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CHARACTER EVOLUTION IN POLYKRIKOID DINOFLAGELLATES[1]

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A synthesis of available data on the morphological diversity of polykrikoid dinoflagellates allowed us to formulate a hypothesis of relationships that help explain character evolution within the group. Phylogenetic analyses of new SSU rDNA sequences from *Pheopolykrikos beauchampii* Chatton, *Polykrikos kofoidii* Chatton, and *Polykrikos lebourae* Herdman helped refine this hypothetical framework. Our results demonstrated that "pseudocolonies" in dinoflagellates evolved convergently at least three times independently from different *Gymnodinium*-like ancestors: once in haplozoans; once in *Ph. beauchampii*; and at least once within a lineage containing *Ph. hartmannii*, *P. kofoidii*, and *P. lebourae*. The Gymnodiniales *sensu stricto* was strongly supported by the data, and the type species for the genus, namely *Gymnodinium fuscum* (Ehrenb.) F. Stein, formed the nearest sister lineage to a well-supported *Polykrikos* clade. The best synapomorphy for the *Polykrikos* clade was the presence of two nuclei irrespective of zooid number. Two unidentified *Gymnodinium* species formed the nearest sister clade to *Ph. beauchampii*, which has four nuclei and four zooids per pseudocolony. The chain-forming dinoflagellate *G. catenatum* L. W. Graham branched closely to the clade containing all members of *Polykrikos* and *Pheopolykrikos*, suggesting that an ancestral capacity toward chain formation existed before the evolution of pseudocolonies in this group. Our results also clarified the phylogenetic significance of nematocysts, ocelloids, and photosynthesis in reconstructing the evolution of polykrikoids and warnowiids. The molecular phylogenies exposed taxonomic problems associated with *Polykrikos*, *Pheopolykrikos*, and *Gymnodinium*, and suggested that a revision for some of these genera is warranted.

Key index words: **character evolution; dinoflagellate; Dinophyceae; *Gymnodinium*; *Pheopolykrikos*; phylogeny; *Polykrikos*; small subunit ribosomal RNA**

Abbreviations: **AU, approximately unbiased; GTR model, general-time-reversible model; HKY model, Hasegawa–Kishino–Yano model; MCMC, Monte-Carlo–Markov chains; ML, maximum likelihood; *Ti*:*Tv*, transition/transversion ratio; WNJ, weighted neighbor joining**

The dinoflagellate genus *Polykrikos* was erected by Bütschli (1873), with the type species *P. schwartzii* Bütschli. The most distinctive feature of this athecate genus is the formation of multinucleated pseudocolonies comprised of an even number of zooids that are otherwise similar in morphology to individual dinoflagellates in external view. However, despite every zooid having its own cingulum and pair of flagella, the zooid sulci are fused together. A pseudocolony often has half the number of nuclei because it has zooids. Trichocysts, nematocysts, taeniocysts, mucocysts, and plastids have all been reported from different members within the group. The genus currently comprises four species: *P. schwartzii*, *P. kofoidii*, *P. lebourae*, and *P. grassei* Lecal. The first three species are relatively well described and distinguishable from one another (Chatton 1914, Kofoid and Swezy 1921, Herdman 1923, Kofoid 1931, Balech 1956, Dragesco 1965, Hoppenrath 2000, Matsuoka et al. 2000, Nagai et al. 2002); however, the taxonomic separation between *P. schwartzii* and *P. kofoidii* is difficult because characters like size, zooid number, and cingular displacement are overlapping in these two species (Kofoid and Swezy 1921, Nagai et al. 2002, Throndsen et al. 2003). The most reliable distinguishing character between them is the presence of striated ribs on the posterior-most zooid or "hyposome" of the pseudocolony in *P. kofoidii* (Nagai et al. 2002). To the best of our knowledge, *P. grassei* has only been observed and recorded once (Lecal 1972), and doubts about the identity of *P. grassei* have been expressed in the literature (Greuet and Hovasse 1977, Sournia 1986). The reexamination of this species is critical from a phylogenetic point of view because it is described to possess an ocelloid (Lecal 1972). These complex organelles otherwise exist only in warnowiid dinoflagellates (Greuet 1987).

The second genus of polykrikoid dinoflagellates is *Pheopolykrikos*, which was first described by Chatton (1933), with the type species *Ph. beauchampii*, 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 (Chatton 1933, 1952). *Pheopolykrikos beauchampii* is photosynthetic and appears to lack the ability to phagocytize (Chatton

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1933). When emending the genus *Pheopolykrikos*, Matsuoka and Fukuyo (1986) transferred *P. hartmannii* M. H. Zimm. (see also Hulburt 1957) into *Pheopolykrikos* for several reasons: the number of nuclei and zooids is the same, there is a single-cell life-cycle stage, and the cells are photosynthetic. Furthermore, these authors emphasized the different cyst morphologies present in the two genera and the possibility of different excystment conditions. It is noteworthy that no cyst stage is known for the type species of *Pheopolykrikos*, namely *Ph. beauchampii*, and therefore, the value of the cyst morphology to separate the two genera remains to be demonstrated.

Polykrikos barnegatensis G. W. Martin is described as a two-zooid pseudocolony with one large nucleus, with plastids, and without nematocysts (Martin 1929). It has subsequently been synonymized with *P. hartmannii* (Chatton 1952), but as Hulburt (1957) pointed out, the "species" differ in the number of nuclei (one in the former, and two in the latter). Because Martin (1929) based the description of the new species on the observation of only one living cell, *P. barnegatensis* is in need of reinvestigation. This uncertainty led us to disregard this species in the following discussion.

There are also different views about the generic classification of polykrikoid dinoflagellates. Loeblich (1980) transferred *Ph. beauchampii* into the genus *Polykrikos*; Dodge (1982) and Sournia (1986) recognized only the genus *Polykrikos* and treated *Pheopolykrikos* as a junior synonym. As mentioned previously, Matsuoka and Fukuyo (1986) retained *Pheopolykrikos* as a separate genus from *Polykrikos*. Fensome et al. (1993) were of the opinion that *Pheopolykrikos* and *Polykrikos* were distinctive enough to be classified into different families—*Pheopolykrikos* in the Gymnodiniaceae, and *Polykrikos* in the Polykrikaceae. Steidinger and Tangen (1997) distinguished the two genera but classified them both into the family Polykrikaceae. Table 1 summarizes the diversity of morphological characteristics in polykrikoid dinoflagellates as described in the literature; we interpret some of the features described therein as follows: a report indicating that *P. schwartzii* is sometimes photosynthetic (Matsuoka and Fukuyo 1986) has not been demonstrated, so we are apprehensive about the validity of this claim. We interpret the 16-zooid pseudocolonies in *P. schwartzii* and the 8-zooid pseudocolonies in *P. kofoidii* as early dividing stages. The single-cell stage described by Morey-Gaines and Ruse (1980) in *P. kofoidii* is unconvincing because the mechanism behind the segregation of the two nuclei in this species was not addressed, and Nagai et al. (2002) were not able to verify this observation.

