3 Ciliates – protists with complex morphologies and ambiguous
4 early fossil record

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ABSTRACT

Since ciliates rarely possess structures that easily fossilize, we are limited in our ability to use paleontological studies to reconstruct the early evolution of this large and ecologically important clade of protists. Tintinnids, a group of loricate (house-forming) planktonic ciliates, are the only group that has a significant fossil record. Putative tintinnid fossils from rocks older than Jurassic, however, possess few to no characters that can be found in extant ciliates; these fossils are best described as '*incertae sedis* eukaryotes'. Here, we review the Devonian fossil *Nassacysta reticulata* and propose that it is likewise another *incertae sedis* eukaryote due to the lack of any unambiguous ciliate characters. Future tintinnid fossil descriptions would be most helpful if: (i) neutral terminology is used in the descriptions but ciliate-specific terminology in the interpretations; (ii) the current ciliate classification is used, although fossil data may expand or modify classifications based on modern forms; (iii) close collaboration with specialists studying extant ciliates is done; and (iv) editors include an expert of extant ciliates in the review process.

Keywords: Acritarch, Ciliophora, Taxonomy, Tintinnids

1. Introduction

Ciliates (Alveolata, Ciliophora) are morphologically extremely diverse in comparison with most other protists (Dunthorn and Katz, 2008; Lynn, 2008). Species and higher taxa are primarily distinguished by variations in cell shape and size, the numbers and patterns of oral and somatic cilia, and the ultrastructure of the fibres associated with the somatic basal bodies underlying the cilia. These minute characters are observed in live and silver-stained specimens, as well as with electron microscopy (Foissner, 2014; Lynn, 2008). Ciliates generally lack hard structures. The prospect for the fossilization of most of the cellular characters that are needed for unambiguous identification and description is therefore extremely low, although overall soft-cell shapes can be fossilized under a few conditions such as in amber (Ascaso et al., 2005; Martín-González et al., 2008; Poinar et al., 1993; Schmidt et al., 2006; Schönborn et al., 1999).

Tintinnid ciliates (Spirotricha, Choreotrichida, Tintinnina) have external loricae (Figure 1), which are more easily fossilized and have a long, but patchy, history in the rock record (Colom, 1948; Lipps et al., 2013; Tappan, 1993; Tappan and Loeblich, 1968). These fossils reliably identified as tintinnids—at least in terms of having the same lorica shape and composition as extant species—first appeared in the Jurassic and range to the Recent (Lipps et al., 2013; Remane, 1985; Rüst, 1885). Some other ciliates (e.g., colpodeans, peritrichs, and trachelophyllids) have loricae, cysts and scales that can potentially be fossilized (Lynn, 2008), although many other protist groups have similar structures as well (e.g., testate amoebae and foraminifera; Hausmann et al., 2003). The hydrocarbon gammacerane was posited to be a molecular fossil of ciliates

as its precursor, tetrahymanol, was thought to be found only in *Tetrahymena* (Summons and Walter, 1990); but tetrahymanol was recently discovered in a variety of organisms (Takishita et al., 2012).

As a community of ciliate researchers who largely study extant ciliates, we look forward to any new and accurately identified ciliate fossil, especially if it originates from pre-Jurassic rocks. Because most genes in ciliates suffer from extensive paralogy and excessive nucleotide substitutions, we are limited in our ability to use molecular data from extant species to infer early evolution and deep relationships (Dunthorn et al., 2014; Lynn, 2008). Publication of new ciliate fossils therefore can have profound effects on how we interpret the history of this large and ecologically important clade of protists.

2. Non-Ciliate Proterozoic and Paleozoic microfossils

Molecular clock analyses place the origin of ciliates sometime in the Proterozoic (Berney and Pawlowski, 2006; Eme et al., 2014). Several fossils from Proterozoic rocks have recently been described as ciliates (Bosak et al., 2011; Li et al., 2007; Li and Zhang, 2006; Li et al., 2009). As discussed elsewhere (Dunthorn et al., 2010; Lipps et al., 2013), these fossils have characteristics that were immediately recognized by researchers working on extant ciliates, as well as a ciliate fossil expert, as being non-ciliate in origin. These purported Proterozoic ciliate fossils are best described as *incertae sedis* eukaryotes or perhaps even inorganic in origin in a few cases. They are not ciliates.

A more recent example of a problematic description is that from a new set of fossils from Middle Devonian (Givetian) sedimentary rock in the Ghadamis Basin (ca. 383-389 MYA) of Libya that has been named *Nassacysta reticulata*, and interpreted as being a ciliate (Figure 5; Steemans et al., 2014). These fossils are organic-walled and 564–1,617 μm in length. They are composed of two structures: “an outer sheath looking like a fish trap open at its narrowest tip; and an inner rounded body enclosed in a thin membrane” (Steemans et al., 2014). The sheath itself is composed of threads about 10 μm wide that form a reticulate structure with polygonal to subcircular meshes. Some of the threads have flat, distally enlarged projections. A membranous structure is also described, but it only covers the threads. The inner round bodies are interpreted to be resting cysts (Steemans et al., 2014).

