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Re-classification of *Pheopolykrikos hartmannii* as *Polykrikos* (Dinophyceae) based partly on the ultrastructure of complex extrusomes

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Abstract

Athecate, pseudocolony-forming dinoflagellates have been classified within two genera of polykrikoids, *Polykrikos* and *Pheopolykrikos*, and different views about the boundaries and composition of these genera have been expressed in the literature. The photosynthetic polykrikoid *Pheopolykrikos hartmannii*, for instance, was originally described within *Polykrikos* and is now known to branch closely with several *Polykrikos* species in molecular phylogenetic analyses of ribosomal gene sequences. In this study, we report the first ultrastructural data for this species and demonstrate that *Ph. hartmannii* has all of the features that characterize the genus *Polykrikos*, including the synapomorphic “taeniocyst-nematocyst complex”. We also demonstrate that the ultrastructure of the chloroplasts in *Ph. hartmannii* conforms to the usual peridinin-containing chloroplasts of most photosynthetic dinoflagellates, which improves inferences about the origin(s) and evolution of photosynthesis within the genus. After taking into account all of the ultrastructural data on polykrikoids presented here and in the literature, this species is re-classified to its original status as *Polykrikos hartmannii*.

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Keywords: Chloroplast; Dinoflagellate; Peridinin; Polykrikoids; Taeniocyst-nematocyst complex; Ultrastructure

Introduction

Athecate, pseudocolony-forming dinoflagellates fall within two genera of polykrikoids – *Polykrikos* Bütschli and *Pheopolykrikos* Chatton – and there are different views about the generic classification of some species; for a detailed summary and discussion see Hoppenrath

and Leander (2007a). Some authors have recognized only the genus *Polykrikos* and treat *Pheopolykrikos* as synonymous (Dodge 1982; Sournia 1986), while other authors have separated the two genera into different families (Fensome et al. 1993). Molecular phylogenetic analyses of ribosomal gene sequences have demonstrated that the type species of *Pheopolykrikos*, namely *Ph. beachampii* Chatton, branches as a lineage that is only distantly related to a well-supported *Polykrikos* clade within the *Gymnodinium* sensu stricto clade (Hoppenrath and Leander 2007a, b; Hoppenrath et al. 2009). These studies have also shown that *Pheopolykrikos hartmannii* (Zimmermann) Matsuoka et Fukuyo

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branches as the nearest sister lineage to the *Polykrikos* clade (Hoppenrath and Leander 2007a, b; Hoppenrath et al. 2009).

The genus *Pheopolykrikos* was first described by Chatton (1933) and subsequently emended by Matsuoka and Fukuyo (1986). *Pheopolykrikos* is different from *Polykrikos* in having the same number of nuclei as zooids and being able to disassociate into single cells/zooids (Chatton 1933, 1952). The type species, *Ph. beauchampii*, is photosynthetic and appears to lack the ability to phagocytize prey cells (Chatton 1933). When emending the genus, Matsuoka and Fukuyo (1986) transferred *Polykrikos hartmannii* Zimmermann into *Pheopolykrikos* because (1) the number of nuclei and zooids is the same, (2) there is a single-celled lifecycle stage, and (3) the cells are photosynthetic.

Polykrikos hartmannii (as *P. Hartmanni*) was originally described as a two-zooid pseudocolony containing two nuclei, chloroplasts, and nematocysts (Zimmermann 1930; Figs 1A, B). Earlier, Martin (1929) described *Polykrikos barnegatensis* as a two-zooid pseudocolony with only one central nucleus and chloroplasts but without nematocysts (Fig. 1C). Chatton (1952) subsequently synonymized *Polykrikos hartmannii* with *P. barnegatensis*; however, he provided a drawing of the species that showed two nuclei (Fig. 1D). Interestingly, this drawing also shows the presence of an acrobase, which was not described for the species at that time and was not mentioned in the text. Hulburt (1957; Fig. 1E) did not follow Chatton's interpretation when describing his observations of *P. hartmannii*; Hulburt reported the presence of nematocysts in some cells and emphasized that the two species differ in the number of nuclei contained within the pseudocolony. Because the description of *P. barnegatensis* was based on the observation of only one cell, Hoppenrath and Leander (2007a) regarded the identity of this species as uncertain. Matsuoka and

Fukuyo (1986) transferred *P. hartmannii* into the genus *Pheopolykrikos*, as mentioned above, in part because these authors reported the absence of nematocysts. Based on the results of molecular phylogenetic analyses, Hoppenrath and Leander (2007a) suggested that *P. hartmannii* should be reclassified as a *Polykrikos* species, but only after ultrastructural data from this species become available to test this conclusion. The ultrastructural investigation reported here was conducted not only to resolve this particular systematic problem but also to better understand the evolutionary history of polykrikoids in general.

