

## Structure of Organic Spines in the Rhizarian Protist *Belonocystis tubistella* Rainer, 1968, and a Description of *Belonocystis quadrangularis* n. sp. (Cercozoa, Insertae Sedis)

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**Summary.** Several Ontario (Canada) populations of the “heliozoan-like” protist *Belonocystis* Rainer, 1968 were studied to help elucidate the taxonomic position of this genus relative to other rhizarian protists with fine pseudopodia. The Ontario material presently includes two distinct species – *B. tubistella* Rainer and *B. quadrangularis* n. sp., described here as a new species based on cell morphology and microstructure of the spiny investiture encasing its cell. The organic nature of the spiny structures of both species has been confirmed by their dissolution in 6% sodium hypochlorite solution. Median cell diameters of *B. tubistella* and *B. quadrangularis* were 10.5  $\mu\text{m}$  and 7  $\mu\text{m}$ , respectively. As revealed by transmission electron microscopy, the spine bases in *B. tubistella* consisted of a complex structure of four curved ribs interconnected by a membranous sheet replete with closely-appressed, large circular to elliptical pores, while in *B. quadrangularis* the spine bases consisted of a simple four-cornered “pyramid” of struts that supported the main shaft of the spine. These findings strengthen the status of the genus *Belonocystis* and its placement outside the realm of the Centrohelida and Rotosphaerida.

**Key words:** Clathrulinidae, Desmothoracida, Rhizaria, pseudoheliozoa, spined protist.

**Abbreviations:** EM – electron microscope, electron microscopical; TEM – transmission electron microscope; LM – light microscope; St. Dev. – standard deviation; min. – minimum; max. – maximum; Coef. Var. – coefficient of variation.

### INTRODUCTION

Rainer (1968) described the new genus *Belonocystis* – a rotosphaerid-like organism with a single type of spine, which, because he believed it had organic spines, he assigned to the Pseudoheliozoa Penard, 1904. *Belonocystis* is apparently not rare, having been found at

two locations in Idaho, USA (William Bourland, personal communication, 9 March, 2011), from several locations in the Netherlands (Ferry Siemensma, personal communication, 9 March, 2011), and from Germany by Michael Plewka (images of *B. tubistella* Rainer at <http://www.mikroskopie-forum.de/index.php?topic=7223.0>).

Until now, there was some question about Rainer’s assumption that spiny elements surrounding the cell of the only known species, *B. tubistella*, were in fact organic and thus contrasted with rotosphaerid genera such as *Pinaciophora* that possess siliceous spine-scales (see

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e.g. Mikrjukov (1999)). The only evidence provided by Rainer (1968) for organic spines in *B. tubistella* was his report that they appeared to be fragile and prone to distortion on being dried. Also, until now, all specimens of *B. tubistella* have been examined only by light microscopy; no chemical testing or examination by electron microscopy (EM) has been done to confirm the organic nature of its spiny investiture, or to describe their ultrastructure. Such studies would be helpful in furthering the discussion of the higher classification of *Belonocystis*.

In the early 1980's I collected specimens of two heliozoan-like protists from ponds in Ontario, Canada, either of which could have been referred to *B. tubistella* on the basis of light microscopy undertaken at that time, with reference Rainer's (1968) description of this taxon. Electron microscopy, however, clearly revealed that spine structure was much different in these two organisms and they therefore represented two distinct species. Only now, after having recently rediscovered one of these species from another Ontario location and subsequently "resurrected" the old data I had collected some 30 years ago, am I formally offering an interpretation of the scale structure in *B. tubistella* and presenting a description of the new species, *Belonocystis quadrangularis*.

## MATERIALS AND METHODS

Samples containing specimens of *Belonocystis tubistella* were collected in May of 1981 (Pond No. 4; KN#V-475) from the edge of a beaver pond (Wood Township, west side of Hwy 169 opposite the road to Barlochan, Ontario, Canada at 45°00'N, 79°31'W) by submerging a 1-L screw-cap glass bottle to near the bottom sediments, and in November of 2010 (KN#V-2129) by submerging a 500 mL wide-mouth polycarbonate bottle to near the bottom sediments of a shallow (20 cm deep) *Typha* marsh located within the drainage basin of Sturgeon Lake, Ontario (44°29'06"N, 78°37'43"W). Samples containing specimens of *Belonocystis* sp. (described here as *B. quadrangularis* n. sp.) were collected in a similar fashion from three permanent ponds in south-central Ontario as follows: Pond #7, 24 October, 1983 (KN#V-553), Snowdon Township, Haliburton County, 44°49'28"N, 78°32'00"W; Pond #16, 13 May, 1984 (KN#V-582), Esquesing Township, Halton Hills, 43°33'45"N, 79°54'20"W; Halton Regional Forest pond, 15 Sideroad, 26 September, 1982 (KN#V-495), Esquesing Township, Halton Hills, 43°34'55"N, 79°58'30"W.

