

MORPHOLOGY AND MOLECULAR PHYLOGENY OF A NEW MARINE SAND-DWELLING
PROROCENTRUM SPECIES, *P. TSAWWASSENENSE* (DINOPHYCEAE,
PROROCENTRALES), FROM BRITISH COLUMBIA, CANADA¹

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A new marine benthic, sand-dwelling *Prorocentrum* species from the temperate region of the Pacific coast of British Columbia, Canada, is described using LM and EM and molecular phylogenetic analyses. The cells have a broad oval shape, 40.0–55.0 µm long and 30.0–47.5 µm wide, and a wide U-shaped periflagellar area on the right thecal plate. The left thecal plate consists of a straighter apical outline in the form of a raised ridge. Five to six delicate apical spines in the center of the periflagellar area are present. The nucleus is located in the posterior region of the cell, and a conspicuous pusule is located in the anterior region of the cell. The cells have golden-brown chloroplasts with a compound, intrachloroplast pyrenoid that lacks a starch sheath. The thecal plates are smooth with round pores of two different sizes. The larger pores are arranged in a specific pattern of radial rows that are evenly spaced around the plate periphery and of irregular rows (or double rows) that form an incomplete “V” at the apical end of the plates. Large pores are absent in the center of the left and right thecal plates. The intercalary band is striated transversely and also has faint horizontal striations. Trichocysts and two types of mucocysts are present. The molecular phylogenetic position of *Prorocentrum tsawwassenense* sp. nov. was inferred using SSU rDNA sequences. This new species branched with high support in a *Prorocentrum* clade containing both benthic and planktonic species.

Key index words: benthic; dinoflagellate; Dinophyceae; morphology; phylogeny; *Prorocentrum*; small subunit ribosomal RNA; taxonomy

Abbreviations: DSP, diarrhetic shellfish poisoning; GPP, Gymnodiniales-Peridinales-Prorocentrales; ML, maximum likelihood

Prorocentrum species are distributed worldwide in marine plankton and sediments. Compared to the planktonic *Prorocentrum* species diversity, only a few benthic species had been described until 1990.

Since that time the investigations by Faust revealed a high diversity of benthic, marine, warm-water *Prorocentrum* species (Faust 1990, 1993a,b, 1994, 1997). The number of benthic *Prorocentrum* species in temperate, cold-water environments seems to be either relatively low or poorly characterized (Dragesco 1965, Dodge 1982, Larsen 1985, Dodge and Lewis 1986, Paulmier 1992, Hoppenrath 2000a). Nonetheless, benthic *Prorocentrum* species not only inhabit the interstitial spaces of marine sediments (Lebour 1925, Dragesco 1965, Larsen 1985, Dodge and Lewis 1986, Faust 1994, Hoppenrath 2000a), but they are also epiphytic on macroalgal surfaces (i.e., phycophilic), floating detritus, and corals (Fukuyo 1981, Steidinger 1983, Taylor 1987, Faust 1990, 1993a,b, 1997, Grzebyk et al. 1994, Morton and Faust 1997, Morton 1998).

Ehrenberg (1834) described the genus *Prorocentrum* with the type species *P. micans*. A second prorocentroid genus was described later, *Exuviaella* with the type species *E. marina* (Cienkowski 1881). The presence or absence of an apical spine was the fundamental distinction between these two genera. Abé (1967) informally proposed that the two genera should be merged, and Dodge (1975) formally made *Exuviaella* a junior synonym of *Prorocentrum*; this view has been widely accepted, despite an attempt to reinstate the genus *Exuviaella* about a decade ago (McLachlan et al. 1997). The order Prorocentrales currently includes two genera: *Prorocentrum* and *Mesoporus*.