Steemans et al. (2014) interpreted *Nassacysta* to be tintinnid-like based on the presence of its vase-shaped lorica and the occurrence of an internal resting cyst. *Nassacysta* was therefore placed within the ciliates under “Tintinnidia? Kofoid and Campbell, 1929”, although this name has not been published as valid; they may have either meant Tintinnida Kofoid and Campbell, 1929 or Tintinnina Kofoid and Campbell, 1929.

Several problems exist with the interpretation of *Nassacysta* as being tintinnid-like. First, the fossils are larger than the vast majority of extant tintinnids, 564–1,617 μm vs. rarely more than 300 μm in length (Dolan, 2010). The problem of being too large was sufficient for Steemans et al. (2014) to reject an affinity with dinoflagellate cysts, chitinozoa (an extinct group of chitinous, flask-shaped microfossils that may be related to metazoa), thecamoebae (testate amoebae), miospores (spores or pollen grains less

than 200 μm across) like *Retispora lepidophyta*, gymnosperm pollen, and acritarchs, but not for tintinnids.

Second, Steemans et al. (2014) described the *Nassacysta* fossils as being vase-shaped, with a broad base and narrowing at the tip with apparent breaks in the outer net-like structure. While almost all tintinnid loricae have a vase-like shape, importantly they have a narrower base and a broader apical aperture with a distinct opening rim, where the cells can easily move in and out (Agatha et al., 2013)—exactly opposite of *Nassacysta*. Only two extant tintinnids (*Tintinnidium mucicola* and *Tintinnopsis amphora*) have a somewhat narrowed apical portion, but neither has a reticulate lorica and both have typical lorica apertures. Steemans et al. (2014) interpreted the typical lorica opening rim as weak and thus torn off sometime during the fossilization in all specimens found, resulting in these distinct breaks. The supposition that *Nassacysta* are tintinnid-like necessitates an unjustified further assumption of apical apertures with distinct rims that were never observed. A more parsimonious explanation for the missing tintinnid-like lorica opening rims in every *Nassacysta* fossil found is that they never existed.

Third, reticulate loricae occur only in about seven tintinnid species belonging to the genus *Dictyocysta* (Figure 2), whose lorica apertures are, like those of the other one thousand known tintinnid species, wider than the cell's apical oral region and are surrounded by a distinct rim. The outer sheath of *Nassacysta* differs from the loricae in *Dictyocysta* in several respects: the size and structure of the threads, 10 μm wide and with a distinct middle lamella as shown in Figures 9 and 11 in Steemans et al. (2014) vs. less than 5 μm wide and composed of minute tubules; the shape of the windows, oblong

198 polygonal vs. circular to elliptical; and the presence of distally flattened and broadened
199 projections (“sticks”; present vs. absent) (Agatha, 2010; Laval-Peuto, 1994; Steemans
200 et al., 2014).

201 Fourth, Steemans et al.’s (2014) interpretation of the enclosed globular structures
202 in *Nassacysta* necessitates a simultaneous cyst formation in all cells right before
203 commencement of the fossilization process. This interpretation fails to explain the
204 absence of empty loricae, which are typically found in Recent sediments and sediment
205 traps in much greater abundances than loricae enclosing resting cysts (Boltovskoy et
206 al., 1996; González et al., 2004; Ling, 1992; Suzuki and Taniguchi, 1995). Accordingly,
207 there is no evidence that they were active, swimming cells prior to encystment as
208 suggested by Steemans et al. (2014). A more parsimonious explanation for these
209 structures is that they perhaps represent wind-blown seeds of extinct plants or extant
210 contaminants.

211 Fifth, Steemans et al. (2014) interpreted some of the laterally fused *Nassacysta* as
212 having sex by stating: “when modern tintinnids are agglutinated, they are associated by
213 joining their oral pole one to the other, while our specimens are laterally associated.
214 However, the tintinnid life cycle is not well known (Tappan, 1993). Lateral fusion has
215 been observed among other ciliate-like dinoflagellates during sexual reproduction...”.
216 This statement about tintinnid sex is problematic for several reasons. Tintinnids typically
217 fuse only in their anterior cell portions (close underneath their apical cell ends where
218 their mouths are) to exchange haploid products of their micronuclei (called conjugation);
219 this exchange is how ciliates have sex (Dunthorn and Katz, 2010; Lynn, 2008). Although
220 the life cycles of most tintinnids have not been examined in detail, a cytoplasmic bridge

must be formed between mating cells to allow for genetic exchange (Figure 3); such exchange by soft cell structures cannot occur through the hard wall of the loricae. Additionally, ciliates and dinoflagellates are placed together in the Alveolata, but we are unaware of organisms that may be described as a “ciliate-like dinoflagellate”.

These obvious differences and the lack of unambiguous ciliate characters, which were already partially recognized by Steemans et al. (2014), immediately show the non-ciliate nature of these fossils to those who work on extant ciliates. These differences are more than enough to exclude placing *Nassacysta* among the tintinnids specifically and in the ciliates generally; these fossils are best considered an *incertae sedis* eukaryote.

