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Supplemental Material

Fossilized reproductive modes reveal a protistan affinity of Chitinozoa

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Supplemental Material includes a brief introduction of the materials and methods, 4 supplementary figures (Figures S1–S4), 2 supplementary tables (Tables S1–S2), and captions for the three movies (Videos S1–S3.

MATERIALS AND METHODS (EXTENDED)

The biological affinity of chitinozoans has puzzled paleontologists for decades. The lack of modern analog, and the unique morphological structures make it even difficult to sort this extinct micro-organic group. However, the sporadically observed abnormal specimens, which turn out to be the key evidence recording the reproduction moments of chitinozoans, provided a new window to explore the nature of chitinozoans.

The organic tests of chitinozoans are usually preserved as flattened in shale and mudstone, which is unable to investigate their inner structures. Specimens from limestone which are usually preserved in three-dimensional and with less morphological malformations thus are targeted for this study. To better understand the inner structures of those crucial specimens, the NIR, FIB-SEM and micro-CT systems are applied.

All chitinozoan specimens studied in the present study were hand-picked from limestone residues after diluted acetic acid treatment instead of the widely adopted standard palynological method (Paris, 1981). The advantage of the former is that it requires less washing and sieving, which helps to prevent the fragile reproductive specimens from disaggregating. Specimens were fixed on stubs with diluted Canada balsam, gelatin solution and Elmer's milky white glue for further tests. Terminology adopted in the present study follows Paris et al. (1999).

The NIR system adopted is Olympus R51X with a flash 4.0 camera. The NIR images were stacked by Zerene Stacker software and the original images are available from one of the corresponding authors (Y.L.) on request. The FIB-SEM analyses were made at the State Key Laboratory of Dielectric Superlattice, School of Physics, Nanjing University (Strata FIB 201) and at the Jinjian Lab in Guangzhou (Auriga Compact). The micro-CT scans were conducted at the Nanjing Institute of Geology and Palaeontology, Chinese Academy of Sciences (NIGPAS, Zeiss Xradia 520 versa) and the China University of Geosciences, Wuhan, China (Bruker-microCT system SkyScan 1172F). The micro-CT data are processed with VG Studio software. The EDS analyses were made under FESEM at NIGPAS (MAIA3 GMU).