The taeniocyst-nematocyst complex is perhaps the best synapomorphy for the *Polykrikos* clade, and species that possess these complex extrusomes are expected to be close relatives; by contrast, the evolutionary pattern of photosynthesis within the *Polykrikos* clade has been more difficult to reconstruct (Hoppenrath and Leander 2007a, b; Hoppenrath et al. 2009). Current data indicate that the most recent ancestor of the *Gymnodinium* sensu stricto clade [type species: *Gymnodinium fuscum* (Ehrenberg) Stein] possessed the usual peridinin-containing chloroplasts found in most photosynthetic dinoflagellates and that photosynthesis was lost in heterotrophic *Polykrikos* species (Hoppenrath and Leander 2007a). The marine benthic *Polykrikos* species, *P. lebourae* Herdman, is phylogenetically nested within heterotrophic *Polykrikos* species, but possesses chloroplasts of yet unidentified origin that were probably acquired via a separate and more recent endosymbiotic event (Hoppenrath and Leander 2007b). This hypothesis suggests that the ancestral peridinin-containing chloroplasts were reduced, or lost, early in the evolution of the *Polykrikos* clade and subsequently replaced with a different kind of chloroplast in *P. lebourae* (Hoppenrath and Leander 2007a, b). Complicated evolutionary scenarios involving the gain and loss of photosynthesis/chloroplasts, like the one described above, appear to

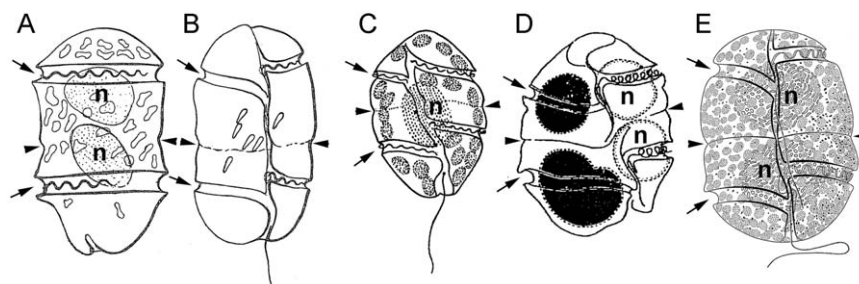


Fig. 1. Reproduced line drawings. (A, B) *Polykrikos hartmannii* from Zimmermann 1930. (A) Dorsal view showing the two nuclei (n) and chloroplasts. (B) Ventral view showing nematocysts. (C) *Polykrikos barnegatensis* from Martin 1929. Ventral view, chloroplasts and one central nucleus (n) are visible. (D) *Polykrikos barnegatensis* (= *P. hartmannii*) from Chatton 1952. Ventral view showing the two nuclei (n) and the acrobase. (E) *Polykrikos hartmannii* from Hulburt 1957. Ventral view showing the two nuclei (n) and chloroplasts. Note in all drawings the two transverse furrows (arrows) and the visible border between the two zooids (arrowheads) of the pseudocolony.

have happened several times independently within dinoflagellates (e.g., Saldarriaga et al. 2001, 2004).

Because *Ph. hartmannii* is photosynthetic and branches as the nearest sister lineage to the *Polykrikos* clade, we were interested in determining the ultrastructural features of this species, especially details associated with the chloroplasts and complex extrusomes. Our aims in this paper were to (1) demonstrate whether or not *Ph. hartmannii* possesses all of the characteristics associated with the *Polykrikos* clade, such as the synapomorphic taeniocyst-nematocyst complex, and (2) improve our understanding of the early evolutionary history of photosynthesis within the clade.

Material and Methods

Collection, isolation, and culturing of the species

A sample containing *Pheopolykrikos hartmannii* was collected from the Rhode River, MD at the Smithsonian Environmental Research Center (SERC) dock (N38°53.1' W76°32.5') on July 31, 2007. A horizontal plankton tow was taken from the surface layer using a 35 µm-mesh net. The sample was held at ambient temperature and transported to the lab. It was screened using a 250 µm-mesh Nitex sieve to remove large zooplankton, and diluted with seawater to enhance viability. Cells were visualized through a dissecting microscope and individually picked using a mouth-pipette. After three washing steps, specimens were placed in 15 psu f/2-medium (Guillard and Ryther 1962) charged with 5% soil extract and grown at medium light conditions (cool-white fluorescent lamps, ~100 mmol photons * m⁻² * s⁻¹) at 17 °C (at UBC) or 20 °C (in MD).

Light microscopy

Cells were observed and micromanipulated with a Leica DMIL inverted microscope. For DIC light microscopy, micropipetted cells were placed on a glass specimen slide and covered with a cover slip. Images were produced with a Zeiss Axioplan 2 imaging microscope connected to a Leica DC500 color digital camera.

Transmission electron microscopy

Cells of *Pheopolykrikos hartmannii* were mixed with fixative containing 5% glutaraldehyde and 0.2 M sucrose in 0.2 M sodium cocodylate buffer (pH 7.2) and pre-fixed at room temperature for one h. Cells were aggregated into a pellet by centrifugation at 1000 g for 5 min and rinsed three times with the 0.2 M buffer. Cells

were then post-fixed with 1% (w/v) osmium tetroxide in 0.2 M the buffer at room temperature for 30 min and subsequently dehydrated through a gradual series of ethanol concentrations (1 h at 30%, 30 min at 50%, 15 min each at 70%, 85%, 90%, 95%, and 100%). The ethanol was substituted with acetone (the transition fluid) using 15 min washes of 1:1 acetone:ethanol and 100% acetone. The dehydrated cells were then infiltrated with acetone-Epon 812 resin mixtures (2:1 for 1 h, 1:1 for 1 h, 1:2 for 1 h) and 100% resin overnight. Ultra-thin serial sections were collected on copper, formvar-coated slot grids and stained with 2% (w/v) uranyl acetate and lead citrate (Reynolds 1963) before being observed using a Hitachi H7600 electron microscope.