In this paper, we reexamine polykrikoid morphology and provide molecular phylogenetic data using SSU rDNA sequences from three polykrikoid species, namely *Ph. beauchampii*, *P. kofoidii*, and *P. lebourae*. These sequences, together with a published sequence from *Ph. hartmannii*, represent most of the known morphological diversity observed in polykrikoid dinoflagellates. Our results allowed us to formulate a robust hypothesis of relationships that helps explain character evolution within the group.

MATERIALS AND METHODS

Organisms and light microscopy. Near-surface plankton samples were collected in the morning hours with a small net (mesh size 20 μm) at the docks of the Bamfield Marine Sciences Center, Vancouver Island (BC, Canada), in June and August of 2005. Immediately after sampling, single cells of *P. kofoidii* and *Ph. beauchampii* were identified at magnifications of ×40 to ×250 (Fig. 1) and isolated from the mixed plankton sample by micropipetting. Sand samples containing *P. lebourae* were collected with a spoon during low tide at Centennial Beach, Boundary Bay (BC, Canada), in October 2005. The sand samples were transported directly to the laboratory, and the flagellates were separated from the sand by extraction through a fine filter (mesh size 45 μm) using melting seawater ice (Uhlig 1964). The flagellates accumulated in a petri dish beneath the filter and were then identified at magnifications of ×40 to ×250 (Fig. 1). Cells were isolated by micropipetting for the preparations described below.

Cells were observed directly and micromanipulated with a Leica DMIL inverted microscope (Wetzlar, Germany) connected to a PixeLink Megapixel color digital camera (PL-A662-KIT, Ottawa, ON, Canada). For DIC LM, micropipetted cells were placed on a glass specimen slide and covered with a coverslip. Images were produced directly with either the PixeLink Megapixel color digital camera or a Zeiss Axioplan 2 imaging microscope (Carl–Zeiss, Oberkochen, Germany) connected to a Leica DC500 color digital camera (Wetzlar, Germany).

Molecular phylogenetic analysis. Individually isolated pseudocolonies (five for *P. kofoidii*, nine for *Ph. beauchampii*, and four for *P. lebourae*) were individually washed four times in filtered (eukaryote free) seawater. All pseudocolonies of one species were deposited into a 1.5 mL Eppendorf tube (DiaMed Lab Supplies Inc., Mississauga, ON, Canada), resulting in one multispecimen sample for each of the three species. Genomic DNA was extracted using a standard hexadecyltrimethylammonium bromide (CTAB) extraction protocol (Zolan and Pukkila 1986) or by placing washed pseudocolonies in distilled water that was directly used for PCR. The PCR was carried out using puReTaq Ready-To-Go PCR Beads (Amersham Biosciences, Piscataway, NJ, USA). The PCR amplification protocol using universal eukaryotic primers consisted of an initial denaturing period (95°C for 2 min); 35 cycles of denaturing (92°C for 45 s), annealing (50°C for 45 s), and extension (72°C for 1.5 min); and a final extension period (72°C for 5 min; Leander et al. 2003). The PCR products corresponding to the expected size were gel isolated and cloned into the PCR2.1 using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA). Clones were sequenced with the ABI big-dye reaction mix (Applied Biosystems, Foster City, CA, USA) using the vector primers and internal primers oriented in both directions. Two new sequences (from two different clones) were generated from *Ph. beauchampii*, two new sequences (from two different clones) were generated from *P. kofoidii*, and one new sequence (from one clone) was generated from *P. lebourae* (GenBank accession codes DQ371291–DQ371295).

The SSU rDNA sequences were aligned with other alveolate sequences using MacClade 4 (Maddison and Maddison 2000), forming a 70-taxon alignment and a 34-taxon alignment. Maximum-likelihood (ML), ML-distance, and Bayesian methods under different DNA substitution models were performed. All gaps were excluded from the alignments before phylogenetic analysis. The α-shape parameters were estimated from the data using the Hasegawa–Kishino–Yano (HKY) and a γ distribution with invariable sites (70-taxon alignment: four

TABLE 1. Morphological features of *Pheopolykrikos* and *Polykrikos* species and their habitat.

	Ph. beauchampii[a]	*Ph. hartmannii*[b]	*P. schwartzii*[c,d]	*P. kofoidii*[c,e,f,d]	*P. grassei*[g]	*P. lebourae*[h,i,j,k]
Pseudocolony length (µm)	100–120	60–100	40–180	30–120	112–152	38–90
Pseudocolony width (µm)	60–75	42–59	25–75	20–60	12–18	20–55
# of zooids in the main life cycle stage (our interpretation)	4	2	8	4	4	8
# of zooids from the literature	1 or 4	1 or 2	2, 4, 8, or 16[c]	2–8[c], 2–8[e], 1–4(8[h])[f], 2–16[d]	2 or 4	8
# of nuclei in the main life cycle stage (our interpretation)	4	2	4	2	2	2
# of nuclei from the literature	1 or 4	1 or 2	1, 2, 4, or 8[c,d]	1–4[c], 1–8[d]	1 or 2	2
Zooid-borders/annulus	Yes	Yes	Yes	Yes	Yes	No
Cingular displacements	Yes[l]	Yes	Yes[c], no[d]	Yes[c,d]	Unknown	Yes, slightly
Pseudocolony compression	Dorsoventral	Dorsoventral	Dorsoventral	No[c], dorsoventral[f]	No	Strong-oblique lateral
Plastids	Yes	Yes	No	No	No	Yes[h,i,j,k] or no[h,k]
Apical groove/acrobase	Unknown	Loop shaped[m]	Loop shaped	Loop shaped	Unknown	Unknown
Striated ribs on "hyposomes"	No	No	No	Yes	Unknown	No
Nematocysts	Tentatively yes	No[b,n] or yes[n]	Yes	Yes	Yes	Yes[j,k] or no[h,i,k]
Ocelloid	No	No	No	No	Yes	No
Cysts	Unknown	Yes	Yes	Yes	Unknown	Unknown
Cyst shape	Unknown	Subspherical	Elliptical	Ovoid	unknown	Unknown
Habitat	Planktonic, marine, brackish	Planktonic, marine	Planktonic, marine	Planktonic, marine	Planktonic, brackish	Benthic, marine

[a]Chatton (1933).
[b]Matsuoka and Fukuyo (1986).
[c]Nagai et al. (2002).
[d]Kofoid and Swezy (1921).
[e]Matsuoka et al. (2000).
[f]Morey-Gaines and Ruse (1980).
[g]Lecal (1972).
[h]Herdman (1923).
[i]Balech (1956).
[j]Dragesco (1965).
[k]Hoppenrath (2000).
[l]visible on image.
[m]Takayama (1985).
[n]Hulburt (1957).

