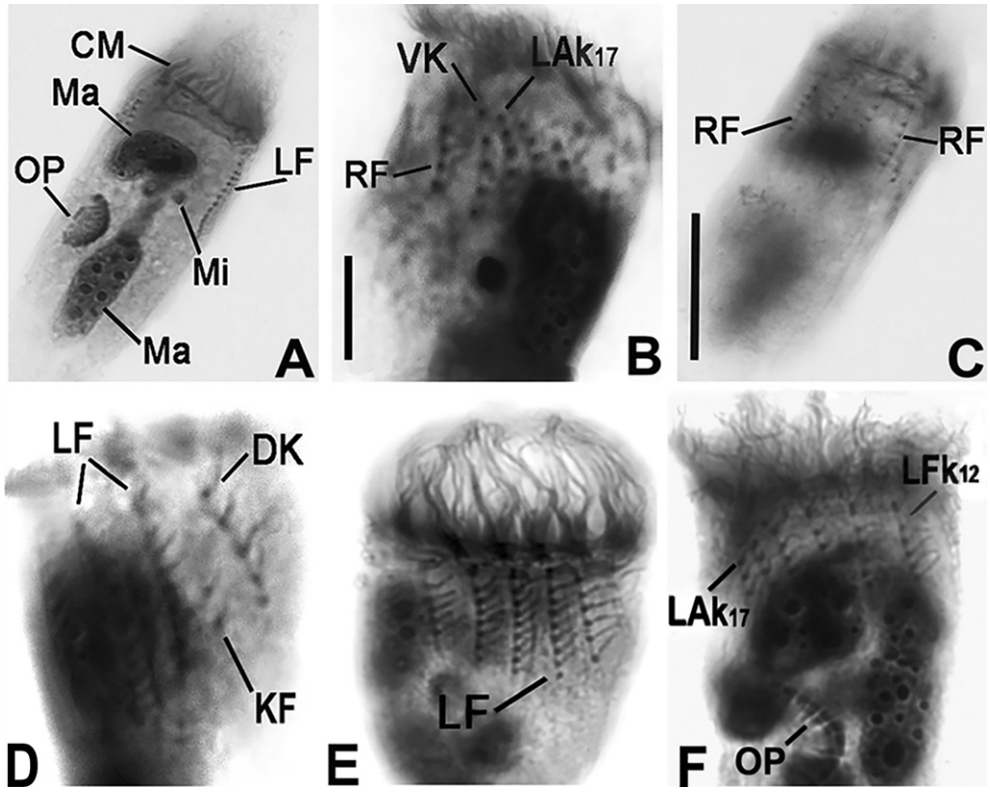

Figure 4 Protargol-impregnated specimens. (**A, B**) Ventrolateral view. (**C, F**) Lateral view. (**D, E**) Dorsolateral view. CM = collar membranelle; DK = dorsal kinety; LA = lateral field; LF = left field; Ma = macronuclear nodule; Mi = micronucleus; OP = oral primordium; KF = kinetal fragment; RF = right field; VK = ventral kinety; k_n = kinety number associated with ciliary map in Fig. 3B. Scale = 10 μm (A, C); 5 μm (B, D, E, F).

with the other genus of this family, *Eutintinnus*: a hyaline, elongated lorica opened on both ends (Kofoid and Campbell 1939) and a ciliary pattern including a short, monokinetidal ventral kinety, a right and a left ciliary field with monokinetidal kineties having one dikinetid anteriorly, a lateral field with fully monokinetidal kineties (see next paragraph), and at least one dorsal kinety (Choi et al. 1992). Genetic data based on SSU rDNA support both the erection of a new genus and its placement in Eutintinnidae, as *Dartintinnus* forms a distinct branch that is sister to *Eutintinnus* in phylogenetic analyses (Fig. 5), and the two genera differ by 5.5–6.5% in