Results

In culture, we observed mainly two-zoid pseudocolonies (Figs 2A-G) but also one-zoid stages (Fig. 2H) and spiny round cysts (Figs 2I-K). Two-zoid pseudocolonies always had two nuclei, two descending transverse furrows (syn.: cinguli) with a transverse flagellum, two longitudinal furrows (syn.: sulci) with a longitudinal flagellum, a visible border between the two zooids, and many small spindle-shaped to oval golden-brown chloroplasts (Figs 2A-G). The acrobase (syn.: apical groove) was loop-shaped (Fig. 2B). Single zooids had an extremely large nucleus (Fig. 2H). Taeniocyst-nematocyst complexes were often difficult to observe in the light microscope because of the obscuring effect of the chloroplasts. Only a few taeniocyst-nematocyst complexes were ever observed in the pseudocolonies, and in most cases, only one taeniocyst-nematocyst complex was contained within one zoid (Figs 2C, D, E, F); however, three complexes were observed in the posterior zoid of one pseudocolony (Fig. 2H). This is the first light microscopical documentation of taeniocyst-nematocyst complexes in *Polykrikos hartmannii*. The cell shown in Hoppenrath et al. (2009) was taken from the same sample/isolate.

The pseudocolonies were highly vacuolated in all our transmission electron micrographs. The nuclei were of the typical dinokaryotic type with large permanently condensed chromosomes (Fig. 3A). Nuclear chambers with close set nuclear pores were not detected (Fig. 3B). The mitochondria had tubular cristae (Fig. 5F). The ultrastructure of the chloroplasts conformed to the peridinin-containing chloroplast with pyrenoids found in most photosynthetic dinoflagellates (Fig. 3C); the chloroplast had three outer membranes (Fig. 3D) and thylakoids in stacks of three (Fig. 3E). The pyrenoid was not traversed by thylakoids and had no starch sheath (Fig. 3C). Three types of extrusomes were present. Trichocysts were enveloped by a single membrane and

