Fossilized reproductive modes reveal a protistan affinity of Chitinozoa

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ABSTRACT

Reproduction is a key aspect of evolution, but the process is rarely preserved in the fossil record. Organisms fortuitously preserved undergoing reproduction provide an exceptional window illuminating the biology of extinct taxa, especially those with unknown phylogenetic position. Here we report exceptional specimens of chitinozoans (enigmatic Palaeozoic organic-walled microfossils) preserved as “test-in-test” morphology, which have previously been interpreted as teratological forms. Application of advanced imaging techniques on newly recovered and reexamined Ordovician materials enabled documentation of critical morphological details of the test’s inner ultrastructure for the first time. The results show that the newly observed spongy material and dendritic structure on or inside the chitinozoan test as well as the test wall itself are all made of clustered rounded spherical particles. Morphological details suggest that those specimens represent key stages of new asexual reproductive strategies, hitherto undescribed, which produce either one or several offspring at a time. This observation challenges the prevailing hypothesis that chitinozoans are eggs of cryptic extinct marine metazoans. Instead, it is more plausible that they represent a new isolated group of protists.

INTRODUCTION

Chitinozoans are a group of enigmatic marine microfossils widespread in Ordovician to Devonian rocks (ca. 481–359 Ma; the contentious Cambrian data reported by Shen et al. [2013] require further study). They are bottle-shaped radially symmetric organic-walled tests, varying from ~50 to 2700 μm in length and having an opening sealed with an apertural plug (either operculum or prosome). Chitinozoans have been intensely studied due to their application in high-resolution biostratigraphy (e.g., Paris, 1990; Nõlvak and Grahn, 1993; Verniers et al., 1995) and in providing essential insights into deep-time global environmental changes (e.g., Steemans et al., 2009; Vandenbroecke et al., 2010, 2015). However, the biological affinity of chitinozoans has remained contentious. Two main hypotheses have been put forward since the first description by Eisenack (1931). A relationship with various groups of protists was favored until the 1980s (Miller, 1996, and references therein), but none of the proposed affinities was supported by closely similar modern analogs. It was also difficult to explain how the hermetically sealed test of chitinozoans would have permitted contact between the inner chamber and the environment (Paris, 1981; Taugourdeau, 1981). This observation, and the discovery of “cocoon” clusters (Kozlowski, 1963; Paris and Nõlvak, 1999), led to the development of the hypothesis that chitinozoans are eggs, egg cases, or reproduction stages of a cryptic group of marine metazoans. The “egg hypothesis” was subsequently reinforced by the perception that there was no fossil record of immature individuals nor evidence of any reproductive process in chitinozoan assemblages (Jaglin and Paris, 1992). A recent study has, however, questioned the prevailing egg hypothesis by documenting a large morphological variation in chitinozoan populations that is inconsistent with the variation found in modern and fossil eggs of aquatic invertebrates, and thus suggested that chitinozoans were independent microorganisms (Liang et al., 2019).

The present work focuses on rare, exceptionally preserved specimens that have previously been regarded as teratological forms. Analyzed by a combination of novel imaging techniques, important details about their morphology and inner ultrastructure are revealed for the first time. This critical new information provides exciting new insights into the reproductive strategies and biological affinity of this enigmatic group. By demonstrating a protist affinity for chitinozoans, our study provides a greater understanding of the diversity and evolution of skeletonized zooplankton during a critical interval of Earth history, the Great Ordovician Biodiversification Event (ca. 485–443 Ma; Servais and Harper, 2018).