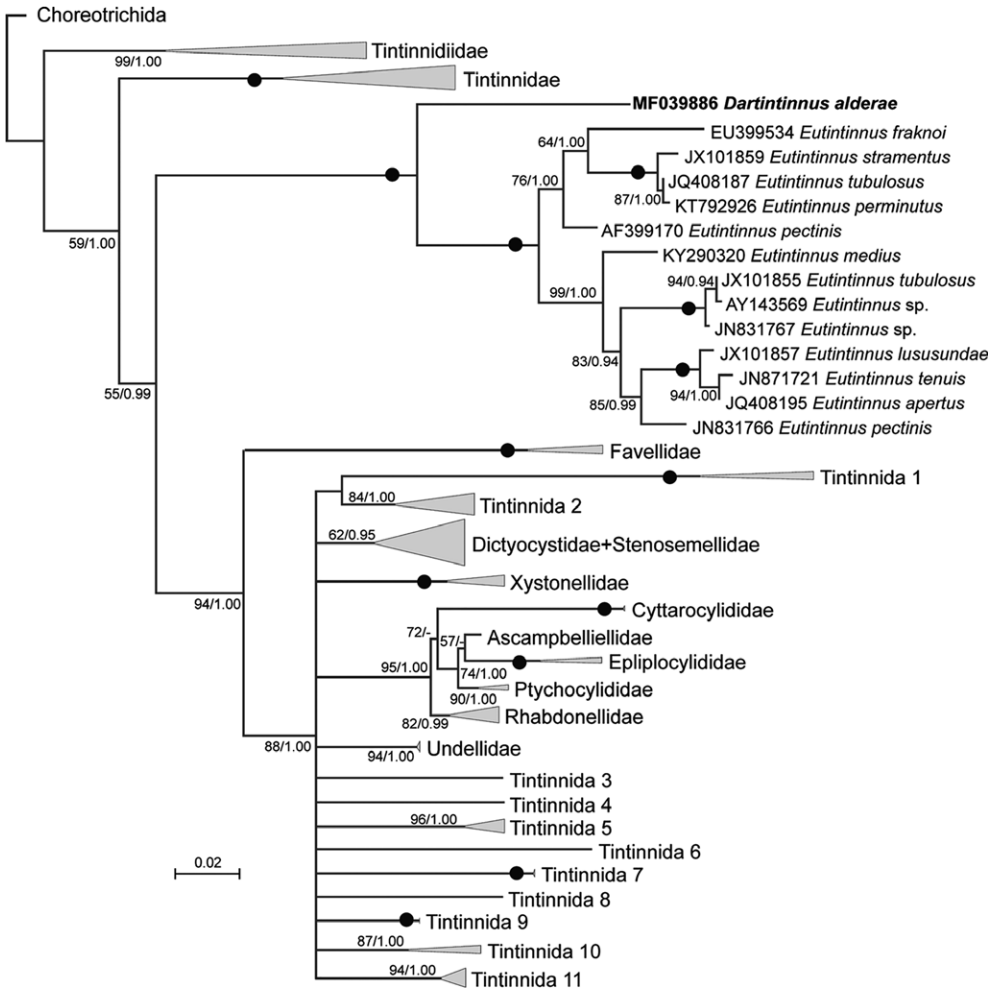

Figure 5 Phylogenetic tree inferred from SSU rDNA sequences indicates that *Dartintinnus alderae* has a sister relationship with *Eutintinnus*. Collapsed lineages are according to Santoferrara et al. (2017). RAxML bootstrap support and MrBayes posterior probability values are shown (only if > 50% and > 0.90, respectively). A black circle indicates full support in both analyses.

their sequence (a genus-level divergence in tintinnids; Santoferrara et al. 2013).