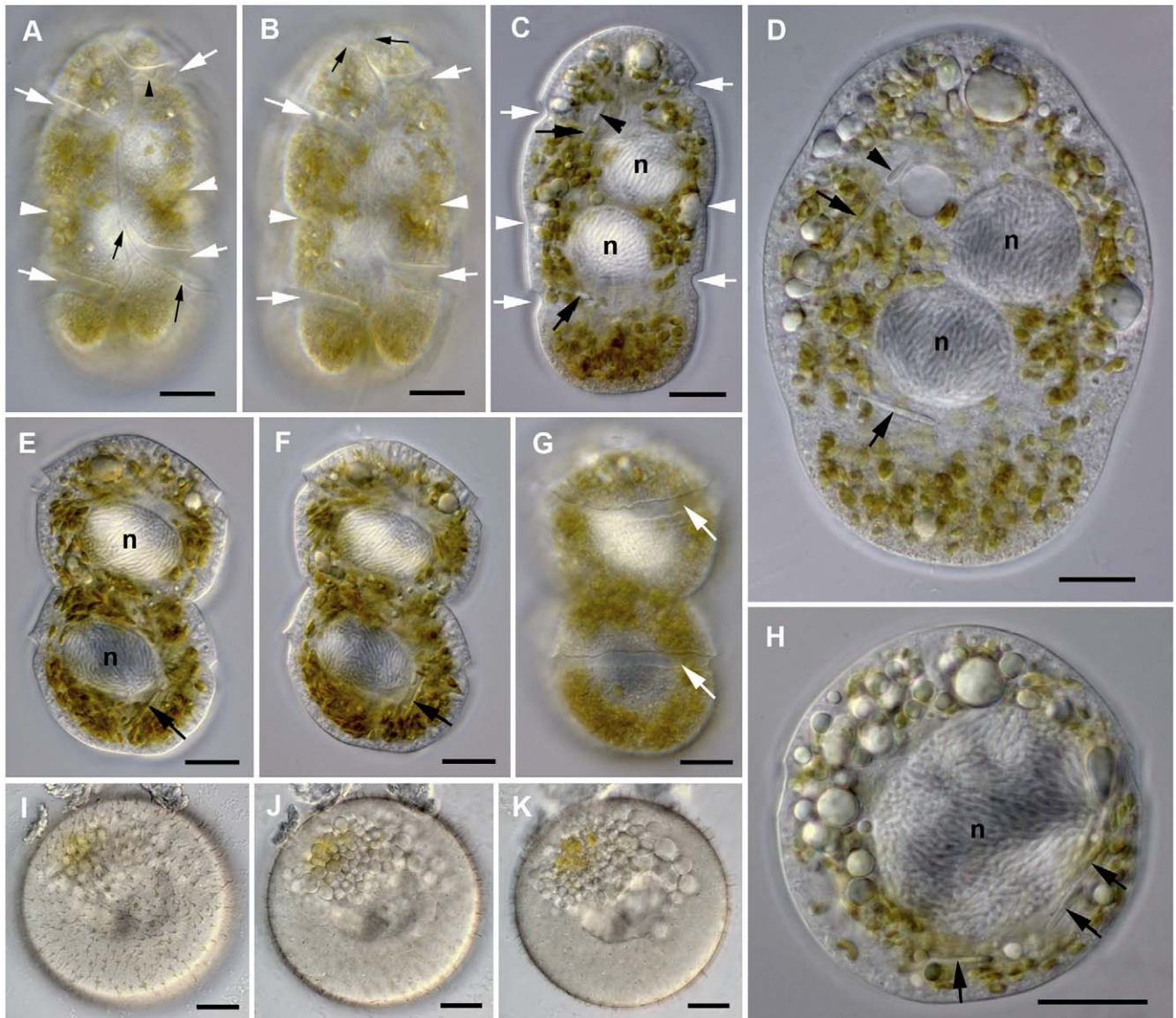


Fig. 2. Light micrographs of *Polykrikos hartmannii*. (A-C) Same pseudocolony in different focal planes. Note the two transverse furrows (white arrows) and the visible border between the two zooids (white arrowheads) of the pseudocolony. (A) Ventral view. The upper transverse flagellum (black arrowhead) and the lower longitudinal flagellum (black arrows) are visible. (B) Ventral view. The acrobase (black arrows) is visible. (C) Mid cell focus. Note the two nuclei (n) and the many small brown chloroplasts. Two nematocysts (black arrows) and a taeniocest (black arrowhead) are visible. (D) Same cell as shown in A-C after contusion under the cover slip making it easier to recognize the nematocyst-taeniocest complex in the upper part of the pseudocolony. (E-G) Same pseudocolony in different focal planes. (E) Mid cell focus. Note the two nuclei (n), the brown chloroplasts, and a nematocyst in the lower zooid of the pseudocolony (black arrow). (F) Slightly different mid cell focus showing a second nematocyst in the lower zooid (black arrow). (G) Dorsal view showing the two transverse furrows of the pseudocolony (white arrows). (H) Single cell/zooid stage with very large nucleus (n) and three nematocysts (black arrows). (I-K) Spiny resting cyst in different focal planes. Scale bars = 10 μm .

were composed of a column-like body with a neck ending in an apical, cap' (Fig. 4A). In transverse section, the trichocyst body was quadrangular (Fig. 4A inset). Long, mature nematocysts were enveloped by a membrane (arrowheads) and were composed of an anterior operculum (o) and a posterior capsule (c),

also named posterior body (Figs 4B, D). The operculum consisted of an unidentified complex of central structures (Fig. 4C). The capsule contained a stylet-like structure (asterisk) in an inner anterior chamber (a) and a single coiled filament in an external posterior chamber (p) (Figs 4B-F). The filament was