The Prorocentrales are morphologically characterized as dinoflagellates with theca consisting of two major plates separated by a sagittal suture and tiny platelets in the periflagellar area, without a cingulum and sulcus, but with two “typical” dinoflagellate flagella arising from one pore. The highly derived morphology of prorocentroid species suggests that they should comprise a strongly supported monophyletic group in molecular phylogenetic analyses. Only species in the genus *Prorocentrum* have been investigated at the molecular phylogenetic level, and it has been shown that these species branch from within the Gymnodiniales–Peridinales–Prorocentrales (GPP) complex (Saldarriaga et al. 2004). Unexpectedly, phylogenetic trees inferred from SSU and LSU rDNA indicate that *Prorocentrum*

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species split into two clades that do not appear closely related (Zardoya et al. 1995, Grzebyk et al. 1998, Litaker et al. 1999, Pearce and Hallegraeff 2004, Saldarriaga et al. 2004, Murray et al. 2005). Although this tree topology could be interpreted to reflect the polyphyly of *Prorocentrum*, the shared morphological features of *Prorocentrum* species argue strongly against this, as has been previously pointed out by Grzebyk et al. (1998). Moreover, the topological backbone from which the two *Prorocentrum* clades emerge is poorly resolved. Grzebyk et al. (1998) invoked homoplasy as an attempt to explain the lack of resolution of the dinoflagellate GPP complex. The separation of the two *Prorocentrum* clades in dinoflagellate phylogenies most likely reflects methodological artifacts associated with taxon sampling and phylogenetic analyses of alveolate ribosomal sequences, which generally lack sufficient phylogenetic signal at deep levels in the hierarchy (Silberman et al. 2004). So far, only one published phylogenetic tree, inferred from LSU rDNA, was able to weakly recover a monophyletic group containing all *Prorocentrum* species in the analysis (Saldarriaga et al. 2004).

Prorocentrum species are distinguished from one another by their cell shape and size, the micromorphology and ornamentation of the thecal plates (the two "lateral" valves) and the intercalary band, and by the architectural details of the periflagellar area (Dodge 1975, Taylor 1980, Faust et al. 1999). Furthermore, the presence and type of pyrenoid (e.g., covered by a conspicuous starch sheath), and the presence of trichocysts and/or mucocysts (also named "vesicles containing diffuse fibrous material" in Dodge and Bibby 1973) are probably useful for species identification.

Benthic species of *Prorocentrum* have attracted special attention because several toxin producers have been recognized (see Faust et al. 1999, Faust and Gullede 2002 for summaries). Therefore, new benthic *Prorocentrum* species are generally labeled "potentially toxic," and their unambiguous identification is crucial for further investigations, such as toxin analyses. Here we characterize a new benthic *Prorocentrum* species that was discovered in sandy sediments in the temperate region of the Pacific coast of British Columbia, Canada. Moreover, we integrate our results into a growing framework built from all other *Prorocentrum* species described from marine benthic environments.

MATERIALS AND METHODS

Collection of organisms. Sand samples containing dinoflagellates were collected with a spoon during low tide at Centennial Beach, Boundary Bay, British Columbia, Canada, in July of 2005 (see also Hoppenrath and Leander 2006). 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 (Uhlir 1964). The flagellates accumulated in a petri dish

beneath the filter and were then identified at $\times 40$ to $\times 250$ magnifications. Cells of this new species were isolated by micropipetting for the preparations described below.

LM. Cells were observed directly and micromanipulated with a Leica DMIL inverted microscope (Wetzlar, Germany). For DIC light microscopy, micropipetted cells were placed on a glass specimen slide and covered with a coverslip. Images were produced with a Zeiss Axioplan 2 imaging microscope (Carl Zeiss, Oberkochen, Germany) connected to a Leica DC500 color digital camera.

SEM. A mixed-extraction sample and a raw culture were fixed overnight with two drops of acidic Lugol's solution. Cells were transferred onto a 5 μm polycarbonate membrane filter (Corning Separations Div., Acton, MA, USA), washed with distilled water, dehydrated with a graded series of ethanol, and critical-point-dried with CO_2 . Filters were mounted on stubs, sputter-coated with gold, and viewed under a Hitachi S4700 scanning electron microscope. Some SEM images were presented on a black background using Adobe Photoshop 6.0 (Adobe Systems, San Jose, CA, USA).

TEM. Cells were concentrated in a microfuge tube by micropipetting and slow centrifugation. The pellet of cells was prefixed with 2% (v/v) glutaraldehyde in seawater at 4°C for 30 min. Cells were washed twice in filtered seawater (30–35 salinity) before postfixation in 1% (w/v) OsO_4 in seawater for 30 min at room temperature. Cells were dehydrated through a graded series of ethanol, infiltrated with acetone-resin mixtures (acetone, 2:1, 1:1, 1:2, resin), and embedded in Epon resin (Epon 812; Electron Microscopy Sciences, Hatfield, PA, USA). The block was polymerized at 60°C and sectioned with a diamond knife on a Leica Ultracut UltraMicrotome. Thin sections were poststained with uranyl acetate and lead citrate and viewed under a Hitachi H7600 transmission electron microscope.