All samples were examined in living (unfixed, unpreserved) states within a few hours of collection. Subsamples concentrated by sedimentation were transferred to 25 mL plastic lined screw-capped glass vials, fixed with 2–3 drops of Lugol's iodine-potassium iodide solution and preserved with a few drops of 37% formaldehyde

solution. Fixed specimens of *Belonocystis* were isolated with a micro-pipette and transferred through several distilled water washes to Collodion®-coated copper location grids before examination at 20 KV in a transmission electron microscope (TEM). Some specimens were shadowed at an angle of about 20° with Pd-Pt alloy in an evaporator/vacuum chamber before TEM examination.

Confirmation of the organic nature of the spines of both of these taxa was determined from specimens that had been isolated and washed as described above and allowed to dry on a No. 1 cover slip. This preparation was inverted on a standard glass slide and anchored at the corners with Canada Balsam. The specimen was located with a low-power (16 x) objective and then observed with a high-power (100 x) phase-contrast objective while a 6% solution of sodium hypochlorite was introduced as a droplets at the edges of the cover slip. Enough hypochlorite solution was added to totally envelop and permeate the previously dried specimen. The dissolution of the spines was observed over the course of a few minutes.

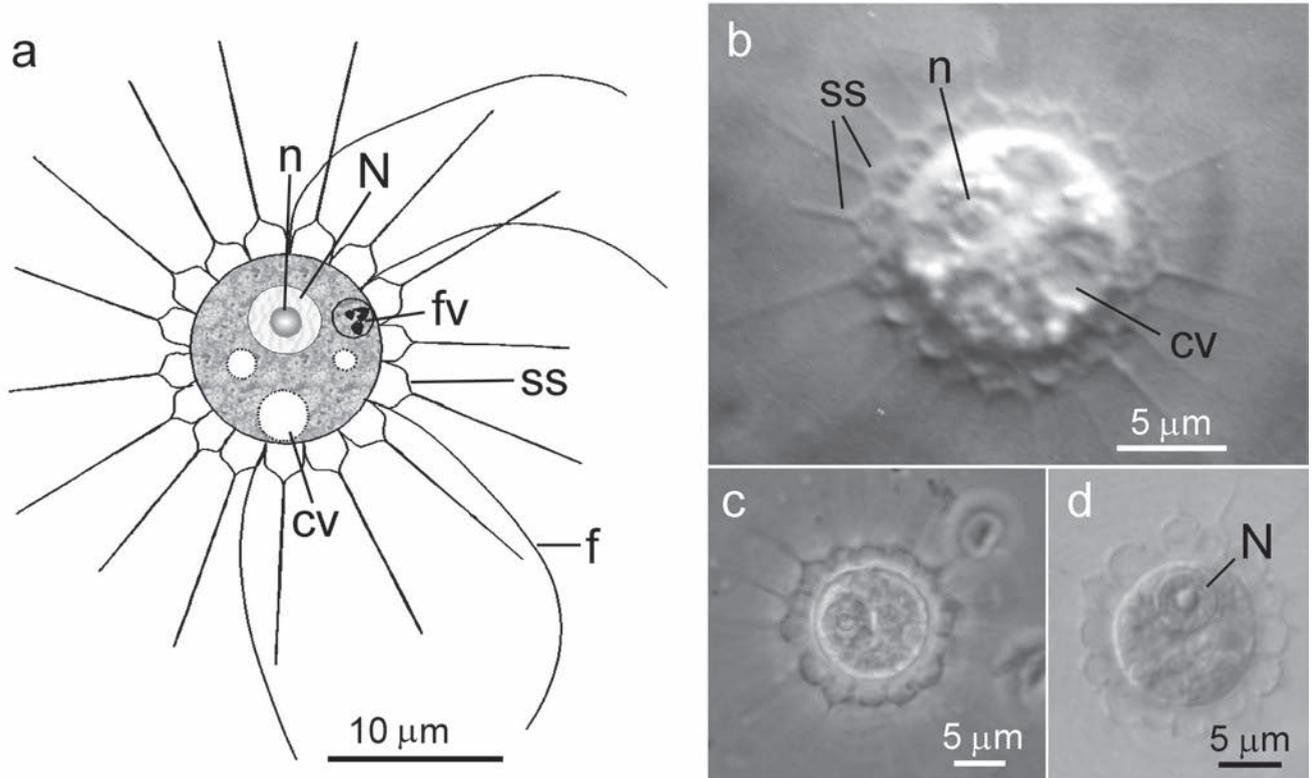
The diameters of 50 cells of each of the two species under investigation were measured at 1000 x (100 x phase contrast object and 10 x eyepieces) with an inverted microscope during systematic (non-overlapping) transects of aqueous sample preparations. Relevant measures of central tendency and standard deviations were calculated and the Mann-Whitney *U*-test (CoHort Software 1995) was used to determine the likelihood of no statistically significant difference between the median cell diameters of the two species.