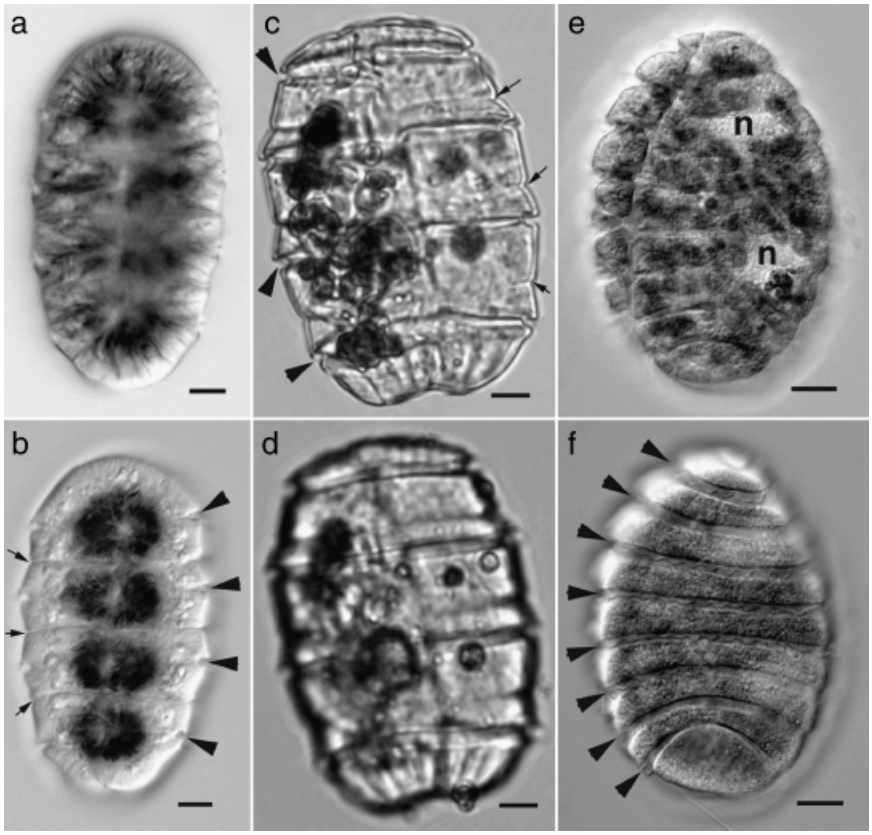

FIG. 1. Light micrographs showing the species that were isolated for DNA extraction. (a, b) *Pheopolykrikos beauchampii*. (a) Normal pseudocolony appearance with plastids being distributed over the zooid periphery. (b) The same pseudocolony with the plastids concentrated around the nuclei, making the four nuclei visible. Note the four transverse furrows (arrowheads) and the visible borders between the zooids (arrows). (c, d) *Polykrikos kofoidii*. (c) Median focus showing the four transverse furrows (arrowheads), the borders between zooids (arrows), and colored food particles. The two nuclei are not clearly visible. (d) The same pseudocolony with focus on the dorsal cell side, showing the characteristic striated ribs on the hyposome of the most posterior zooid. (e, f) *Polykrikos lebourae*. (e) Normal pseudocolony appearance of the phototrophic form of this species showing many plastids being distributed over the zooid periphery and two nuclei (n). (f) The same pseudocolony with focus on the eight transverse furrows (arrowheads). Borders between zooids are not visible in this species. Scale bars, 10 μm.

rate categories, $\alpha = 0.32$, transition/transversion ($Ti:Tv$) = 2.53, fraction of invariable sites = 0.15; 34-taxon alignment: eight rate categories, $\alpha = 0.29$, $Ti:Tv = 2.51$, fraction of invariable sites = 0.41). Heuristic γ-corrected ML trees (analyzed using the parameters listed above) were constructed with PAUP* 4.0 (Swofford 1999) using the HKY model for base substitutions on the 70-taxon alignment and using the general-time-reversible (GTR) model for base substitutions on the 34-taxon alignment (Posada and Crandall 1998, Swofford 1999); 10 random sequence additions were used in the latter analysis. The ML bootstrap analyses were performed on the 34-taxon alignment with PAUP* 4.0 (Swofford 1999) on 100 resampled data sets using HKY (without random sequence additions) and the α-shape parameter and $Ti:Tv$ estimated from the original data set.

The ML distances for both SSU rDNA data sets were calculated with TREE-PUZZLE 5.2 using the HKY substitution matrix (Strimmer and Von Haeseler 1996). A distance tree was constructed with weighted neighbor joining (WNJ) using Weighbor (Bruno et al. 2000). Five hundred bootstrap data sets were generated with SEQBOOT (Felsenstein 1993). Respective distances were calculated with the shell script "puzzleboot" (M. Holder and A. Roger, http://www.tree-puzzle.de) using the α-shape parameter, and $Ti:Tv$ estimated from the original data set and analyzed with Weighbor.

We also examined both data sets with Bayesian analysis using the program MrBayes 3.0 (Huelsenbeck and Ronquist 2001). The program was set to operate with GTR, a γ distribution, and four Monte-Carlo–Markov chains (MCMC; default temperature = 0.2). A total of 2,000,000 generations were calculated with trees sampled every 100 generations and with a prior burn-in of 200,000 generations (2000 sampled trees were discarded). A majority-rule consensus tree was constructed from 18,000 post-burn-in trees with PAUP* 4.0. Posterior probabilities correspond to the frequency at which a given node is found in the post-burn-in trees.

Five alternative topologies differing in the relative position of *Ph. beauchampii* were generated with McClade. Approximately unbiased (AU) tests were performed with CONSEL (Shimodaira and Hasegawa 2001) using the likelihoods calculated with TREE-PUZZLE 5.2 (Strimmer and Von Haeseler 1996) with the same models and parameters indicated above.

GenBank accession numbers: See Supplementary Material Appendix S1.

RESULTS AND DISCUSSION

Polykrikoids evolved from within the Gymnodiniales sensu stricto. We generated SSU rDNA sequences from multispecimen samples of *P. kofoidii*, *Ph. beauchampii*, and *P. lebourae* (phototrophic form). We found 10 base differences between the two clones of *Ph. beauchampii* and six base differences between the two clones of *P. kofoidii*. Although other sources of variation cannot be entirely ruled out (e.g., PCR artifacts), we think that the majority of these base differences can be attributed to natural variation.