Molecular phylogenetic analysis. Cells from a raw culture were washed with filtered (eukaryote-free) seawater and deposited in a 1.5 mL Eppendorph tube (Dia-Med Lab Supplies Inc., Mississauga, ON, Canada). Genomic DNA was extracted by using a standard hexadecyltrimethylammonium bromide (CTAB) extraction protocol (Zolan and Pukkila 1986). The PCR was carried out using puReTaq Ready-To-Go PCR Beads (Amersham Biosciences, Piscataway, NJ, USA), and the PCR amplification protocol using universal eukaryotic primers described in Hoppenrath and Leander (2007a) and in 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). A clone was sequenced with ABI big-dye reaction mix (Applied Biosystems, Foster City, CA, USA) using the vector primers and internal primers oriented in both directions. One new sequence from *P. sawwassenense* sp. nov. was completely sequenced using both vector primers and two internal primers oriented in both directions (GenBank accession code EF657885).

The SSU rDNA sequences were aligned with other alveolate sequences using MacClade 4 (Maddison and Maddison 2000), forming a 58-taxon alignment. Maximum-likelihood (ML) and Bayesian methods under different DNA substitution models were performed with the programs PHYML (Guindon and Gascuel 2003) and MrBayes (Huelsenbeck and Ronquist 2001), respectively. All gaps were excluded from the alignment prior to phylogenetic analysis (1,623 aligned sites). For ML, the alignment of nucleotide sequences was analyzed using a general-time-reversible (GTR) model of substitution (Posada and Crandall 1998) considering corrections for site-to-site rate variation (γ) with eight categories of rate variation and proportion of invariable sites. Five hundred bootstrap replicates were performed with the same parameters described above.

We also examined the 58-taxon data set with Bayesian analysis with the following parameters: GTR, a gamma 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 (2,000 sampled trees were discarded). A majority-rule consensus tree, including branch lengths, was constructed from 18,000 postburn-in trees. Posterior probabilities correspond to the frequency at which a given node is found in the post-burn-in trees.

GenBank accession numbers are available in the supplementary material (Appendix S1).

RESULTS

Prorocentrum tsawwassenense sp. nov. Hoppenrath et B. S. Leander

Description: Cellulae photosyntheticae, ovales, 40.0–55.0 µm longae et 30.0–47.5 µm latae. Nucleus in regione postica cellulae. Pyrenoides in regione centrale cellulae. Valvae levis, poris numerosi. Area apicalis valvae dextra U-formata. Area apicalis valvae sinistra collare. Area periflagellaris 7–9 platalatis apicalibus formata. 5 aculei apicalis. Balteus intercalaris transversale et horizontale striatus.

Type locality: Centennial Beach, Boundary Bay, British Columbia, Canada (49°0.0' N, 123°8.0' W).

Holotype: Figure 2A.

Iconotype/isotype: Figure 4.

Etymology: The species has been named after the town of the type locality, Tsawwassen.

General morphology. The cells have a broad oval shape with a widely U-shaped (syn.: arc-shaped) periflagellar area on the right thecal plate (syn.: valve) in right valve view (Figs. 1, A–E, and 2, A and C). Cells are 40.0–55.0 µm long and 30.0–47.5 µm wide. The periflagellar area forms a wide arc and only slightly excavates the right thecal plate (Fig. 1, C–E). The periflagellar area is nearly straight at the apical margin of the left thecal plate (Fig. 1B), which consists of a raised edge or ridge (Fig. 1B). In “apical” view, the shape of the periflagellar area is a triangle. Delicate apical spines in the center of the periflagellar area are sometimes detectable with the light microscope (Fig. 1C). The round to oval nucleus is located in the posterior region of the cell (Fig. 1, A, D, and E), and a pusule is usually visible in the anterior region of the cell (Fig. 1, A and D). Healthy cells have golden-brown chloroplasts (Fig. 1, A and B), but under starved conditions, the cells turn pale and are packed with colorless and colored granules (Fig. 1, C–E). A pyrenoid is not visible with the light microscope.