## RESULTS

### *Belonocystis tubistella* Rainer, 1968; Figs 1a–d, 2a–h

Cells were spherical, 8–16 µm in diameter and moved slowly among the detritus of the microscopic preparation, facilitated by their fine filopodia (Fig. 1a) which were difficult to see, even with high magnification phase-contrast microscopy. Cells contained an eccentrically located nucleus of about 4 µm in diameter with a prominent nucleolus (Fig. 1a, b, d). From one to four functional contractile vacuoles were present, ranging in size up to about 4 µm in diameter, with size apparently dependent upon the stage of the diastole-systole cycle.

The most distinctive feature of these cells was the radiating series of spines with their broad tent-shaped bases (Fig. 1). Spines were 10–15 µm long, consisting of a hollow shaft that was widest (0.1–0.3 µm diameter) near the base and tapered to a very fine distal point (Fig. 2a–c). The bases of the shafts were divided into four curved arms that formed a basal tent-like structure covered by a membranous sheet that was perforated with circular to elliptical pores in close proximity forming a mesh-work appearance to the spine bases (Fig.



**Fig. 1.** *Belonocystis tubistella*. **a** – diagrammatic representation of a living cell; **b–d** – images of living cells. N – nucleus, n – nucleolus, fv – food vacuole, cv – contractile vacuole, f – filose pseudopod, ss – spine.

2e, g, h). In the living, uncollapsed state, the top portion of the spine base appeared funnel-shaped with sloping sides that abruptly straightened to nearly parallel sides where scale bases abutted one another (Figs 1a, b, 2d). Scale bases were 2–3  $\mu\text{m}$  wide and pores in the sheet mesh-work structure forming the bases were 0.1–0.3  $\mu\text{m}$  in diameter.

***Belonocystis quadrangularis* n. sp.; Fig. 3a–j**

**Diagnosis:** Cells spherical, 5–9  $\mu\text{m}$  in diameter with a prominent eccentric nucleus 3–4  $\mu\text{m}$  in diameter and 1–3 contractile vacuoles up to 3  $\mu\text{m}$  in diameter. Cells, lacking chloroplasts, phagotrophic, motility by fine pseudopodia, covered with a single type of organic spine consisting of a long, tapered, hollow shaft with a pyramid-shaped base comprised of four arms or struts 1.6–1.8  $\mu\text{m}$  long, originating from an irregularly shaped nodal swelling at the base of the main spine shaft. Base of the main spine shaft distal to the basal