SUPPLEMENTARY FIGURES

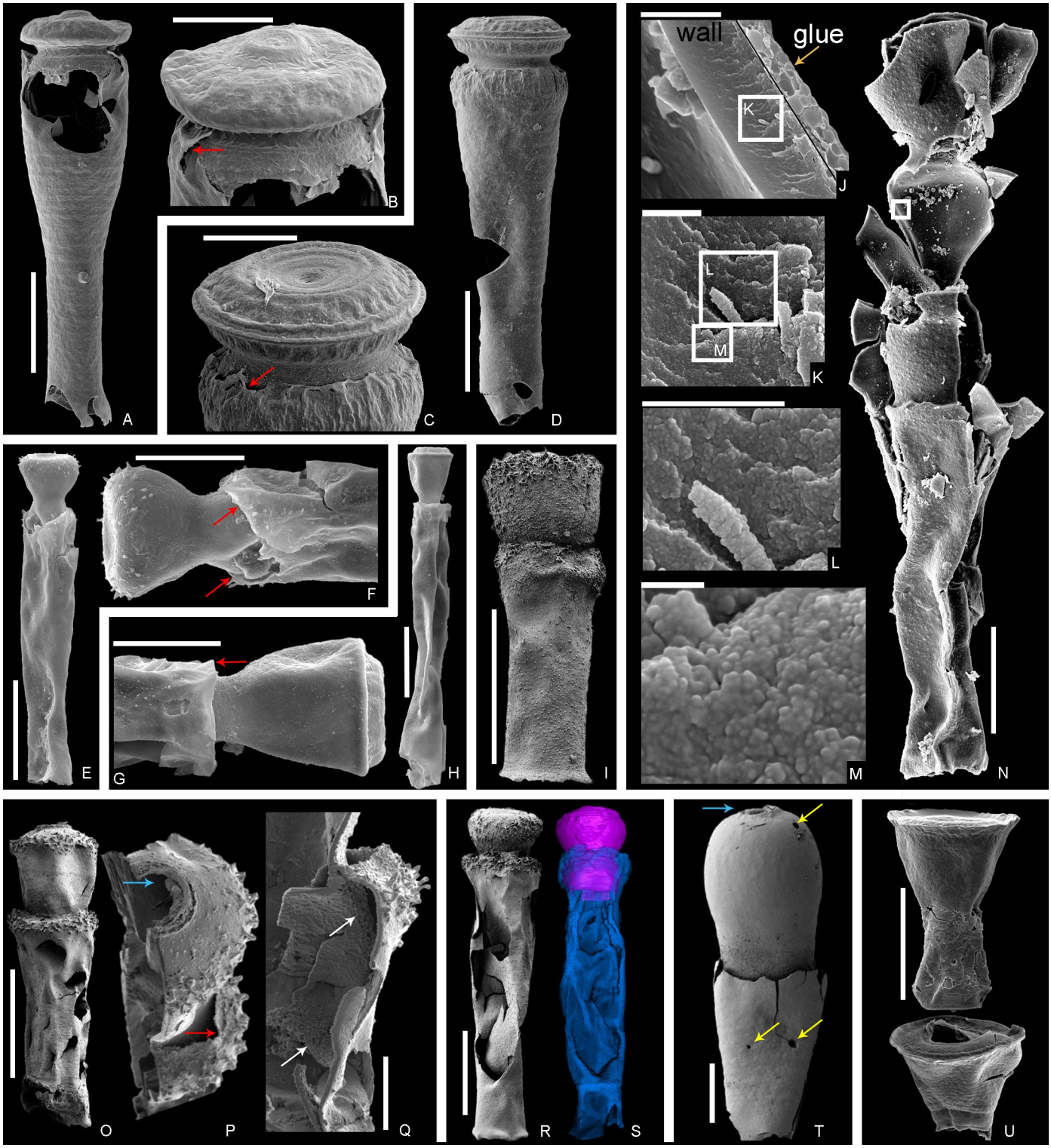

Figure S1. SEM images of reproductive specimens. A–H, N, SEM images of specimens shown in Figs. 1A–C, 1E, 3. **I, O–U**, Other reproductive specimens. Red arrows indicate the test wall of parental individuals. Orange arrow denotes the glue left on the test surface. Blue arrows show the hollow on the base of the offspring specimens. White arrows indicate the spongy

material at the contact area on the outer surface of the offspring. Yellow arrows show tiny holes that are common in chitinozoan tests (e.g., Fig. 8 in Wrona, 1980), which may be a result of predation or parasitism as Grahn (1981a) and Porter (2016) suggested. **J**–**M**, High-resolution enlargements show that the condensed test wall is made of closely arranged spherulitic material. **R**–**S**, Colored micro-CT image of a reproductive specimen shows clearly that the neck of the offspring is inside the chamber of the parental individual. **U**, Separated individuals of a reproductive *Cyathochitina* when transferred to the stub for SEM. Scale bars represent 100 µm, except for **B, C, F and G** (50 µm), **J** (5 µm), **K and L** (1 µm), and **M** (200 nm).