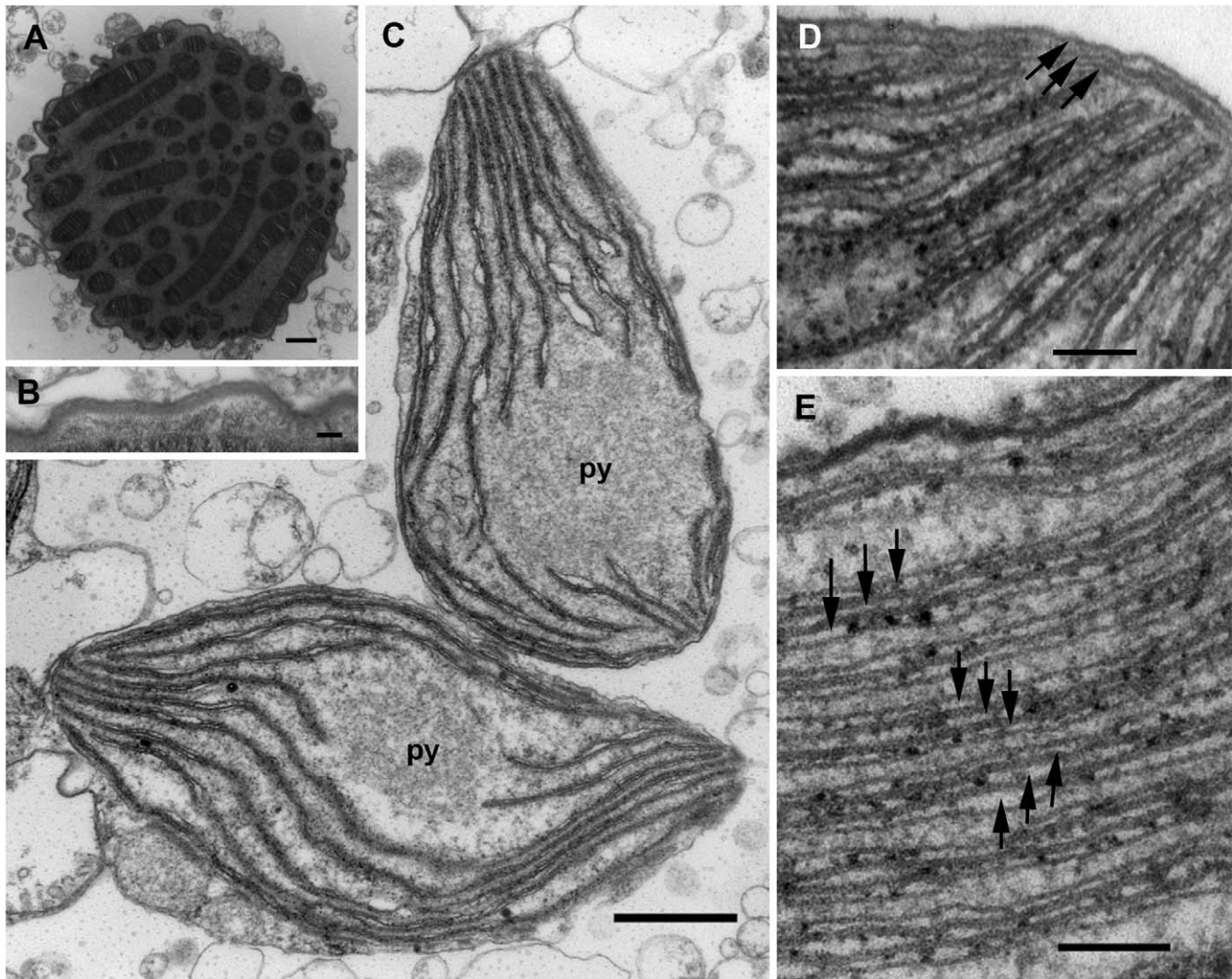


Fig. 3. Transmission electron micrographs of *Polykrikos hartmannii*. (A) Nucleus with condensed chromosomes. Scale bar = 2 μm . (B) Detail of the nuclear membrane. Scale bar = 100 nm. (C) Two spindle-shaped typical dino-chloroplasts with thylakoids in stacks and pyrenoid (py) not traversed by thylakoids. Scale bar = 500 nm. (D) Detail of a chloroplast showing the three outer membranes (arrows) typical for peridinin-containing dino-chloroplasts. Scale bar = 100 nm. (E) Detail of a chloroplast showing thylakoids in stacks of three (arrows) typical for peridinin-containing dino-chloroplasts. Scale bar = 100 nm.

connected to the anterior chamber (Fig. 4C). The association of the nematocyst with a taeniocyst (T) within the chute (ch) is visible in Figs 4B, D. Mature taeniocysts were enveloped by a membrane and consisted of a densely stained posterior body (bo) with conical neck (n) (Figs 5A-G). In transverse section, the neck (syn.: collar region) consisted of concentric lamellae within the taeniocyst body (Figs 5A, F, G). These concentric structures have also been named ‘medulla’. A round posterior amorphous zone (syn.: ‘posterior crown’) could not be detected. Only fragments of the taeniocyst head were visible (Figs 5B-D). Mucocysts were not detected. These are the first transmission electron microscopic observations for *P. hartmannii*.

Discussion

A strongly supported *Polykrikos* clade – with *P. hartmannii* diverging as the sister lineage to a clade consisting of the other species – was previously demonstrated with phylogenetic analyses of SSU rDNA sequences; the *Polykrikos* clade branched robustly within the *Gymnodinium* sensu stricto clade (Hoppenrath and Leander 2007a, b; Hoppenrath et al. 2009). Moreover, although the complete *Polykrikos* clade was not demonstrated (or denied) with analyses of LSU rDNA sequences (Hoppenrath et al. 2009; Kim et al. 2008), these data suggested that *P. kofoidii* and *P. schwartzii* are closely related to one another (Hoppenrath et al. 2009; Kim et al. 2008); this