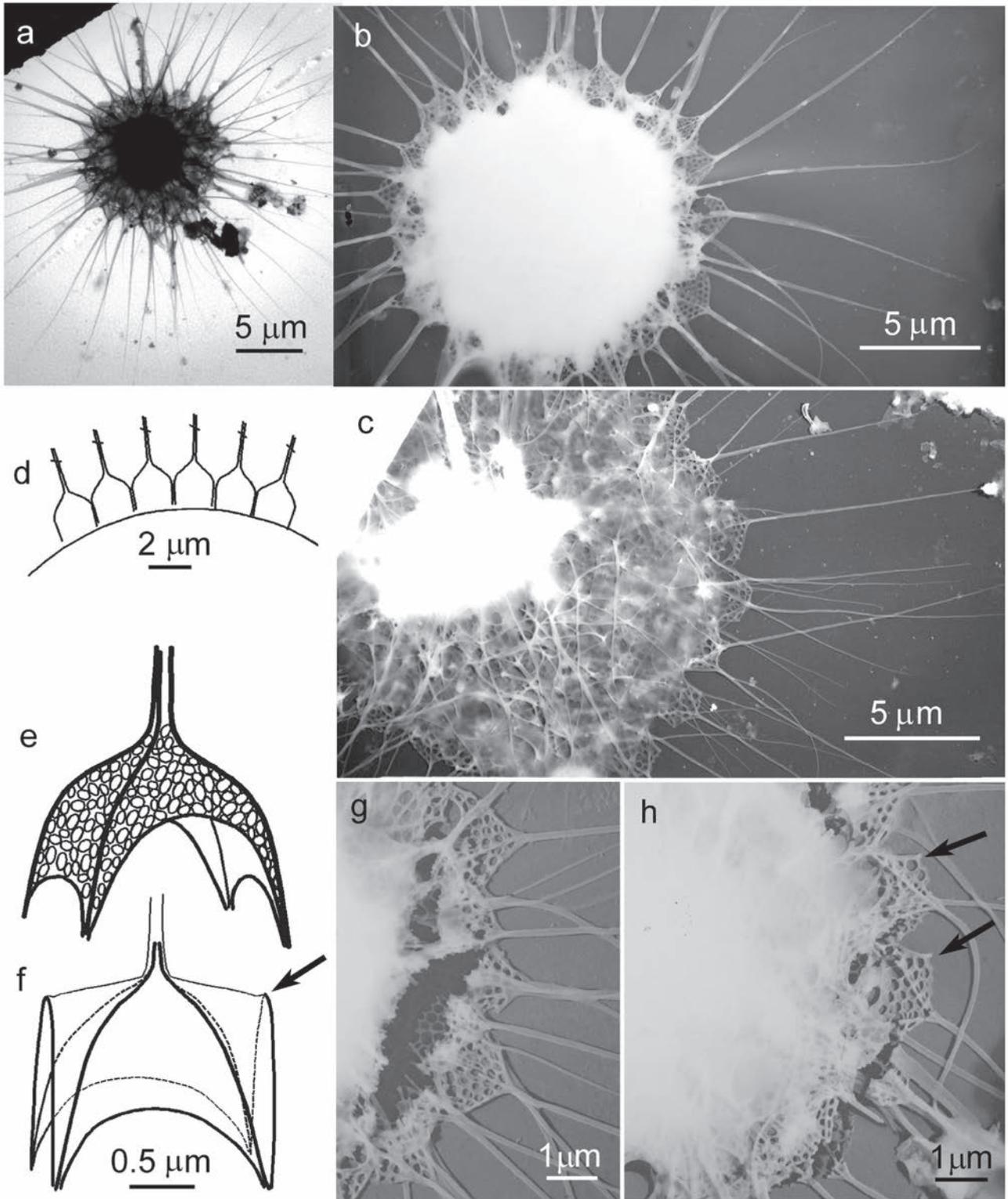
struts, 0.23–0.30  $\mu\text{m}$  in diameter and tapering over a total length of 15–20  $\mu\text{m}$  to a distal tip that is about 0.07  $\mu\text{m}$  in diameter.

**Etymology:** The specific epithet (*quadrangularis*) denotes the 4-sided pyramidal structure of the spine bases of this taxon comprised of four spreading struts serving to anchor the spine near the surface of the cell (*L. quadrangularis* – four-sided, quadrangular).