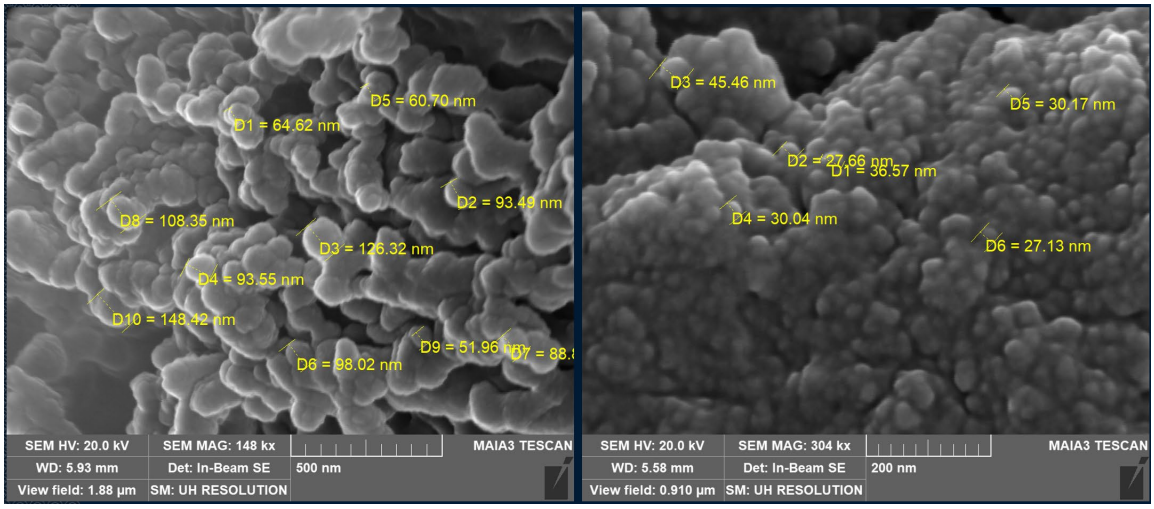

Figure S2. Ultrastructure of the sponge structure (left) and the test wall (right). Both the sponge structure and the test wall are made of clustered spheres with a diameter of around several tens to hundred nanometers and less than fifty nanometers (named as spherulitic material in this study), respectively.

Figure S3. Energy-dispersive X-ray spectroscopy (EDS) tests showing that all the elements of both the sponge and the test wall are the same.

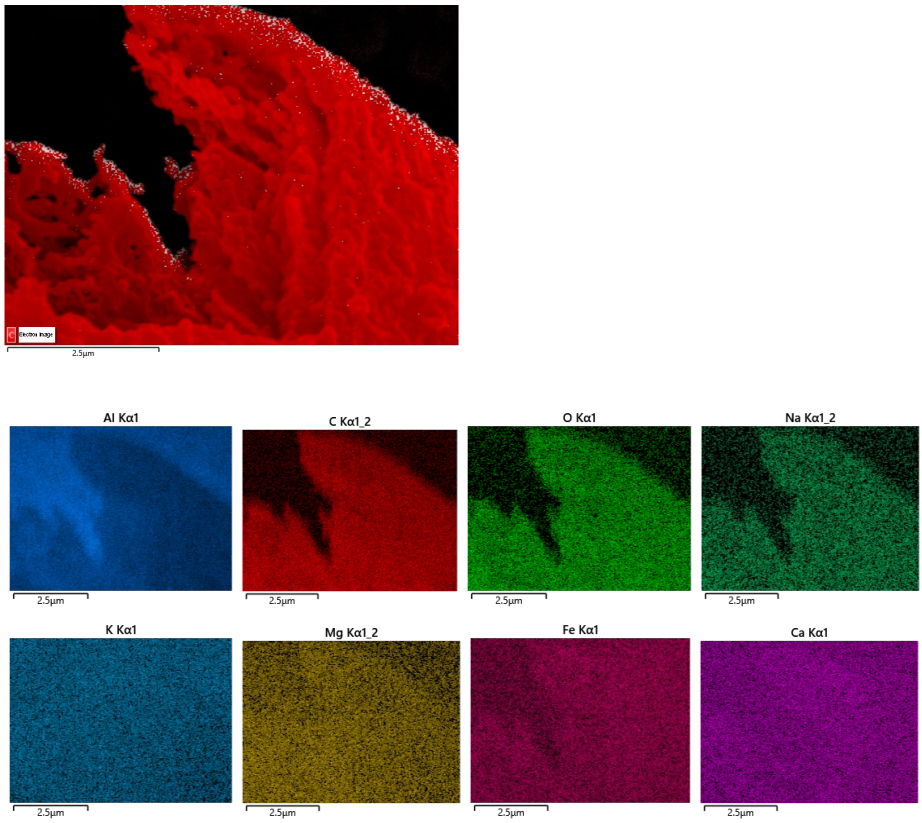

Figure S3.1. EDS analysis on the area of Fig. 3G.