Beyond lorica collapsibility, main differences between *Dartintinnus* and *Eutintinnus* include the lorica geometry (resembling an isosceles tetrahedron when the cell is contracted or a cylinder, respectively) and two cell features (Fig. 3, 4 and Table 2). First, the number of macronuclear nodules is two in *D. alderae* and usually four in *Eutintinnus* (Choi et al. 1992). Second, the somatic ciliature of *D. alderae* contains a single dorsal kinety with fewer than 10 kinetids, while there are usually two longer dorsal kineties (up to 76 kinetids) in the *Eutintinnus* species with known ciliary patterns (*E. angustatus*, *E. pectinis* and *E. tenuis*; Choi et al. 1992). Only *E. angustatus* rarely presents a single (or up to three) dorsal kineties (Choi et al. 1992). The kinetal fragments detected in a minority of *D. alderae* specimens as well as in *E. pectinis* and *E. tenuis* (Choi et al. 1992) cannot be inferred as additional dorsal kineties at this time. On the other hand, *D. alderae* has a lateral ciliary field, which is apparently also present in some *Eutintinnus* species. Between the ventral kinety and the left field, *E. tenuis* has three to four fully monokinetidal kineties and *E. angustatus* has one such kineties, consistent with a clear or an incipient lateral field, respectively (Choi et al. 1992). The family diagnosis is updated here to accommodate the features of the new genus and those overlooked for *Eutintinnus* (see Taxonomic Summary).

Anterior collapsibility of the lorica had been described only in *Nolaclusilis,* the sole genus of the family Nolaclusilidae (Sniezek et al. 1991; Snyder and Brownlee 1991). Out of the two known *Nolaclusilis* species, *N. hudsonicus* presents two cell projections attached to the lorica wall, which are suggested to cause the active collapse of the lorica when specimens contract (Sniezek et al. 1991). In *N. bicornis*, it is instead believed that the collapse is hydrodynamic, i.e. due to water expelled from the lorica by the contracting cell (Snyder and Brownlee 1991).

Dartintinnus does not display cell projections, or any other mechanism that is indicative of an active lorica collapse. Instead, and apparently as a result of lorica flimsiness, the posterior end is generally collapsed, while the anterior end folds to accommodate the cell extending beyond or retracting inside the lorica (Fig. 1, 2 and Movie S1). *Dartintinnus* and *Nolaclusilis* species also differ in that the latter have a bell-shaped lorica opened only at the anterior end, and a ciliary pattern lacking a dorsal kinety, a lateral field, and a buccal membranelle (which are all observed in *Dartintinnus*; Fig. 3, 4). Genetic comparison is not possible at the moment as Nolaclusiliidae DNA sequences are not available. Although the inclusion of this family may change the topology of the tintinnid phylogenetic tree (Fig. 5), *Dartintinnus* is expected to maintain a sister relationship with *Eutintinnus* given the higher affinity in ciliary patterns.

Comparison between *Dartintinnus alderae* and similar species

To the best of our knowledge, the most similar species to *D. alderae* is *Eutintinnus tubus*, which was sampled in brackish waters from a salt marsh in New York, USA, and was described based on the lorica only (Stokes 1893). Despite its similarity in lorica width (oral and aboral diameters estimated from the figure included in the original description are 13–16 and 5–6 μm, respectively), *E. tubus* has a longer, subcylindrical lorica (80–100 μm) and a different behavior: the author describes the cell movement within and beyond the lorica, but there is no reference to lorica collapse (Stokes 1893). The approximately thirty remaining *Eutintinnus* species are mostly marine and present larger, cylindrical or subcylindrical loricae (Balech 1968; Brandt 1906; Campbell 1942; von Daday 1887; Entz 1885; Hada 1932; Jörgensen 1924; Kofoid and Campbell 1929; Müller 1776; Ostenfeld 1899; Wailes 1925).

One undescribed species from the Black Sea is similar to *D. alderae* in lorica size, geometry, and collapse (reported as *Nolaclusilis* sp.; Gavrilova and Dolan 2007; Gavrilova and Dovgal 2016). However, cytological and DNA sequencing studies are needed to confirm if they correspond to the same species. Another potential report related to *D. alderae* may come from Chesapeake Bay, USA, where Snyder and Brownlee (1991) mentioned "one small *Eutintinnus*[-like] species [that closes] its aboral opening".