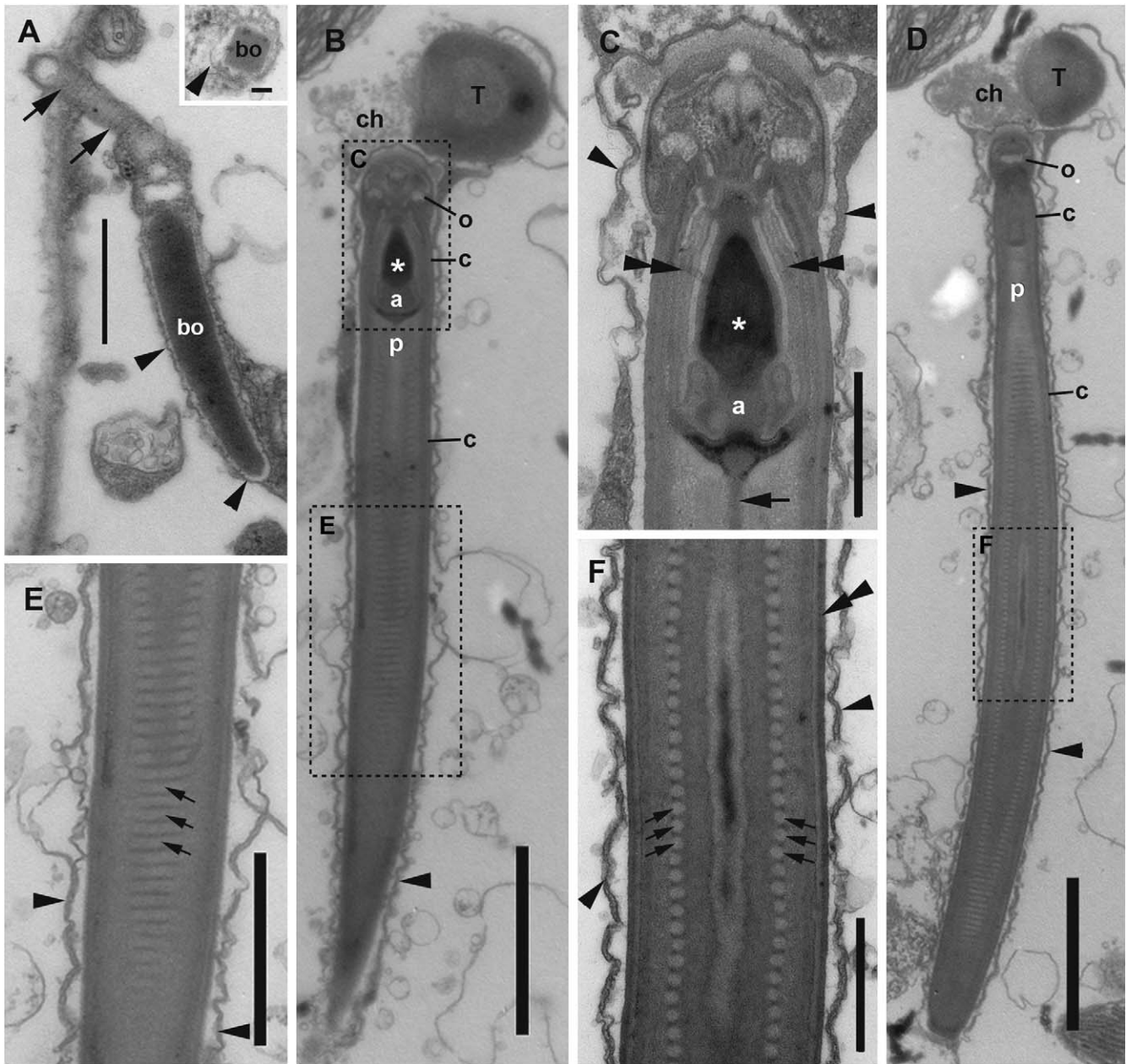


Fig. 4. Transmission electron micrographs of *Polykrikos hartmannii*. Trichocysts and nematocysts. (A) Longitudinal and transverse section of a trichocyst. They were enveloped by a single membrane (arrowhead) and composed of a column-like body (bo) with a neck (arrows). Scale bar = 500 nm. In transverse section (inset, scale bar = 100 nm) the trichocyst body was quadrangular. (B-F) Nematocysts. (B) Part of a nematocyst enveloped by a membrane (arrowhead) in longitudinal section. It was composed of an operculum (o) and a capsule (c) that consisted of an anterior chamber (a) with a stylet-like structure (asterisk) and a posterior chamber (p). The nematocyst in association with a taeniocyst (T) within the chute (ch). See C and E for the dotted boxes. Scale bar = 2 μm. (C) Detail in the dotted box of B showing the operculum and anterior chamber (a) with 'wall' (double arrowheads), stylet-like structure (asterisk), and connected filament (arrow). Scale bar = 500 nm. (D) Nematocyst enveloped by a membrane (arrowhead) consisting of operculum (o) and capsule (c) in longitudinal section. Capsule composed of an anterior (a) and posterior (p) chamber. Nematocyst in association with a taeniocyst (T) within the chute (ch). See F for the dotted box. Scale bar = 2 μm. (E) Detail in the dotted box of B showing the coiled filament in tangential section – visible as parallel lines (arrows) – in the posterior chamber. Note the enveloping membrane (arrowheads). Scale bar = 2 μm. (F) Detail of the posterior chamber in the dotted box of D showing the densely coiled filament in cross section – visible as dotted lines (arrows). The enveloping membrane (arrowheads) and the capsule 'wall' (double arrowhead) are visible. Scale bar = 500 nm.