The phylogenetic position of these polykrikoid species within the dinoflagellate clade was analyzed by means of a global alignment of 70 taxa representing the bulk of dinoflagellate diversity. These molecular phylogenetic data demonstrated that polykrikoids were all members of the Gymnodiniales clade, including the *Gymnodinium sensu stricto* (*s.s.*) species together with *G. fuscum*, the type species of the genus (Fig. 2).

In order to better demonstrate the branching order and branch support within this clade, we focused our attention on a smaller alignment consisting of 34 mainly athecate taxa representing the different Gymnodiniales subclades, with *Polarella glacialis* and *Symbiodinium microadriaticum* as outgroup taxa (Fig. 3). Our inferred phylogenies demonstrated strong support for the *Gymnodinium s.s.* clade (Figs. 2 and 3). Moreover, a strongly supported "*Polykrikos*" clade formed the sister group to *G. fuscum* (Fig. 3). *Pheopolykrikos hartmannii* (unpublished sequence from GenBank, AY421789) was most distantly related to the other species within the *Polykrikos* clade (Fig. 3). *Pheopolykrikos beauchampii* was part of another clade consisting of two unidentified dinoflagellate taxa, the so-called *Pheopolykrikos* clade (Fig. 3). Although the Bayesian posterior probability for the *Pheopolykrikos* clade was modest (0.94), bootstrap support for this node was low (Fig. 3). Nonetheless, the relatively distant relationship between *Ph. beauchampii* and *Ph. hartmannii* is discordant with the morphology-based taxonomic separation of *Pheopolykrikos* from *Polykrikos*.

In order to gain an additional insight into how well the data supported the phylogenetic separation of *Ph. beauchampii* from the polykrikoid clade (consisting of *Ph. hartmannii*, *P. kofoidii*, and *P. lebourae*), we performed AU tests on five alternative topologies differing in the relative position of these taxa. The topologies examined were similar to the tree shown in Fig. 3 (34-taxon data set), except for the position of *Ph. beauchampii*, which was alternatively placed as (1) a sister branch to the clade consisting of *G. fuscum* and the *Polykrikos* clade (Fig. 4a), (2) a sister branch to the *Polykrikos* clade (Fig. 4b), (3) a sister branch to *G. fuscum* (Fig. 4c), (4) a sister branch to the clade consisting of *P. kofoidii* and *P. lebourae*, and (5) a sister branch to *Ph. hartmannii*. The topology placing *Ph. beauchampii* as the sister lineage to the clade consisting of *G. fuscum* and the *Polykrikos* clade received the highest AU scores ($P = 0.979$) and is consistent with the relationships inferred from the phylogenetic analyses (Table 2; Figs. 3 and 4a). The remaining four topologies (Fig. 4, b–e) were rejected by the AU tests at the 5% threshold (Table 2). These data provide additional confidence in the topology shown in Fig. 3 and strengthen the modest bootstrap values and Bayesian posterior probabilities supporting nodes B, C, and F.

Only one other molecular phylogenetic study (SSU) including a *Polykrikos* species has been published (Saunders et al. 1997), but this particular sequence was never deposited in GenBank because the authors of the sequence questioned its validity. This sequence was identified as *P. schwartzii*, and it branched as the sister lineage to *G. mikimotoi* Miyake et Kominami ex Oda, now *Karenia mikimotoi* (Miyake et Kominami ex Oda) Ge. Hansen et Moestrup, in the analyses conducted by Saunders et al. (1997). The *Karenia* lineage was not closely related to the *Gymnodinium s.s.* clade in either the analyses of Saunders et al. (1997) or in ours (Figs. 2 and 3). These phylogenetic data, combined with the absence of a confident identification, led us to conclude that the *P. schwartzii* sequence from Saunders et al. (1997) is not reliable.

Daugbjerg et al. (2000) defined clades of the Gymnodiniales on the basis of ultrastructural and partial LSU rDNA sequence data and erected new genera for them. In that paper, Hansen and Moestrup emended the genus *Gymnodinium* and characterized it as follows: "Unarmoured unicellular or colony-forming dinoflagellates with horseshoe-shaped apical groove running in an anticlockwise direction. Nuclear envelope with vesicular chambers. Cingulum displacement one or more cingulum widths. Nuclear or dorsal fibrous connective present" (Daugbjerg et al. 2000, p. 305). These authors suggested that morphological characters known for *Polykrikos* support a phylogenetic relationship between this genus and *Gymnodinium s.s.* (Daugbjerg et al. 2000). For instance, like *Gymnodinium*, the apical groove of *P. schwartzii* (as *P. kofoidii* in Takayama 1985) and *P. kofoidii* is loop shaped (syn. horseshoe shaped) and runs in a counterclockwise direction (Takayama 1985, Nagai et al. 2002). Moreover, *Ph. hartmannii* (as *P. hartmannii*) was shown to have a similarly shaped apical groove (Takayama 1985). Although the apical groove of *Ph. beauchampii* and *P. lebourae* is unknown, we predict from their phylogenetic position that the apical groove is also horseshoe shaped and runs in a counterclockwise direction (unpublished LM data for *P. lebourae* are so far consistent with this interpretation). Moreover, the distinctive morphology of the nuclear envelope in *P. kofoidii* has been inferred to be homologous to the type found in *Gymnodinium* species (Dodge and Crawford 1969, Bradbury et al. 1983, Daugbjerg et al. 2000, Hansen et al. 2000). Little is known about the ultrastructure of *Ph. beauchampii*. Roberts (1991) discussed details of the flagellar apparatus, which is different from that in *P. kofoidii* (see Roberts 1991, fig. 19.11). Although no ultrastructural data for either *Ph. hartmannii* or *P. lebourae* have been published, the origin of polykrikoid dinoflagellates from within the *Gymnodinium s.s.* clade in our molecular phylogenetic analyses is perfectly consistent with current ultrastructural data.