Evolutionary patterns in tintinnids

Tintinnids have evolved into two main lineages, the mostly limnetic Tintinnidiidae (with ventral organelles in their ciliature and a flexible, agglomerated lorica) and the mostly marine remaining taxa (with a ventral kinety and a generally stiff lorica that is totally-, partially- or non-agglomerated), as suggested by the most recent integral (Agatha and Strüder-Kypke 2013, 2014) and phylogenetic (Santoferrara et al. 2017) reviews. The marine branch transitioned from a low-complexity ciliary pattern including only the ventral kinety and the right and left ciliary fields (Nolaclusiliidae, presumably Tintinnidae), to the introduction of dorsal kineties and a lateral ciliary field (Eutintinnidae, Favellidae), to the more complex pattern with a posterior kinety (known in Dictyocystidae, Ptychocylididae, Stenosemellidae and some *Tintinnopsis* species).

In this context, the novel genus *Dartintinnus,* with one dorsal kinety, suggests a transition between the lack of a dorsal kinety in *Nolaclusilis* and the presence of two dorsal kineties generally observed in *Eutintinnus*. A single dorsal kinety also occurs in species with more complex ciliary patterns, such as *Tintinnopsis brasiliensis* (Cai et al. 2006) and *Schmidingerella arcuata* (Agatha and Strüder-Kypke 2012). In these taxa, the single dorsal kinety was either caused by loss of one such kinety from *Eutintinnus* (Agatha and Strüder-Kypke 2013) or maintained from the Eutintinnidae ancestor. The lateral ciliary field, present in *D. alderae* (Fig. 3, 4), apparently in *E. angustatus* and *E. tenuis* (Choi et al. 1992), as well as in *Favella* and all the genera known to have the most complex ciliary pattern (Agatha and Strüder-Kypke 2013, 2014), probably appeared in Eutintinnidae. In this context, the apparent lack of a lateral field in *E. pectinis* (Choi et al. 1992) needs confirmation. Also unresolved is whether the lorica collapsibility, which may be active in *Nolaclusilis* but passive in *Dartintinnus*, evolved sequentially or independently in these genera.

Lineage discovery in the XXI century

Protists, which have a long history of descriptions via microscopy, are in a new golden age of discovery due to a dramatic increase in the use of molecular methods for environmental surveys. These methods have pointed at novel lineages (some of them now formally described) in groups such as dinoflagellates (Guillou et al. 2008), stramenopiles (Massana et al. 2004), haptophytes (Liu et al. 2009), and ciliates (Orsi et al. 2012). Contrary to lineages of unexplored habitats, relatively small size, or those lacking conspicuous morphological features, tintinnid families and genera are assumed to be mostly known, at least based on lorica morphology. Here, we have shown that there are still novel taxa to be discovered, even in the well-studied tintinnids.

Some reasons for the delayed discovery of *D. alderae* may include: (i) the thin and relatively small lorica, which is likely under-represented in samples taken with plankton nets, (ii) the hyaline lorica, which may not be detected at low magnification, and (iii) the relative rarity of tintinnid studies in riverine waters as compared to marine environments. For example, in the Northeast USA, *D. alderae* was found in riverine waters that have been much less frequently targeted for plankton studies, especially compared with the adjacent waters of the Long Island Sound estuary, where regular sampling efforts since 2002 have failed to detect this species (G. B. McManus, unpublished data). Within the salinity gradient along the Thames River, we detected *D. alderae* mainly in mesohaline waters, at

salinities of 11–17 (Fig. S1, S2 and Table 1). Specimens with similar lorica features (although not confirmed as the same species; see above) were detected in the Black Sea at salinities of 15–18 (specifically near the confluence of the Chernaya River with Sevastopol Bay and in the Odessa Gulf; Gavrilova and Dolan 2007; Gavrilova and Dovgal 2016), and in the mesohaline portions of the Chesapeake Bay, USA (Snyder and Brownlee 1991). To the best of our knowledge, no other reports are consistent with *Dartintinnus*, although this genus may have been confused with *Eutintinnus* in other surveys.