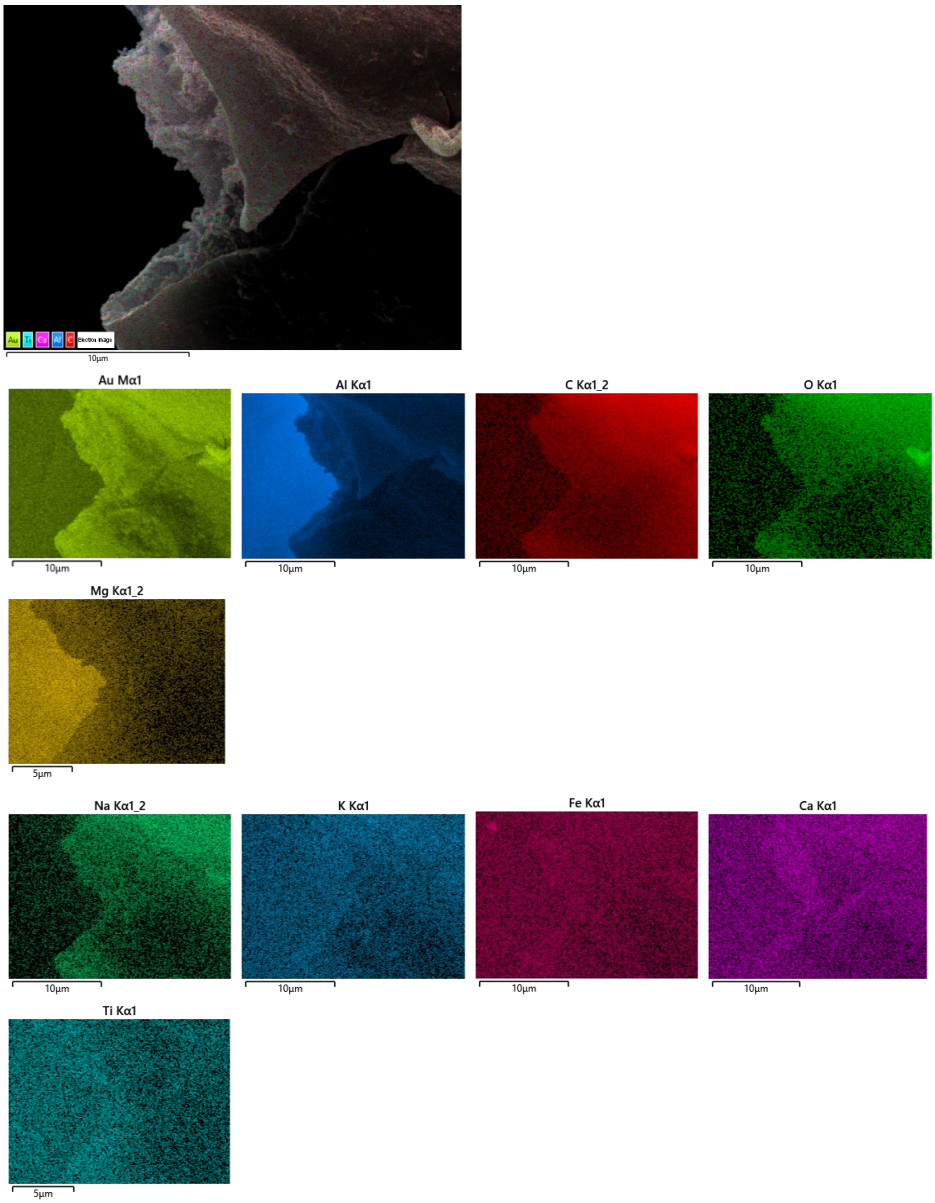

Figure S3.2. EDS analysis on the area of Fig. 3K.