Character evolution in polykrikoids. The pseudocolonial cell organization of *Polykrikos* species is among the most novel features of these dinoflagellates. A similar cellular organization has also been described for parasitic dinoflagellates, such as *Haplozoon* Dogiel, where, for example, *H. axiothellae* Siebert forms a compartmentalized syncytium (Siebert 1973, Siebert and West 1974, Leander et al. 2002). The compartments in the pseudocolonies of *H. axiothellae* each contain at least one nucleus, are partitioned by alveolar membranes, and are enveloped by a continuous plasma membrane. Moreover, each compartment in the pseudocolony contains a ventral pore, and together these form a linear series resembling the monokinety of polykrikoids (Leander et al. 2002). Although on superficial ultrastructural grounds, the pseudocolonies of polykrikoids and haplozoans could

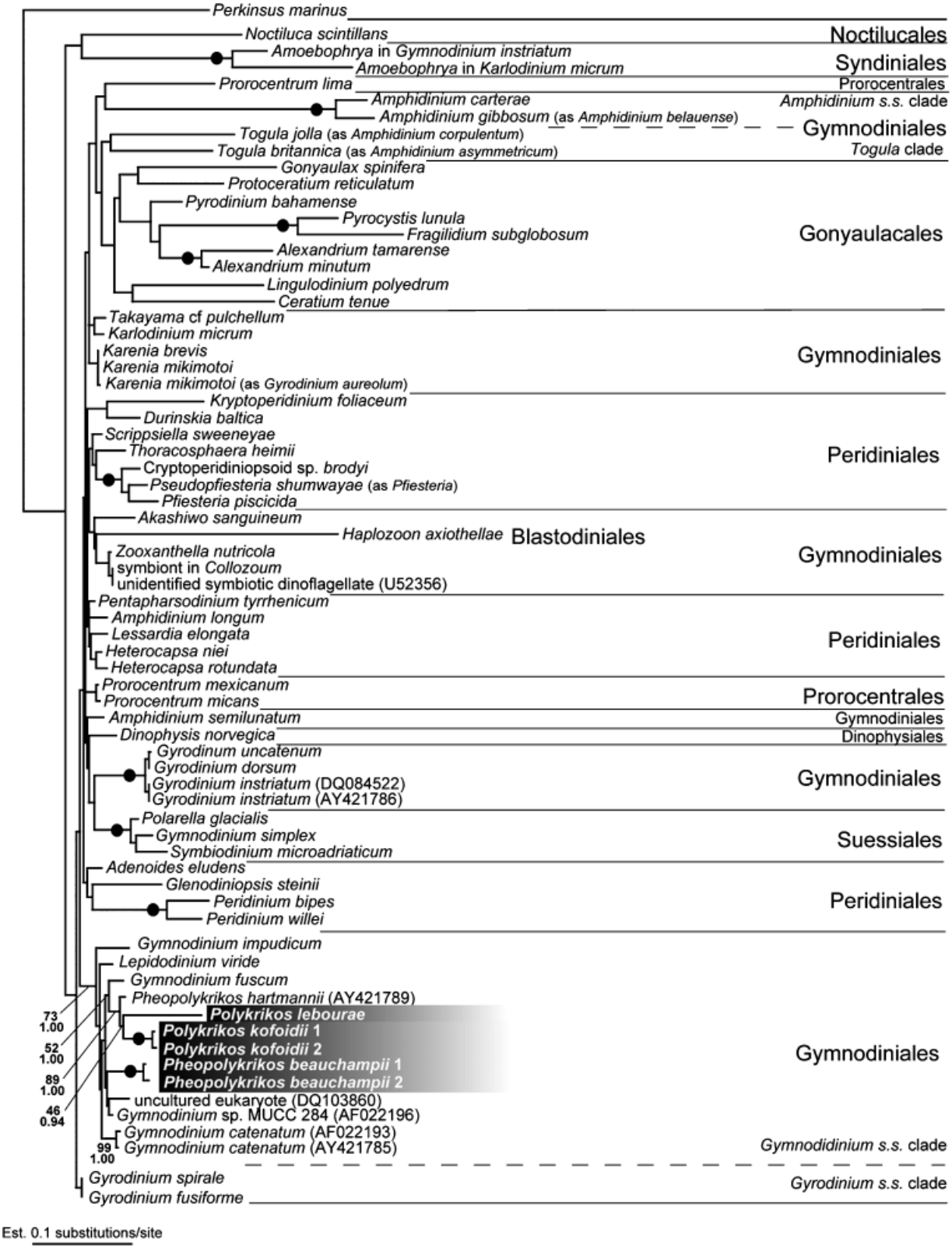

FIG. 2. The γ-corrected maximum-likelihood tree ($-\ln L = 18311.321$, $\alpha = 0.32$, eight rate categories) inferred using the Hasegawa–Kishino–Yano model of substitution on an alignment of 70 SSU rDNA sequences and 1625 unambiguously aligned sites. Numbers at the branches denote γ-corrected bootstrap percentages of 500 replicates using weighted neighbor joining (top) and Bayesian posterior probabilities–general time reversible (bottom). Black dots on branches denote bootstrap percentages and posterior probabilities of 95% or higher. Sequences derived from this study are highlighted in shaded boxes.