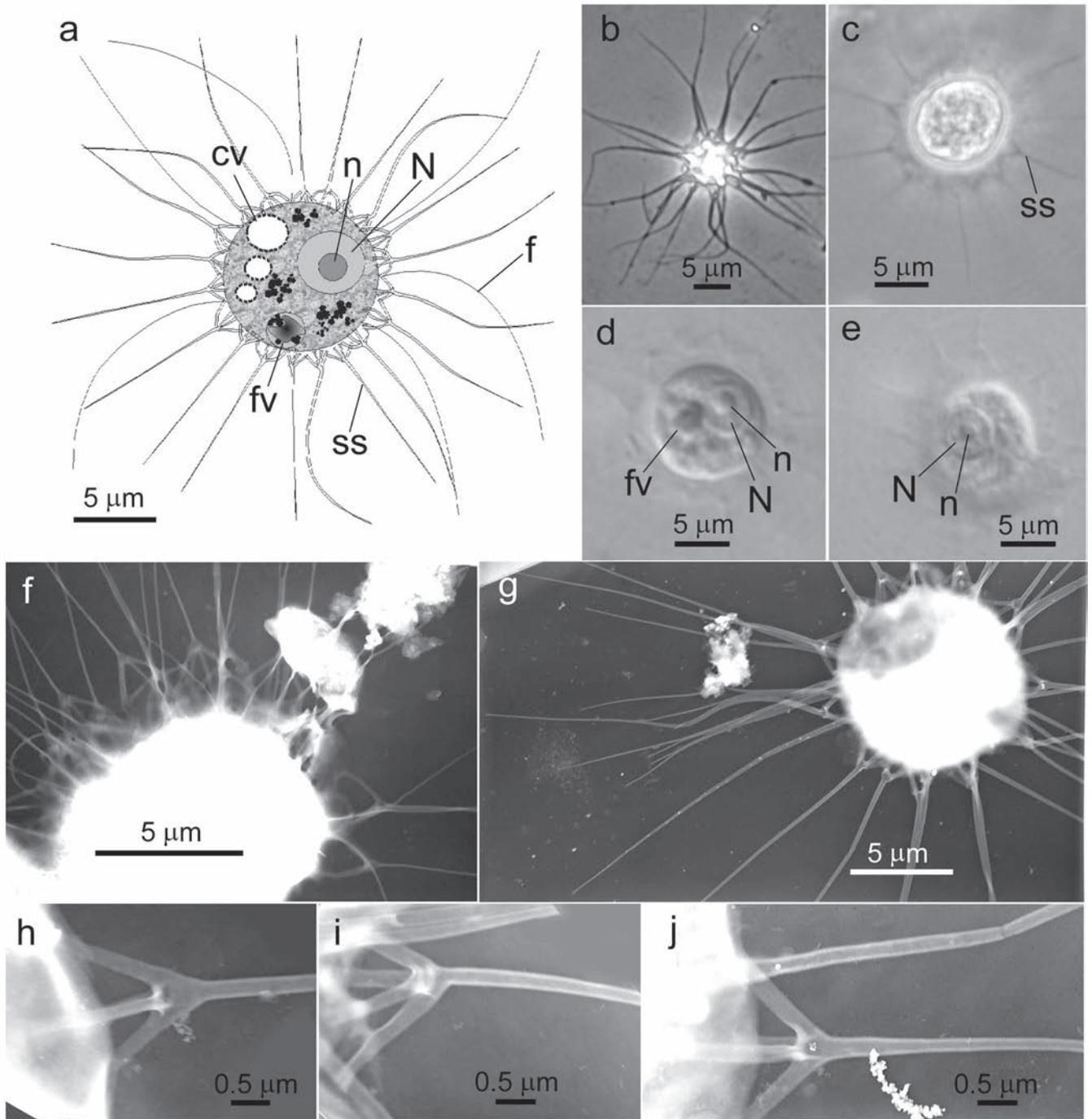
**Type specimen:** Holotype, Fig. 2g. Several paratype specimens mounted in Canada Balsam on a glass slide, is retained at the Canadian Museum of Nature (Invertebrate Zoology Division), Ottawa, Ontario, Canada; Catalogue No CMNI-2012-000x.

**Type locality:** Pond #7, Snowdon Township, Haliburton County, Ontario, Canada 44°49'28"N, 78°32'00"W.

**Material from type locality:** Retained by the author in sample No. V-553, collected by K. Nicholls, 24 October, 1983.



**Fig. 2.** *Belonocystis tubistella* scale structure. **a–c** – whole cells, died, TEM images showing the investiture of radiating spines; **d–f** – diagrammatic depiction of the arrangement (**d**) and structure of spine bases showing meshed structure of an intact scale base (**e**) and the collapsed state of a dried spine base (**f**). The meshed structure is not included in **f**, to visualize better the collapsed shape of the spine; **g–h** – TEM images showing the meshed structure of the spine bases. The arrows in **h** are examples of the distorted (high “shoulder” effect) shape of collapsed spine bases (as illustrated with the arrow in **f**).