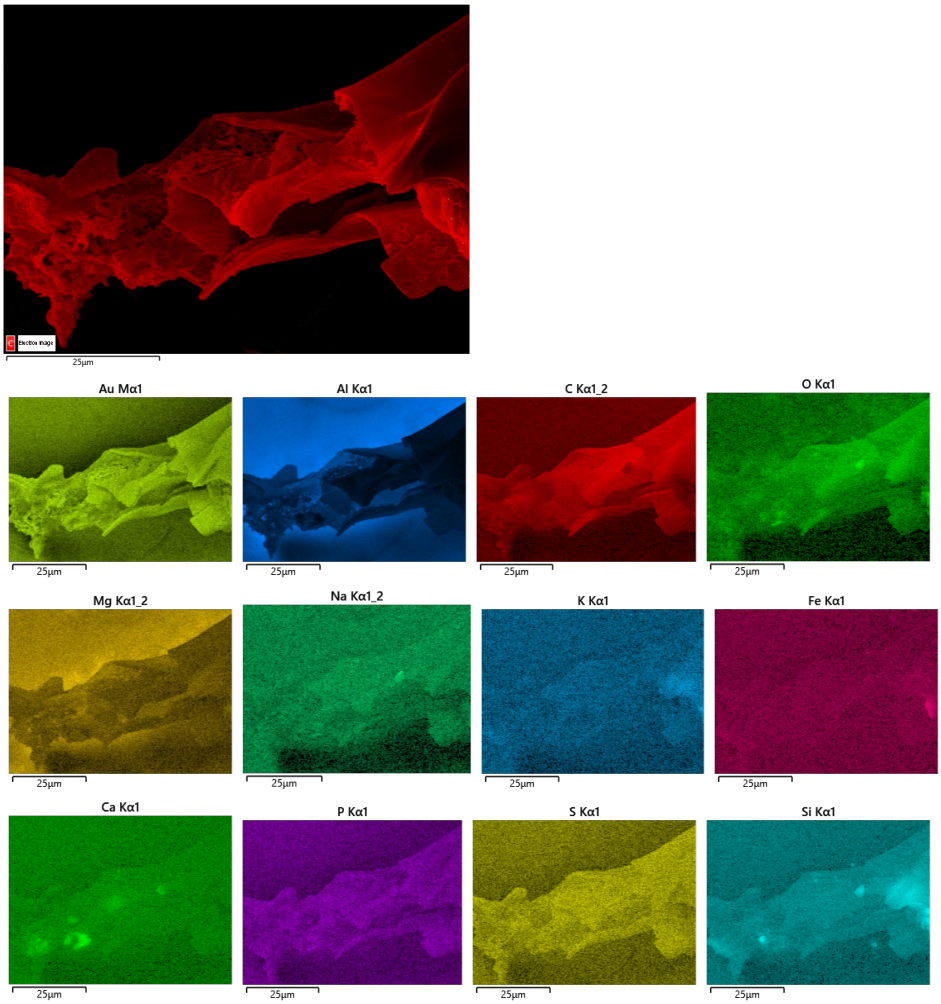

Figure S3.3. EDS analysis on the area of Fig. 3N.