MATERIAL AND METHODS

We discovered or reexamined 20 exceptional chitinozoan specimens among hundreds of thousands of specimens processed from the Middle and Late Ordovician limestone of Estonia, the United States, and Russia (for details

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Figure 1. Vertical and transversal X-ray computed micro-tomography sections of reproductive chitinozoans. (A–E) One individual preserved in a parental chamber, with a 3-D micro-CT view on the left and a vertical section on the right of each specimen. (F,G) A SEM (F) and a 3-D micro-CT (G) view, and longitudinal sections (F2, F3, G1, G2) of linked specimens with multiple individuals. Green arrows show locations of corresponding transversal sections (shown at right with double letters); red and blue arrows indicate parental and offspring tests, respectively. Pink arrow denotes the enclosed base of an offspring test. Scale bars represent 30 μm (AA–FF), 50 μm (F1, GG1–GG9), and 100 μm (others).

Figure 2. Focused ion beam scanning electron microscopy (FIB-SEM) analyses of reproducing chitinozoans. (A–K) Near-infrared microscopy (NIR) image (A) and inner structure (B–K) of an incidentally broken specimen. Shown are spongy materials (white arrows) on the contact area (C, D), thickened wall (yellow arrows) where the primary prosome is located (H, I), ultrastructure of prosome (J), and inside surface (K) on longitudinal section (F) of offspring. Transversal prismatic structure in H and L is caused by the FIB-SEM test, called a curtain effect. (L–U) NIR image (L) and inner structure (M–U) of complete reproductive specimen. Shown are hollow copula (blue arrows) and carina (red arrows) in N and O, fibrous structure (P–S) filled in the carina, differentiated layers of wall (white arrows in Q), and ultrastructure of sponge material (T, U). Scale bars represent 100 μm (A, B, L, M), 50 μm (N, O), 20 μm (D–G, P, Q, T), 5 μm (C, H, I, R), 2 μm (S, U), and 1 μm (J, K).
see Table S1 in the Supplemental Material1). Techniques applied include near-infrared microscopy (NIR), focused ion beam scanning electron microscopy (FIB-SEM), field emission scanning electron microscope, and X-ray computed micro-tomography (micro-CT). Detailed information on our methods is presented in the Supplemental Material.

RESULTS

Sixteen (16) of the 20 exceptional specimens are characterized by a complete test that carries one or several less-complete ones at the aboral end, normally called the base. Under NIR, FIB-SEM, and micro-CT, the necks of the less-complete tests occur inside the chamber of the complete tests (Figs. 1 and 2).

One incidentally broken specimen contains a three-dimensionally preserved individual that represents a typical Belonechitina wesenbergenensis specimen, but with the smallest known size of the species (Fig. 2B; Table S2). The simple single-layered prosome of the upper individual is much thinner than the normal prosome in the lower individual (Figs. 2E–2G). The flexure at which the initial prosome is located is thickened by a less-uniform wall (Figs. 2G–2I).

An image slice near the base of a Cyathochitina campanulaeformis specimen shows that the upper individual occupied a similar base with a smaller but distinct carina (red arrows in Fig. 2O). An elongated membrane—the copula—appears on the center of the base (Fig. 2N), which has never before been reported from the genus Cyathochitina. Unlike a common copula, this one is hollow inside (Fig. 2O). Similar hollows are recognized in most of the exceptional specimens we examined (Figs. 1E–1G; Figs. S1P–S1U). The chamber wall of the upper test seems to be an extension of the lower test layers, whereas the upper test neck is connected with the inner layer of the lower test chamber by spongy material (Figs. 2O–2T). The spongy material also occurs in the contact area of other exceptional specimens (arrows in Figs. 2C, 2D, 3V, and 3W; Fig. S1Q), reminiscent of the granules illustrated in Grahn and Afzelius (1980, their figure 3F). The ultrastructure of the spongy material (Figs. 2U and 3K–3M), the dendritic structure (Figs. 3F and 3R–3T), the prosome (Fig. 2J), and the test wall (Figs. 2K, 3O–3Q, 3X, and 3Y; Figs. S1J–S1M) show that all of

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1Supplemental Material. Extended introduction to the materials and methods, Figures S1–S4, Tables S1 and S2, and supplemental movies (Movies S1–S3). Please visit https://doi.org/10.1130/GEOLO.S.12735848 to access the supplemental material, and contact editing@geosociety.org with any questions. All raw micro-CT data are available on request from liangyan@nigpas.ac.cn.