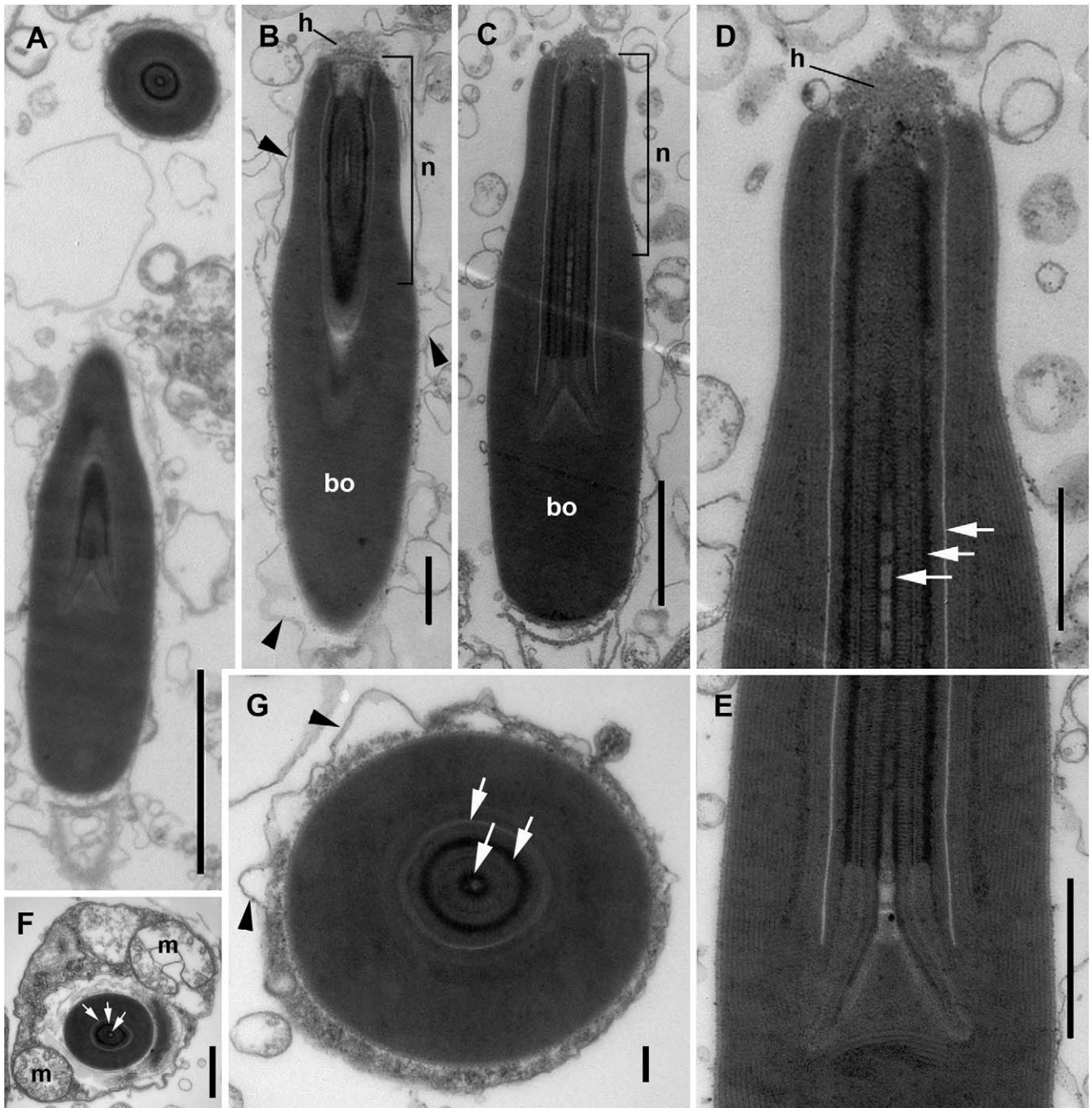


Fig. 5. Transmission electron micrographs of *Polykrikos hartmannii*. Taeniocysts. (A) Taeniocyst in transverse (upper right) and oblique longitudinal section. Scale bar = 2 μ m. (B) Oblique longitudinal section through the neck (n) and body (bo) of a taeniocyst. Note the enveloping membrane (arrowheads) and fragments of the head (h). Scale bar = 500 nm. (C) Longitudinal section through the neck (n) and body (bo) of a taeniocyst. Scale bar = 2 μ m. (D) Detail of the neck of the taeniocyst shown in C, with parts of the head (h). Note the concentric lamellae (arrows). Scale bar = 500 nm. (E) Detail of the upper part of the body of the taeniocyst shown in C (different section). Scale bar = 500 nm. (F) Transverse section through the neck region of a taeniocyst showing the concentric lamellae (arrows). Note the two mitochondria with tubular cristae (m). Scale bar = 500 nm. (G) Transverse section through the neck region of a taeniocyst showing the concentric lamellae (arrows) and the enveloping membrane (arrowheads). Scale bar = 100 nm.

relationship was hypothesized previously based on comparative morphological data (Hoppenrath and Leander 2007a).