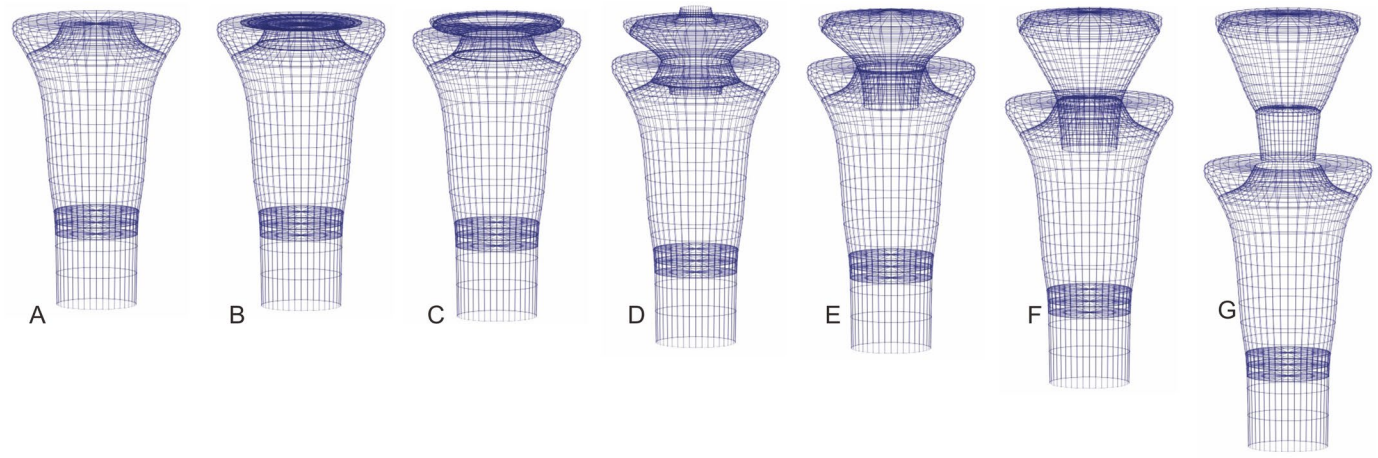

Figure S4. Hypothetical 3D perspective drawing of the reproductive cycle of *Cyathochitina campanulaeformis*. B, The base of the parental individual becomes thickened by the extension of the test wall. **C**, A hollow appears at the center of the offspring's base. **D**, With the continuous extension of the test wall, the chamber of the offspring becomes larger and morphologically resembles the parental individual, while at the same time the inner layer of the parent secretes spongy material to form the neck of the offspring test. **E**, The chamber and neck of the offspring are formed separately and converged. **F**, The primary prosome (a simple single-layered septum) is formed and the hollow at the offspring base becomes enclosed at almost the same time. **G**, The offspring leaves the parent with a hole left in the base, which quickly becomes inwardly enclosed.

SUPPLEMENTAL TABLES

Table S1. Information of 16 studied reproductive specimens and 4 specimens highlighted by a blue shading represent the beginning and the self-healing stage of the reproductive process.

No.	Figure	Species	Sample	Age	Locality	Locality information	Registered number*
1	Fig. 1A; Fig. S1A	*Cyathochitina calix*	M-13459, Kaugatuma core, 458.7 m	Darriwilian	Saaremaa Island, Estonia	Unpublished	GIT-2019-001
2	Fig. 1B; Fig. S1D	*Cyathochitina* sp. 1	M-5880	Darriwilian	Osmussaar, Estonia	Hints et al., 2012	GIT-2019-002
3	Fig. 1C; Fig. S1E	*Belonechitina* sp.	M-13170	Sandbian	Peetri 71-T core, 191.0 m, North Estonia	Männil and Bauert, 1986	GIT-2019-003
4	Fig. 1D	*Cyathochitina campanulaeformis*	M-13497	Sandbian	Kaugatuma core, 429.3 m, Saaremaa Island, Estonia	Unpublished	GIT-2019-004, LOST
5	Fig. 1E; Fig. S1H	*Conochitina* sp. 1	M-13984	Darriwilian	Viki core, 358.7 m, Saaremaa Island, Estonia	Hints et al., 2014	GIT-2019-005
6	Fig. 1F	*Cyathochitina* sp. 2	Männamaa core, 142.9 m	Katian	Hiiumaa Island, Estonia	Nõlvak, 2008	GIT-2019-006
7	Fig. 1G; Fig. S1N	*Lagenochitina* sp. 1	M-5872	Darriwilian	Osmussaar, Estonia	Unpublished	GIT-2019-007
8	Fig. 2A	*Belonechitina wesenbergensis*	HWY99-45 m	Katian	Oklahoma, USA	Goldman et al., 2007	GIT-2019-008
9	Fig. 2M	*Cyathochitina campanulaeformis*	HWY99-48.5 m	Katian	Oklahoma, USA	Goldman et al., 2007	GIT-2019-009, LOST
10	Fig. 3A-C	*Conochitina* sp. 2	M-7964, Kaugatuma core, 349.0 m	Katian	Saaremaa Island, Estonia	Kaljo et al., 2008	GIT-2019-010
11	Fig. 3U	*Laufeldochitina* sp. 1	M-13455, Kaugatuma core, 460.1 m	Darriwilian	Saaremaa Island, Estonia	Unpublished	GIT-2019-011
12	Fig. 4A	*Cyathochitina*	Assamalla	Katian	Assamalla,	Calner et	GIT-2019-