Figure 3. Scanning electron microscopy images of morphological ultrastructure of broken (A–C) and complete (U) reproductive chitinozoan specimens. Shown are enlargement of the broken part in C (N), ultrastructure of nested structure (D–F), sponge material (K–M, V–W), branched structure (R–T), the test wall (G–J, O–Q, X–Y), and a longitudinal micro-CT section (Z) of the upper part of the complete specimen, with red and blue arrows indicating the parental and offspring tests, respectively. Scale bars represent 100 μm (A–C,U,Z), 10 μm (I,N,U,V), 5 μm (K,X), 2 μm (D, E,G,O,S), 1 μm (H,J,L,R,T,W,Y), 500 nm (F,P,Q), and 200 nm (M).
them are made of clustered rounded spherical particles with a diameter of approximately several tens of nanometers to >100 nm (Fig. S2), here named spherulitic material. Energy-dispersive X-ray spectroscopy element mappings showed no difference among those structures (Fig. S3). It turns out that the different forms or structures, i.e., the spongy material, dendritic structure, and condensed test walls, are all made of spherulitic material, but with different sizes and different densities of the particles.

The second type of exceptional specimen features a chain consisting of several small individuals that become progressively shorter distally (i.e., in the aboral orientation). The lowermost individual of Cyathochitina? sp. 1 (white arrow in Fig. 1F) was broken off when the specimen were being manipulated during microtomography. The SEM image clearly shows that the base of the lowermost individual is closed and the individual seems to be detaching from the chain (Fig. 1F). The micro-CT image (Figs. 1F–1F) shows that the second test is also closed while the bases of the other two are open (denoted by pink arrows).

The lowermost individual of another “chained” specimen has a complete but compressed test and is difficult to identify to the genus level (Fig. 1G). The largest diameter of the other three tests becomes slightly but progressively smaller upward. The second test has a cylindrical neck and conical chamber with a distinct flexure under micro-CT and is attributable to the genus Lagenochitina. The uppermost two tests have conical chambers and poorly developed necks. The bases of the individuals are not closed. Transverse sections along the test walls at different points, including a prosome section of the lowermost test (Fig. 1GG), show that the upper three tests are of different stages of “maturity”. Two micro-CT–based videos in the Supplemental Material (Movies S1 and S2) provide a clearer view of this key specimen.

Four additional unusual specimens were studied, including a Cyathochitina distinguished by a thickened but still-enclosed base carrying a shaping carina (Fig. 4A), a Lagenochitina that developed two bases (Fig. 4B), another Cyathochitina characterized by a special base with two base margins and a centered hollow (Fig. 4E), and a Conochitina featuring multiple layers around the hollow (Fig. 4F) that highly resemble an inward self-healing process.

**DISCUSSION**

These exceptional chitinozoans are not a newly discovered phenomenon—similar material has been sporadically reported from different stratigraphic levels and in different taxa, such as Ancyrochitina sp. 1. (Cramer and Díez, 1970), Ancyrochitina cf. brevis (Jaglin and Paris, 1992), Angochitina eisenacki (Cramer and Díez, 1970), Conochitina ex aff. campanulaeformis (Eisenack, 1937), Conochitina sp. (Wrona, 1980), Cyathochitina campanulaeformis (Kozłowski, 1963), and Hercrochitina sp. cf. H. crickmayi (Miller, 1996). Terms such as dimorphic or polymorphic (Taugourdeau and Magloire, 1964), gravid, or sausage-type bead colonies (Cramer and Díez, 1974) were used to describe those specimens, and a rejuvenation stage in vegetative reproduction was proposed (Cramer and Díez, 1970, 1974). However, restricted by the less-advanced techniques and the dominant egg hypothesis, further work was suspended. Compared with undisputed teratological chitinozoans, or simply linked and/or chained clusters which are common in some taxa (e.g., Pierochitina, Linochitina, Calpichitina in Paris et al. [2015]), specimens in this study are distinguished by regular and symmetrical “test-in-test” morphology where a complete test carries one or several less-complete ones at the base.