**Fig. 3.** *Belonocystis quadrangularis*. **a** – diagrammatic representation of a living cell (labelling as in Fig. 1); **b** – dried specimen light microscopic image showing organic spines as radiating elements around the cell; **c** – fixed cell showing cone-shaped bases of the spines; **d–e** – living cells showing nucleus and food vacuole; **f–g** – portions of cells showing investitures of spines (TEM images); **h–j** – TEM images of the four-legged basal structure of the spines.

### Comparative morphology of *B. tubistella* and *B. quadrangularis*

Most *B. quadrangularis* cells were in the 6–8  $\mu\text{m}$  diameter range, while most *B. tubistella* cells were in the 8–14  $\mu\text{m}$  diameter range (Fig. 4). Median values ( $n = 50$ ) were 7  $\mu\text{m}$  and 10.5  $\mu\text{m}$  for these two species, respectively. Based on the distributions of measured cell diameters, the Mann-Whitney *U*-test indicated that the likelihood of these two samples representing the same population (i.e. the same size distribution) was very low ( $P < 0.0001$ ).

Other differences detected with high resolution LM included the differently shaped spine bases. In *B. quadrangularis*, the sides of the triangular bases were tepee-like with more or less straight sides, while in *B. tubistella*, the bases of the spines often appeared to resemble the common structure of a human house with vertical walls and a peaked roof (Fig. 2d). The more evident difference in structure of the spine bases was revealed by EM; the distinctive perforated membrane structure of the *B. tubistella* spine-scale bases was totally absent in all *B. quadrangularis* spines.

The spiny investiture surrounding cells of both *Belonocystis* species dissolved within 10 minutes of flooding with 6% sodium hypochlorite (NaClO) solution.

## DISCUSSION

Rainer (1968) dealt with heliozoan-like organisms possessing fine pseudopodia in three suborders of the order Heliozoa (Actinophrydia, Centroheliidia and Rotosphaeridia) and in two orders (“Desmothoraca” and “Pseudoheliozoa”) of the super-group “Pseudoheliozoa.” Now, four decades later, the generic membership of these groups and the interrelationships and evolutionary origins of the genera assigned to these groups is complex and largely unresolved. It is clear, however, that the order “Pseudoheliozoa” as used by Rainer (1968) to accommodate an eclectic group of seven genera with diverse morphologies and life histories likely has no phylogenetic basis in modern protistan classification systems (Nikolaev *et al.* 2004, Pawlowski 2008).

Owing to the presence of supposedly organic spines instead of the siliceous scales of the centohelids and rotosphaerids, Rainer (1968) placed *Belonocystis* in the “pseudoheliozoan sister-group” to the clathrulinids (Family Clathrulinidae Claus, 1874 = Desmothoracidae Hertwig & Lesser, 1874). The clathrulinids, however,

are characterized by an organic “capsule” that surrounds the cell and consists of polygonal plates joined at their margins and with a central pore visible in LM through which fine pseudopodia emerge (Mikrjukov 2000). When Rainer (1968) established *Belonocystis*, he was well aware of the spined *Hedriocystis spinifera* Brown, 1918 (now *Penardiophrys spinifera* (Mikrjukov 2000)) and included drawings reproduced from Brown’s original publication. The two genera (*Belonocystis* and *Penardiophrys*) are clearly different, but it is possible that other reports of one of the two species (*P. spinifera* or *B. tubistella*) have misidentified it as the other species, owing to the presence of spines in both species, as may have been the case in Mayer’s (1997) report of *Hedriocystis spinifera*.