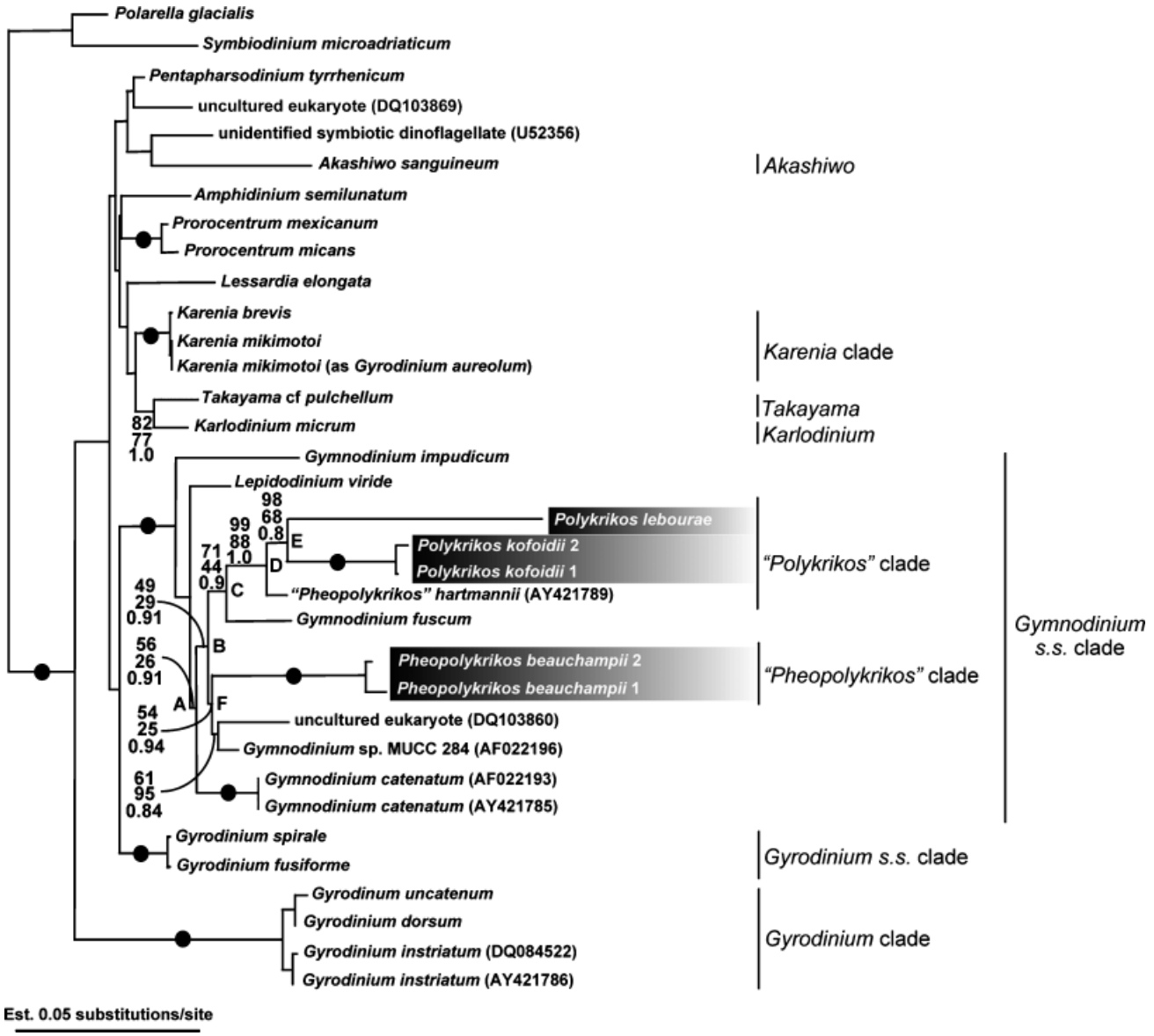

FIG. 3. The γ-corrected maximum-likelihood (ML) tree ($-\ln L = 6534.9393$, $\alpha = 0.29$, eight rate categories) inferred using the general-time-reversible (GTR) model of substitution on an alignment of 34 SSU rDNA sequences and 1687 unambiguously aligned sites. Numbers at the branches denote bootstrap percentages using ML–Hasegawa–Kishino–Yano (top), bootstrap percentages using weighted neighbor joining (middle), and Bayesian posterior probabilities–GTR (bottom). Black dots on branches denote bootstrap percentages and posterior probabilities of 95% or higher. Sequences derived from this study are highlighted in shaded boxes. Letters at nodes refer to hypothetical ancestors of specific clades and correspond to the letters in Fig. 5.

be inferred to be homologous, our molecular phylogenetic data indicate otherwise (Fig. 2). Our results suggest that the pseudocolonies of polykrikoids and haplozoans evolved convergently from within independent gymnodinoid lineages, namely the *Gymnodinium s. s.* for the former, and possibly the *Akashiwo*-like gymnodinoids for the latter (albeit weakly supported by molecular phylogenetic data; Fig. 2).

The chain-forming dinoflagellate *G. catenatum* was among the nearest lineage to the clade containing *Polykrikos* and *Pheopolykrikos* (clade B; Figs. 2, 3, and 5). This putative relationship suggests that the stem group that gave rise to polykrikoids consisted of phototrophic gymnodinoids with an ancestral capacity (or predisposition) toward chain formation. Nonetheless, the more inclusive *Gymnodinium s.s.* clade was very well supported by the molecular phylogenetic data (Figs. 2 and 3). Clades C and F both consisted of a combination of polykrikoids and single zooid *Gymnodinium* species (Figs. 3 and 5). This phylogenetic topology suggests that the pseudocolonies in *Ph. beauchampii*, on the one hand, and the pseudocolonies in *Ph. hartmannii*, *P. kofoidii*, and *P. lebourae* on the other, arose independently by convergent evolution from different *Gymnodinium*-like ancestors

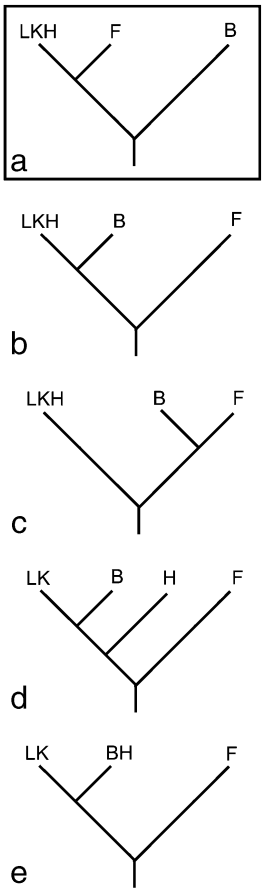

FIG. 4. Topologies used to evaluate five alternative phylogenetic positions of *Pheopolykrikos beauchampii* by performing approximately unbiased (AU) likelihood tests on the 34-taxon data set (see the results in Table 2). Labels at the termini indicate the first letter of specific epithets and are as follows: L, *Polykrikos lebourae*; K, *P. kofoidii*; H, *Pheopolykrikos hartmannii*; F, *Gymnodinium fuscum*; B, *Ph. beauchampii*. The topology most favored by the SSU rDNA phylogenetic analyses and AU likelihood tests (a) is highlighted with a box. Topologies b–e were rejected by the AU tests at the 5% threshhold.

TABLE 2. *P* values for AU likelihood tests of five alternative phylogenetic positions of *Pheopolykrikos beauchampii* (topologies shown in Fig. 4).

Topology	a	b	c	d	e
34-taxon alignment	0.979	0.026	0.028	0.000	0.000

(Figs. 3 and 5). The AU tests provide additional support for this inference (Table 2; Fig. 4).

The major evolutionary innovation along the "*Pheopolykrikos*" lineage is pseudocolony formation with an equal number of zooids and nuclei (Figs. 3 and 5). *Pheopolykrikos beauchampii* consists of four zooids and four nuclei, and we infer that an intermediate form consisting of two zooids and two nuclei was a necessary precursor. Although this intermediate form is known and represented by the extant *Ph. hartmannii*, our molecular phylogenetic data suggest that this particular species is more closely related to "true" *Polykrikos* species than to *Ph. beauchampii* (Figs. 3 and 5). Accordingly, the sister clade to clade F consists of *Ph. hartmannii*, two *Polykrikos* species, and the phototrophic *G. fuscum* (clade C). The pseudocolony-forming species formed a strongly supported subclade (clade D) within clade C (Figs. 3 and 5). The most prominent synapomorphy for clade D is the presence of two nuclei, which remains constant regardless of zooid number (*Ph. hartmannii*, *P. kofoidii*, and *P. lebourae* each have two nuclei, but two, four, and eight zooids, respectively). This stable character state was unexpected and potentially insightful. Doublings of the number of zooids irrespective of the nuclei could have been achieved by an incremental series of incomplete zooid divisions along this lineage. For instance, we infer that members of clade E are derived from an ancestor that doubled the number of zooids without doubling the nuclei, resulting in a lineage with pseudocolonies consisting of four zooids and two nuclei, like that found in *P. kofoidii* (Fig. 5).