In a recent analysis of all the tintinnid sequences available in GenBank (both from morphologically-identified specimens and environmental surveys), some of the most divergent sequences that could not be linked to known lineages were sister to Eutintinnidae (fig. 4A in Santoferrara et al. 2017). At least one of these sequences is closely related to *D. alderae* (99% similarity, environmental sequence AY180046 from seawater in a salt marsh in Massachusetts, USA; Stoeck and Epstein 2003). This exemplifies the huge potential of environmental sequencing to reveal uncharacterized diversity, even for the relatively well-studied tintinnids. Our work also stresses the importance of taxonomic study, not only to describe novel diversity, but also to improve the current classification and evolutionary hypotheses in this ecologically relevant group.

TAXONOMIC SUMMARY

Tintinnida Kofoid and Campbell, 1929
Eutintinnidae Bachy, Gómez, López-García, Dolan and Moreira, 2012
Improved diagnosis. Lorica generally resembling an elongated cylinder with anterior and posterior openings at each end, rarely collapsible, hyaline. Two or four macronuclear nodules and two micronuclei. Somatic ciliature includes a short monokinetidal ventral kinety, a right and a left ciliary field with monokinetidal kineties having one dikinetid anteriorly, usually a lateral field including monokinetidal kineties, and one to three dorsal kineties.

Dartintinnus Smith and Santoferrara n. g.

Diagnosis. Lorica with anterior and posterior openings, both of which are collapsible. Lorica geometry resembles an isosceles tetrahedron when collapsed. Two macronuclear nodules. Somatic ciliature includes a lateral field and a single dorsal kinety. Sequence of the SSU rDNA presents a sister phylogenetic relationship to *Eutintinnus*, with an identity lower than 95%.
Etymology. The prefix *Dart* refers to a sewing method that relies on folds coming to a point to tailor a fabric to the wearer's shape. In an analogous way, the lorica of this ciliate can fold (collapse) at the anterior aperture upon cell retraction. The suffix *tintinnus* references tintinnid affiliation. One of the two consecutive "*t*" letters are elided for convenience.
Zoobank registration number. DC36E0E1-BD20-4AB2-AAAD-472F6ED15E52.
Type species. *Dartintinnus alderae* n. sp.

Dartintinnus alderae Smith, Song, Gavrilova, Kurilov, Liu, McManus and Santoferrara, n. sp.

Diagnosis. Lorica length and width of the anterior end average 48 and 13 μm, respectively. Cell proper has an average length and width of 22 and 12 μm in vivo, and 23 and 13 μm after protargol impregnation, respectively. Ciliary pattern typically includes seventeen kineties: one ventral and one dorsal kinety, and a right, a left and a lateral ciliary field typically containing five, seven and three kineties, respectively, with no more than twelve kinetids each. About 14 collar membranelles (up to three elongated) and one buccal membranelle.
Type locality. Thames River, Connecticut, USA (between 41°31′22.9″N, 72°04′35.5″W and 41°22′47.1″N, 72°05′42.9″W).
Type material. A slide containing both a holotype and several paratypes has been deposited at the American Museum of Natural History, New York, USA, with accession number 66884. An additional slide containing several paratypes is held by author S.A.S.
Gene sequence. A sequence of the SSU rDNA has been deposited in NCBI GenBank with accession number MF039886.
Zoobank registration number. EFC2D9F3-02FD-417A-A08C-177778C9F5BA.
Dedication. Named in honor of Viviana A. Alder (Universidad de Buenos Aires, CONICET and Instituto Antartico Argentino, Argentina) for her contributions to tintinnid research, pioneering work as a woman in Antarctic oceanography, and commitment to the training of future researchers.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Movie S1. The anterior opening of the lorica usually collapses and expands as the cell retracts and extends, respectively.

Movie S2. The lorica shape appears different depending on the angle of observation.

Table S1. Occurrence of *Dartintinnus alderae* in the Thames River, Connecticut, USA.

Figure S1. Sampling area in the Thames River, Connecticut, USA.

Figure S2. Detection of *Dartintinnus alderae* in relation to the salinity and temperature gradients recorded in the Thames River, Connecticut, USA.