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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**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. beauchampii* 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...
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 dissociate 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. Hartmannii*) 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 35μm-mesh net. The sample was held at ambient temperature and transported to the lab. It was screened using a 250μm-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-zooid pseudocolonies (Figs 2A-G) but also one-zooid stages (Fig. 2H) and spiny round cysts (Figs 2I-K). Two-zooid 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 acrobe (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 zooid (Figs 2C, D, E, F); however, three complexes were observed in the posterior zooid of one pseudocolony (Fig. 2H). This is the first light microscopical documentation of taeniocyst-nematocyst complexes in *Pheopolykrikos 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...
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 (arrows) in an external posterior chamber (p) (Figs 4B-F). The filament was
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.
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. *(Pheopolykrikos 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 example, 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 schwartzi* 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. schwartzi* 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. herdmanei*. 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 acrobe 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 acrobe (e.g., Daugbjerg et al. 2000).

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


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