It is possible that the spine bases of both of the *Belonocystis* species studied here are conjoined or tightly associated to the extent that the entire investiture of spiny structures covering the cell might be considered a modified “capsule,” in which case the Clathrulinidae might be a convenient place to relegate this genus. This may be disputed, because Rainer (1968) illustrated a single spine, which suggests that single spines can disassociate; but this depiction (his Fig. 84B) may instead have simply represented his interpretation of the structure of a single spine, since he did not write specifically about finding loose, unattached spines. Siemensma (1981) also included a drawing of a single unattached spine (his Plate 60, A). I have observed many specimens of both *Belonocystis* species in living and in dried states, and not once did I observe single disassoci-

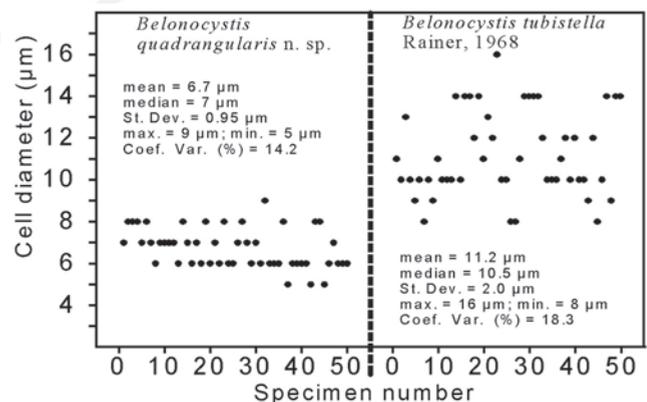


Fig. 4. Measurements of cell diameter (excluding spine-scale investitures) in 50 specimens of *Belonocystis tubistella* and *B. quadrangularis*.

ated spines (as often happens when cells of centrohelids and rotosphaerids bearing siliceous scales are dried).

At the concentration used (6%), sodium hypochlorite solution is effective in oxidizing organic matter “contaminating” diatom frustules (Carr *et al.* 1986) and for dissolving the organic “glue” that binds siliceous idiosomes to the tests of testate amoebae (Nicholls 2006). At this concentration, NaClO does not dissolve the plates forming the thecae of dinoflagellates, but does disassociate them by dissolving the organic glue that binds them together (Nicholls 1998). Dinoflagellate plates are not comprised of silica, but of organic long-chain polyunsaturated glucans or other complex cellulosic compounds (Lau *et al.* 2007) that are not readily susceptible to dissolution by NaClO. Therefore, had the NaClO not dissolved the *Belonocystis* spines, it would not necessarily have meant that the spines were of non-organic constituents. In that case, confirmation of their non-siliceous nature would have required a specific test for Si, like the EDX-ray analysis used to detect the presence of Si in the scales of other protists (Manton and Sutherland 1979, Nicholls 1979). This is important because had either of the two known *Belonocystis* species been shown to have siliceous spines, it would need to be transferred most likely to the Rotosphaeridia as a sister genus to other rotosphaerid genera which differ from *Belonocystis* by having either only plate-scales or both plate-scales and spine-scales of a siliceous nature.

It is worth pointing out the similarity of the structure of the spine base of *Belonocystis tubistella* to that of *Luffisphaera* “form B” of Belcher and Swale (1975). Spines of both of these taxa have swollen bases consisting of a superstructure of membranous mesh supported on arching ribs. Other differences, however, clearly reveal the different origins of these two taxa: the flared distal portion of the spine shaft in *Luffisphaera* “form B” and its elaborately constructed spine base-plate are not features found in *B. tubistella*, and *Luffisphaera* species (for which neither flagella nor pseudopodia are known) are all very small (2.5–3.5  $\mu\text{m}$  in diameter). This phenomenon of co-existing homologous structures in widely disparate phylogenetic lines is relatively common in nature. Other examples include: the resemblance of the spine-scale base of *Belonocystis quadrangularis* with that of the marine haptophyte *Chrysochromulina pelagica* (Estep *et al.* 1984), and the generally similar scale structures of the zooflagellates *Cyathobodo crucifer* (Swale and Belcher 1975) and *Pseudodendromonas insignis* (Hibberd 1985) with that

of the haptophyte *Chrysochromulina elegans* (Estep *et al.* 1984).

Neither Corliss (1994) nor Adl *et al.* (2005), the two most recent comprehensive classification systems for eukaryotic protists, dealt specifically with the genus *Belonocystis*. Patterson *et al.* (2002) assigned *Belonocystis* to a large group of problematic genera under the heading “Amoebae of uncertain affinities.” Further TEM analysis of sectioned material in order to confirm the apparent lack of axial filaments in the filopodia, the lack of an associated centroplast and information on the structure of mitochondrial cristae is needed to suggest possible phylogenetic relationships among *Belonocystis*, *Clathrella*, *Clathrulina*, *Hedriocystis* and *Penardiophrys*.

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