A subsequent zooid-doubling event from ancestor E would produce a lineage with pseudocolonies consisting of eight zooids and two nuclei, like that found in *P. lebourae* (Fig. 5). Apparently, this pseudocolonial form does not seem capable of separating into smaller zooid numbers, which reflects a higher degree of zooid integration. These pseudocolonies also lack visible zooid borders, and unlike other polykrikoids, the zooids are appreciably narrower at the anterior and posterior ends of the pseudocolony. It is plausible, therefore, that this degree of integration is somehow related to the benthic mode of life found in *P. lebourae*; all other known polykrikoids are planktonic and also capable of dissociating into smaller zooid numbers. Moreover, the phylogenetic position of *P. lebourae* demonstrates that this species is deeply nested within a clade of planktonic species, suggesting that the benthic mode of life in *P. lebourae* arose secondarily from planktonic ancestors (Figs. 3 and 5). This stands in contrast to the well-supported inference that, in general, benthic modes of life predate planktonic modes (Leander 2004).

Nonetheless, *P. lebourae* has been described as being both photosynthetic and nonphotosynthetic, and it is not clear whether photosynthesis was lost ancestrally in clade E (comprising *P. kofoidii* and *P. lebourae*) and subsequently regained in *P. lebourae* or whether it was lost in *P. kofoidii* and *P. lebourae* independently. This question can be addressed by investigating the ultrastructure of *P. lebourae* and by acquiring molecular data from the heterotrophic form of this species. At present, we can envision three possible scenarios for explaining photosynthesis in *P. lebourae*: (1) *P. lebourae* has typical peridinin-containing plastids like *G. fuscum*, and the heterotrophic form subsequently lost photosynthesis and represents a different species; (2) *P. lebourae* is ancestrally heterotrophic like *P. kofoidii*, and the photosynthetic form acquired plastids via an endosymbiotic replacement event (e.g., tertiary or serial secondary); or (3) *P. lebourae* is ancestrally heterotrophic and able to temporarily retain plastids (and photosynthesis) via

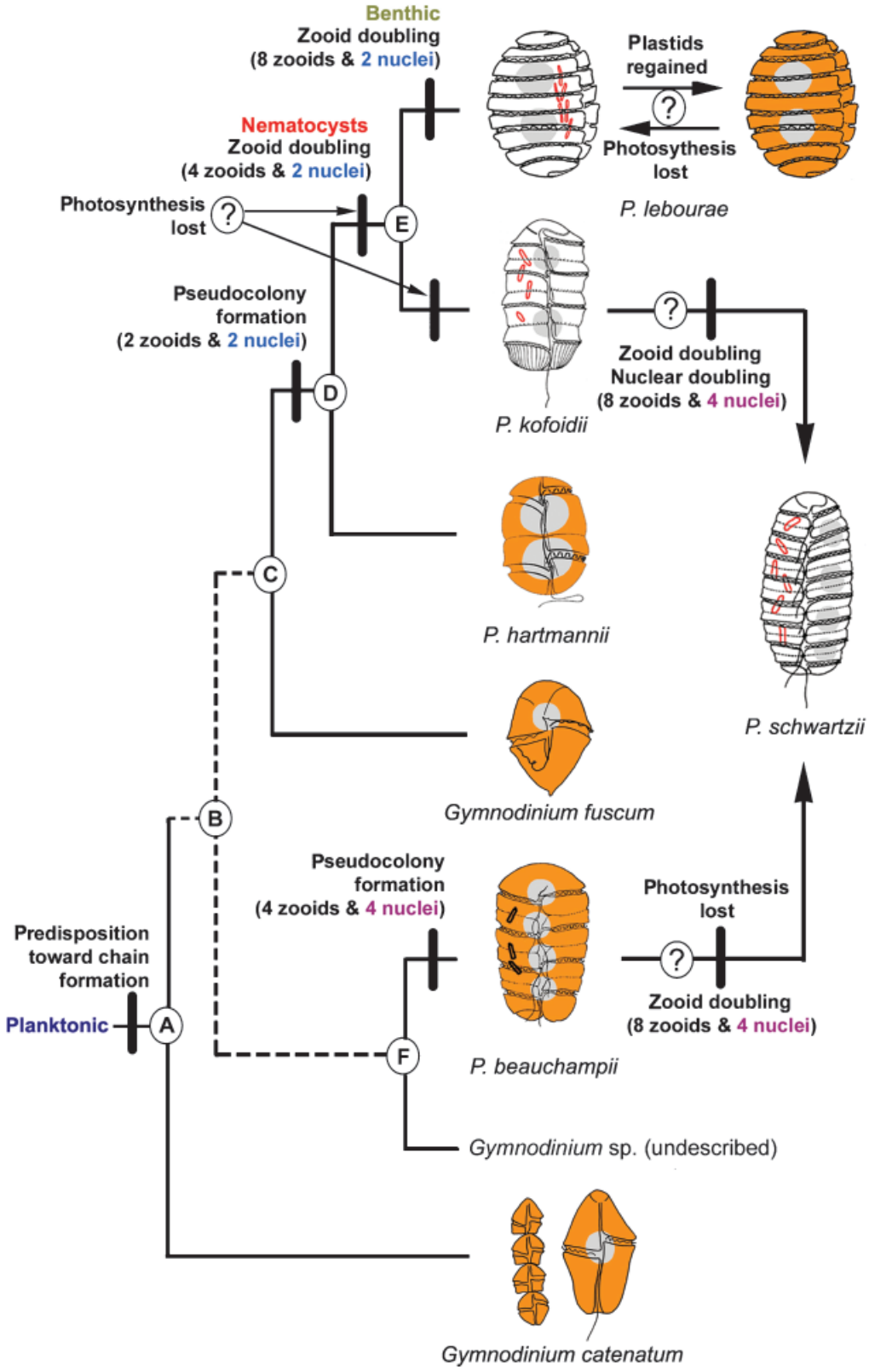

FIG. 5. Synthetic phylogeny of polykrikoid dinoflagellates derived from known morphological diversity and the molecular phylogenetic results of this study. Characters of interest are parsimoniously mapped onto the topological framework; letters at nodes refer to hypothetical ancestors of specific clades (see Results and Discussion). Colored cells indicate species that are capable of photosynthesis. Nematocysts are highlighted in red; black organelles indicate nematocyst-like structures reported in the literature. Dashed lines indicate regions of significant phylogenetic uncertainty. The drawings are modified after Lebour (1925), Martin (1929), Chatton (1933), Hulburt (1957), Greuet (1987), Steidinger & Tangen (1997), Daugbjerg et al. (2000), Larsen (2002).

kleptoplasty. The loss and replacement of plastids is not unusual and has occured several times in dinoflagellate evolution (Saldarriaga et al. 2001). Even though the plastids of *Ph. hartmannii* and *Ph. beauchampii* have not been described, our phylogenetic analyses suggest that they are probably homologous to the peridinin-containing plastids found in *G. catenatum* and *G. fuscum*. However, one should consider that *Lepidodinium viride* is also a member of this well-supported clade, and it has plastids originating from chlorophytes (Watanabe et al. 1990). The possibility that *P. lebourae* is ancestrally heterotrophic is supported by the presence of nematocysts, a character shared with the heterotrophic polykrikoids *P. kofoidii* and *P. schwartzii* (Table 1; Fig. 5). This complex organelle is unlikely to have evolved several times independently, and we infer that the presence of nematocysts is a solid synapomorphy for clade E (Fig. 5). Moreover, the presence of well-developed nematocysts forms the basis for our prediction that molecular sequence data from *P. schwartzii* will demonstrate a closer relationship with clade E than with clade F (Fig. 5).