With advanced imaging techniques, the less-complete tests turn out to be complete but smaller tests. The features preserved in the exceptional specimens resemble an asexual reproductive process, which can be divided into two modes: one parental individual producing one offspring at a time, or producing multiple offspring almost simultaneously.

**Reconstruction of Chitinozan Reproduction**

Considering all the developmental details displayed by the exceptional fossils, the process of chitinozoan reproduction can be reconstructed. In the first mode, the parent produces one offspring at a time in the following sequence (Fig. S4; Movie S3). The base of the parental individual extends to form a second base with a hollow that later appears at the center of it. With the extension of the parental test wall, the offspring chamber becomes larger and morphologically resembles the parental individual. The offspring disconnects from the parental individual after the test is completed, a stage marked by the closure of the hollow at the offspring base and the formation of the primary prosome. The parental individual is hypothesized to be alive after reproduction based on the presence of an inwardly enclosing base.

The full process of the second mode—one parental individual produces multiple offspring in a chain—is not yet fully understood due to the limited number of discovered specimens. However, based on the available evidence from the two chains studied, the basic process, i.e., the way each offspring grows, is likely to be the same as in the first mode. Because multiple offspring are duplicated in a line, one distinct feature is that the individuals closer to the parental individual are more mature than those on the distal end.

The reproductive process could have occurred rapidly, as in the modern Tintinnina (ciliate protists), which can build a new test in a matter of minutes (Laval-Peuto, 1981), thus the preservation of reproducing individuals would be expected to be exceedingly rare. Moreover, due to the fragile nature of the attachment between the parent and offspring tests, as in the case of Belonochitina wesenbergensis and Cyathochitina sp. 1 (Fig. S1U), the likelihood of recovering intact reproducing specimens is further reduced.

All these specimens are interpreted as reproducing chitinozoans; no evidence of nuclear fission or soft tissue division has been detected. Both of the reproductive modes have no perfectly matching modern analog, but both are
characterized by cloning of the parental test, together with all its morphological structures and ornamentations.

Under optical microscopy or SEM, the mode of producing one offspring at a time highly resembles budding—a common asexual reproduction strategy found among some earlybranching animals and plants. It differs from budding in its directed orientation—the clone takes place only at the anti-aperture side, and the neck of the offspring forms inside the parental chamber.

The other mode of producing multiple offspring in a chain resembles strobilation, a rare asexual reproductive process that occurs in certain cnidarians, such as the polyp stage of the jellyfish Aurelia aurita, and helminths (Technau et al., 2015). However, strobilation is the spontaneous transverse segmentation of the body, whereas the multiple lined tests in chitinozoans are more like reduplicate clones of the parental test. When the offspring detached from the parental individual, it was probably a mature, independent individual.

Previously, all the reproductive specimens that exhibit a neck and prosome belong to the Prosomateria according to the latest classification (Paris et al., 1999). No reproductive specimens have been discovered among Desmochitina, a genus characterized by an ovoid chamber sealed by an operculum and lacking a neck, whose tests commonly occur in long chains and sometimes exhibit cocoon-like preservation.

Biological Affinity of Chitinozoans

The morphology, geometric shapes, significant within-population variation, widespread distribution in disparate paleoenvironments, and new evidence for two modes of asexual reproduction make it plausible to link chitinozoans, at least the taxa presented in this study, with protists.

Previous studies (Miller, 1996, and references therein) have also associated chitinozoans with some protist groups, such as rhizopods, ciliates, foraminifera, dinoflagellates, and others, mainly based on test outline comparisons. None of these groups are fully comparable with chitinozoans discovered among Ciliata tintinnina, at least the taxa presented in this study, with protists.

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