		campanulaeformis	251 core, 67.5m		Estonia	al., 2010	012
13	Fig. 4B	*Lagenochitina* sp. 2	Kaugatuma core, 426.0 m	Sandbian	Saaremaa Island, Estonia	Unpublished	GIT-2019-013
14	Fig. 4C	*Cyathochitina campanulaeformis*	HWY99-48.5 m	Katian	Oklahoma, USA	Goldman et al., 2007	GIT-2019-014
15	Fig. 4D	*Conochitina clavaherculi*	Lasnamägi quarry, M-4156	Darriwilian	Tallinn, Estonia	Unpublished	GIT-2019-015
16–18	Fig. S1I, 1O, 1R	*Hercochitina violana*	HWY99-44 m	Katian	Oklahoma, USA	Goldman et al., 2007; Liang et al., 2019	GIT-2019-016–018
19	Fig. S1T	*Euconochitina*? sp. 1	Kaugatuma core, 460.1 m	Darriwilian	Saaremaa Island, Estonia	Unpublished	GIT-2019-019
20	Fig. S1U	*Cyathochitina* sp. *campanulaeformis*	Aleksejevka quarry	Sandbian	Leningrad District, Russia	Unpublished	GIT-2019-020
Note: All specimens are stored at the Department of Geology, Tallinn University of Technology.							

Table S2. Previous size variation on *Belonechitina wesenbergensis*.

L_{min} (μm)	L_{max} (μm)	Measured specimens	L_{max}/L_{min}	Age	Locality	Reference
142	218	7	1.54	Upper Ordovician	Estonia	Eisenack, 1959
107	271	11	2.53	Upper Ordovician	Estonia	Eisenack, 1965
108	117	3	1.08	Upper Ordovician	Baltica erratics	Eisenack, 1968
105	201	40	1.91	Upper Ordovician	America	Jenkins, 1969
146	342	/	2.34	Middle Ordovician	Sweden	Grahn, 1981b*
147	263	/	1.79	Upper Ordovician	Sweden	Grahn, 1982
130	310	50	2.38	Ordovician,	Canada	Martin, 1983
137	154	2	1.12	Middle and upper Ordovician	Estonia	Grahn, 1984
167	324	/	1.94	Upper Ordovician	Poland	Wrona et al., 2001
150	160	2	1.07	Upper Ordovician	Canada	Asselin et al., 2004
130	210	21	1.62	Upper Ordovician	England	Vandenbroucke, 2008
86	224	127	2.60	Upper Ordovician	USA	This study

*The species was later revised as *Belonechitina wesenbergensis* s.l. by Grahn and Nõlvak (2010).

CAPTIONS FOR THE SUPPLEMENTAL VIDEOS

Movie S1. A micro-CT based video showing the attachment and inner structure of the multiple reproductive tests presented in Fig. 1G in an SEM view, shown in a colored view, a separate view, and a transparent view successively.

Movie S2. A Micro-CT based video showing the transverse sections of the multiple reproductive tests presented in Fig. 1G.

Movie S3. Colored hypothetical 3D animation demonstrating the reproductive cycle of *Cyathochitina campanulaeformis*.

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