The phylogenetic position of *P. schwartzii* within this *Gymnodinium s.s.* clade, however, remains speculative. Two possible lineages could have given rise to this species. If we emphasize that the number of nuclei within a pseudocolony is a relatively stable character in polykrikoids, then one can posit that *P. schwartzii* evolved from a *Ph. beauchampii*–like ancestor (each with four nuclei) via zooid doubling, plastid loss, and nematocyst gain (Fig. 5). Interestingly, "nematocysts" of unknown structure have been ambiguously reported in *Ph. beauchampii* (Chatton 1933), and it is not at all clear whether the observed structures actually represent true nematocysts (no images were provided). We find this report to be unreliable and believe that further investigation of *Ph. beauchampii* is necessary before the evolution of nematocysts within polykrikoids can be more confidently inferred.

The nematocysts of dinoflagellates are complex organelles with one or several extrusive filaments that closely resemble the cnidae of cnidarians (including the Myxozoa). These distinctive organelles are restricted not only to some polykrikoids but also warnowiids (Greuet 1987). Although the origin(s) of nematocysts in dinoflagellates and cnidarians is unclear, there is speculation that these organelles could be highly adapted endosymbionts of unknown origin (Hausmann et al. 2003). Nonetheless, the ultrastructure, development, and function of nematocysts in *Polykrikos* have been studied on several occasions (Chatton and Grassé 1929, Greuet 1972, Greuet and Hovasse 1977, Westfall et al. 1983). After many years of speculation, it was shown that nematocysts in *P. kofoidii* were used to catch and engulf prey (Matsuoka et al. 2000). As mentioned previously, we think that the nematocyst–taeniocyst complex of *Polykrikos* species (Westfall et al. 1983, Greuet 1987) is a synapomorphy for clade E, which, by extension, also includes *P. schwartzii* (Figs. 3 and 5). This inference has a direct impact on the possible origin of warnowiid dinoflagellates. Although the nematocysts of warnowiids [e.g., *Nematodinium armatum* (Dogiel) Kofoid et Swezy] are more complicated than those in *Polykrikos* (Morin and Francis 1967, Greuet 1971), the presence of these putatively homologous organelles in both groups of dinoflagellates suggests that warnowiids evolved from within the polykrikoids or *vice versa*. Alternatively, both lineages may have evolved from a nematocyst-bearing common ancestor that lacked the other diagnostic morphological characteristics in *Polykrikos* and warnowiids. Obtaining molecular phylogenetic data from different warnowiid species will shed considerable light on this issue.

Another complex organelle that is restricted to warnowiids and perhaps polykrikoids is the distinctive multilayered photoreceptor called an ocelloid. As mentioned in the Introduction, a *Polykrikos* species, namely *P. grassei*, was described as having an ocelloid (Lecal 1972), but the reliability of this species description has been questioned (Greuet and Hovasse 1977, Sournia 1986). *Polykrikos grassei* is otherwise strikingly similar to *P. kofoidii*. If the ocelloid is a constant cell feature of *P. grassei*, then it is unclear what happens to the organelle when the pseudocolony disassociates into two-zooid stages (Lecal 1972). It is plausible that the ocelloid observed in *P. grassei* was derived from a residual prey cell (a warnowiid) observed by chance in that population. Nevertheless, a reinvestigation of *P. grassei* is required in order to rule out the possibility that a misinterpretation occurred and to help understand the evolutionary relationships between polykrikoids and warnowiids.

Taxonomic implications. The results of our molecular phylogenetic analyses reveal several taxonomic problems associated with the genera *Polykrikos*, *Pheopolykrikos*, and possibly *Gymnodinium*. As circumscribed today, species within *Pheopolykrikos* are polyphyletic (Fig. 3). *Pheopolykrikos hartmannii* is more closely related to the *Polykrikos* species than to *Ph. beauchampii* and should probably be reclassified as *P. hartmannii*. This tentative conclusion is indicated by the quotation marks around *Ph. hartmannii* in Figure 3.

However, alternative taxonomic solutions exist. For instance, "*Pheopolykrikos*" *hartmannii* might represent an independent clade warranting a generic distinction of its own (i.e., the erection of a third genus of polykrikoids). By contrast, Loeblich (1980), Dodge (1982), and Sournia (1986) have considered all polykrikoid species as belonging to one genus, namely *Polykrikos*. Our results demonstrate that this *Polykrikos* clade must also then include *G. fuscum*, which is the type species of the genus *Gymnodinium*. This scenario would cause a succession of problems that do not seem justified. Nonetheless, we think that it is premature to transfer any of the above species to different genera or to describe any new taxa. In order to solve these nomenclatural problems, more ultrastructural and molecular phylogenetic data are needed from polykrikoids, warnowiids, and gymnodinoids. Specifically, *Ph. beauchampii*, *Ph. hartmannii*, and *P. lebourae* need to be

investigated at the ultrastructural level to better understand the structure of their plastids, nematocysts, and zooid compartmentalization. The SSU rDNA sequence of *P. schwartzii* is also needed in order to demonstrate its phylogenetic position and genus affiliation. Once the above data have been accumulated, the taxonomy of polykrikoid dinoflagellates should be revised.

NOTE ADDED IN PROOF

During the review process for this paper, we were able to investigate the morphology and molecular phylogeny of both the photosynthetic and heterotrophic forms of *P. lebourae* in more detail. These data significantly contribute to the hypothetical framework of character evolution presented here. Hoppenrath, M. & Leander, B. S. 2007. Morphology and phylogeny of the pseudocolonial dinoflagellates *Polykrikos lebourae* and *Polykrikos herdmanae* n. sp. *Protist* (in press).

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Supplementary Material

The following supplementary material is available for this article:

Appendix S1. GenBank accession numbers for SSU rDNA sequences used in the phylogenetic analyses of polykrikoid dinoflagellates.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1529-8817.2007.00319.x
(This link will take you to the article abstract).

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