All reliably described *Polykrikos* species, namely *P. schwartzii* Bütschli, *P. kofoidii* Chatton, *P. lebourae* Herdman, and *P. herdmanae* Hoppenrath et Leander,

are characterized by pseudocolonies having (1) a closed loop-shaped acrobase, (2) descending cinguli, (3) a sulcus being connected with the acrobase and reaching the posterior end of the pseudocolony, (4) half or a quarter the number of nuclei as zooids, (5) the ability to disassemble into pseudocolonies with fewer zooids containing only one nucleus, and (6) taeniocyst-nematocyst complexes (Hoppenrath and Leander 2007a, b; Nagai et al. 2002; Takayama 1985). One major evolutionary innovation of these polykrikoid dinoflagellates is the pseudocolonial cell organization derived from a uni-nucleated ancestor, like the closely related *Gymnodinium fuscum* (Hoppenrath and Leander 2007a). Pseudocolony formation is almost certainly the result of incomplete cell division following nuclear duplication. (*Pheo*)*Polykrikos hartmannii* fits within this circumscription except that it has the same number of nuclei as zooids and that the pseudocolonies are capable of disassembling into two single zooids with one nucleus (Chatton 1952; Hulburt 1957; Matsuoka and Fukuyo 1986; Zimmermann 1930; present study). This is inferred to represent an ancestral state for the *Polykrikos* lineage.

The (early) sister relationship between '*Ph.*' *hartmannii* and the remaining members of the *Polykrikos* clade is further supported by morphological evidence. For instance, the two sulci of the zooids in '*Ph.*' *hartmannii* are not fused like that in the other *Polykrikos* species (Hoppenrath and Leander 2007a, b; Takayama 1985). Moreover, unlike other *Polykrikos* species, '*Ph.*' *hartmannii* contains chloroplasts with ultrastructural features that conform to the typical dinoflagellate peridinin-type; a pigment analysis of the culture also demonstrated peridinin as a major carotenoid (unpublished data, pers. comm. Horn Point laboratory). The hypothesis that photosynthesis was lost early in the evolution of the *Polykrikos* clade and later replaced in *P. lebourae* is, therefore, consistent with both comparative morphological data and molecular phylogenetic data (Hoppenrath and Leander 2007a, b).

Hoppenrath and Leander (2007a) found that a prominent synapomorphy of the *Polykrikos* clade, including '*Ph.*' *hartmannii*, is the presence of two nuclei regardless of zooid number. *Polykrikos schwartzii* is an exception to this pattern because this lineage contains four nuclei (and eight zooids) – a character state that is interpreted to be derived from within the *Polykrikos* clade. Another robust synapomorphy for the *Polykrikos* clade is the presence of taeniocyst-nematocyst complexes, a conspicuous multiparted ultrastructural system that has been demonstrated for all *Polykrikos* species described so far (Greuet 1987; Hoppenrath and Leander 2007a, b; Westfall et al. 1983; present study). No other dinoflagellates are known to possess this association of complex extrusomes. *Polykrikos hartmannii* was originally described to possess nematocysts but not taeniocysts

(Zimmermann 1930); Hulburt (1957) reported the presence of nematocysts in some specimens, and Matsuoka and Fukuyo (1986) stated that nematocysts were absent in their specimens. These complex extrusomes were sometimes difficult to observe in our samples of *P. hartmannii* with light microscopy because of the obscuring effect of the chloroplasts, and these difficulties help explain the contradictory observations reported in the past. Nonetheless, this is the first time that taeniocysts have been demonstrated in *P. hartmannii* using either light or transmission electron microscopy.

Nuclear pores opening into nuclear chambers is a distinctive feature found in the genus *Polykrikos* (*P. kofoidii* and *P. lebourae*) and some species of the *Gymnodinium sensu stricto* clade (Bradbury et al. 1983; Ellegaard and Moestrup 1999; Hansen 2001; Hansen and Moestrup 2005; Hansen et al. 2000; Hoppenrath and Leander 2007b). It would be interesting to know whether the most basal species of the *Polykrikos* lineage, *P. hartmannii*, also has nuclear chambers, but we were unable to find any evidence of nuclear pores or chambers.

Matsuoka and Fukuyo (1986) emphasized morphological differences in the resting cysts present in *P. schwartzii* and *P. kofoidii*, on the one hand, and *P. hartmannii*, on the other. This was one of the main justifications for classifying polykrikoid species into different genera. As pointed out before (Hoppenrath and Leander 2007a), resting cyst stages have not yet been described for the type species of *Pheopolykrikos*, namely *Ph. beauchampii*. In addition, resting cysts are not known for either *P. lebourae* or *P. herdmannae*. In our opinion, cyst morphology (like chloroplasts) is probably only a useful taxonomic character at the species level within the genus *Polykrikos*.

In conclusion, '*Ph.*' *hartmannii* has all of the features for the genus *Polykrikos*, including the synapomorphic taeniocyst-nematocyst complexes, and therefore should be re-classified into the genus *Polykrikos* as originally described. The closed loop-shaped acrobase connected to the sulcus is also typical for all known *Polykrikos* species and separates the genus from other *Gymnodinium sensu stricto* taxa that have an open (counterclockwise) loop-shaped acrobase (e.g., Daugbjerg et al. 2000).

Polykrikos hartmannii Zimmermann 1930, Zeitschrift für Botanik 23, p. 438, Figs 8, 9.

Nomenclatural synonym: *Pheopolykrikos hartmannii* (Zimmermann) Matsuoka et Fukuyo 1986, J. Plankton Res. 8, p. 817.

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