3. Moving forward with future putative ciliate fossils

Nassacysta from the Ghadamis Basin (Steemans et al., 2014) now joins other purported ciliate fossils from the Doushantuo Formation (Li et al., 2007), the Huangmailing Formation (Li and Zhang, 2006; Li et al., 2009), and the Tsagaan Oloom Formation (Bosak et al., 2011), that are also immediately recognizable as being not ciliates by researchers working on extant species because of conspicuous morphological differences or the lack of any unambiguous morphological characters that would unite them with ciliates. We cannot identify them any further than as *incertae sedis* eukaryotes.

Although there may be impetus to place a new fossil into a taxon of extant organisms such as ciliates or other protists, especially if it is useful for stratigraphy, sometimes all that can be said is that it is a microfossil of uncertain eukaryotic affinity;

e.g., as Cohen and Knoll (2012) did with an extensive collection of mid-Neoproterozoic scale microfossils. Forcing fossils into taxa without definite criteria to do so impedes prompt understanding of their evolutionary relationships and should be avoided. If a fossil is to be described as an extinct member of the ciliates, we—the active community of taxonomists, phylogeneticists, and ecologists working on extant ciliates—recommend the following steps.

We ask that care be taken in using the current ciliate-specific terminology (e.g., Lynn, 2008) when interpreting the morphological structures in the fossils, which will allow a firm comparison with extant taxa. We emphasize the application of these terms in the interpretation of the fossil structures, not in the description of the fossils that may better be done with plain language. Specifically, the loricae of extant tintinnids are easily recognizable by a combination of the following characters (Agatha, 2010; Agatha et al., 2013; Agatha and Simon, 2012). The lorica is a single subspherical, obconical, obovoidal, cylindrical, bell-shaped, or flask-shaped chamber; the posterior portion of the bowl might be tapered, and a flaring or cylindrical apical collar might be set off against the bowl. The apical opening of the lorica is wide enough to allow an unhampered extension and contraction of the wide apical oral region of the ciliate cells. Its rim is continuous, but might be somewhat irregular or with teeth or gutters. The loricae are derived from cell secretions to which foreign material may attach, resulting in entirely agglutinated loricae, loricae composed of a hyaline collar and agglutinated bowls, or entirely hyaline loricae. Hyaline loricae or lorica portions might have rounded windows, surface ridges, or fins. The resting cysts are ellipsoidal or flask-shaped and have a distinct wall and occasionally an emergence pore (Figure 4). Knoll (2014) noted that

systematic interpretation of fossils in that ancient assemblages could include or even consist entirely of an extinct stem-group (= basal grade); therefore, some of the tintinnid lorica characters listed here may represent derived characters not occurring in fossils of the ancestors.

We ask that authors describing fossils as ciliates should use the current ciliate classification (e.g., Adl et al., 2012; Lynn, 2008) and carefully consider work reporting related evidence from extant species. This may be done by collaborating with someone working with extant species, and it would greatly facilitate a more accurate use of the terminology in the interpretations and taxonomic assignments. Such constructive cross-field collaborations occurred in Porter et al. (2003) with Neoproterozoic testate amoebae fossils and in Schönborn et al. (1999) with Triassic protists in amber.

Additionally, we ask authors and editors who are handling putative ciliate fossil submissions might include an expert of extant ciliates in the review process. In addition to comparing a new fossil with previously described ones, which paleontologists are in the best position to do especially if there have been taphonomic and diagenetic changes, comparisons with extant species are also essential. These steps could at least assist in the interpretations of putative ciliates found in the rock record.

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403 **Figs. 1-4.** Morphology of extant tintinnid ciliates. 1: *Stenosemella pacifica*, a typical
404 tintinnid ciliate (modified from Agatha and Tsai, 2008). Extended live cell projecting its
405 anterior end with the membranelles used for locomotion and filter feeding out of the
406 lorica. The somatic (body) cilia are arranged in specialized fields and rows. The lorica is
407 composed of a small hyaline collar with minute windows and an agglutinated bowl. 2:
408 *Dictyocysta mitra*, one of the few ciliates with a lorica sac and a reticulate lorica wall
409 (modified from Agatha, 2010). The lateral view shows the extended ciliate within the
410 membranous lorica sac that lines the lorica. The lorica sac merges anteriorly into a
411 closing apparatus that forms typical triangular folds in closed condition. 3: Conjugating
412 specimens of the genus *Schmidingerella* (original). The arrow marks the cytoplasmic
413 bridge through which the division products of the micronucleus (migratory nuclei) are
414 exchanged. 4: Resting cyst in the genus *Schmidingerella* (original). Scale bars = 40 μm .
415

416 **Fig. 5.** Two fossils of *Nassacysta reticulata* identified by Steemans et al. (2014) as
417 tintinnid-like ciliates. Since this fossil (holotype left) displays numerous characters
418 dissimilar of tintinnids and ciliates in general, it is best classified as *incertae sedis*
419 eukaryote. Scale bar = 100 μm (incorrectly labelled as 10 μm in the original publication).
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421 permission.

Figure 5