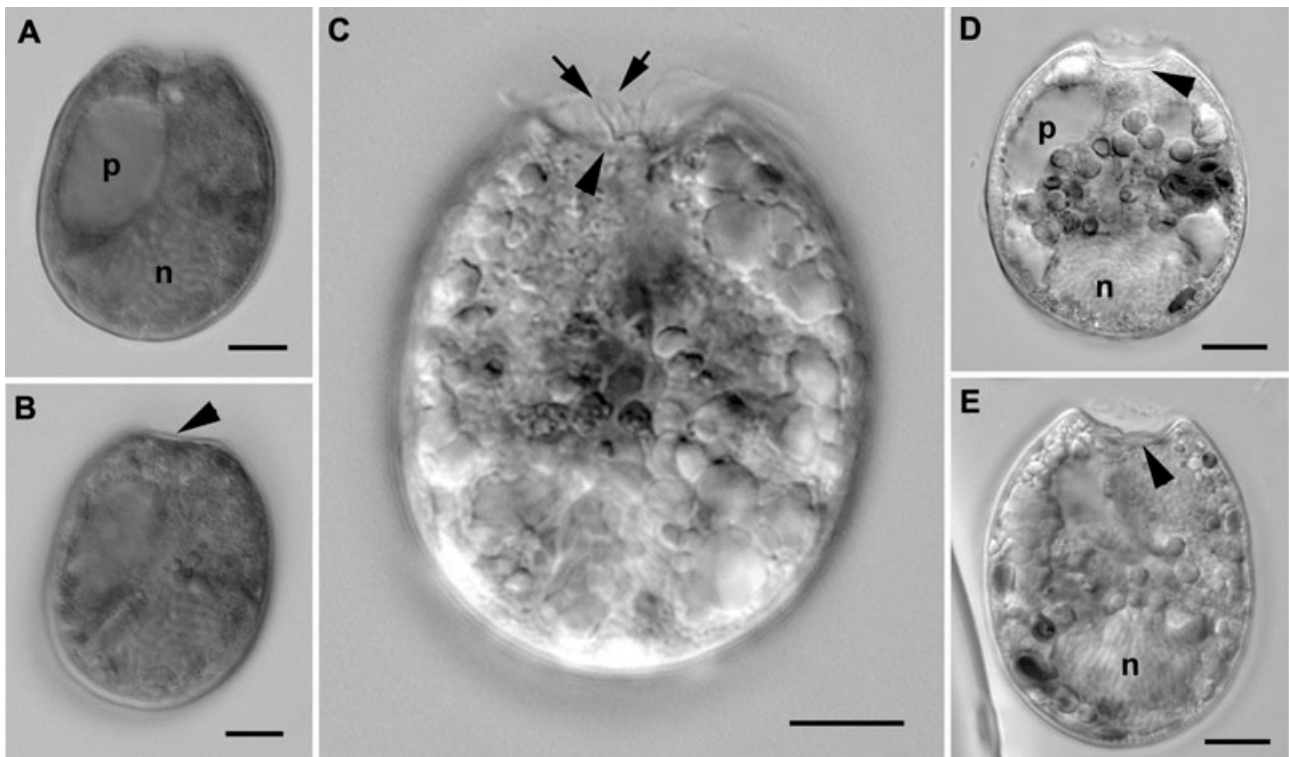


FIG. 1. Light micrographs showing *Prorocentrum tsawwassenense* sp. nov. from a freshly extracted sample (A, B) and from an older raw culture (C–E). (A) Right valve view showing the anterior located pusule (p) and the posterior nucleus (n). (B) Same cell as in (A) with the focal plane on the left valve showing the anterior ridge of the left thecal plate (arrowhead). (C) Right-valve view showing the arc-shaped (U-shaped) excavation of the periflagellar area (arrowhead) and the “spines” in the center of the area (arrows). (D) Midcell focal plane of a more rounded cell showing the anterior pusule (p), the posterior nucleus (n), and the arc-shape periflagellar area (arrowhead). (E) A more elongated oval cell showing the arc-shaped periflagellar area (arrowhead) and the posterior nucleus (n). Scale bars, 10 µm.

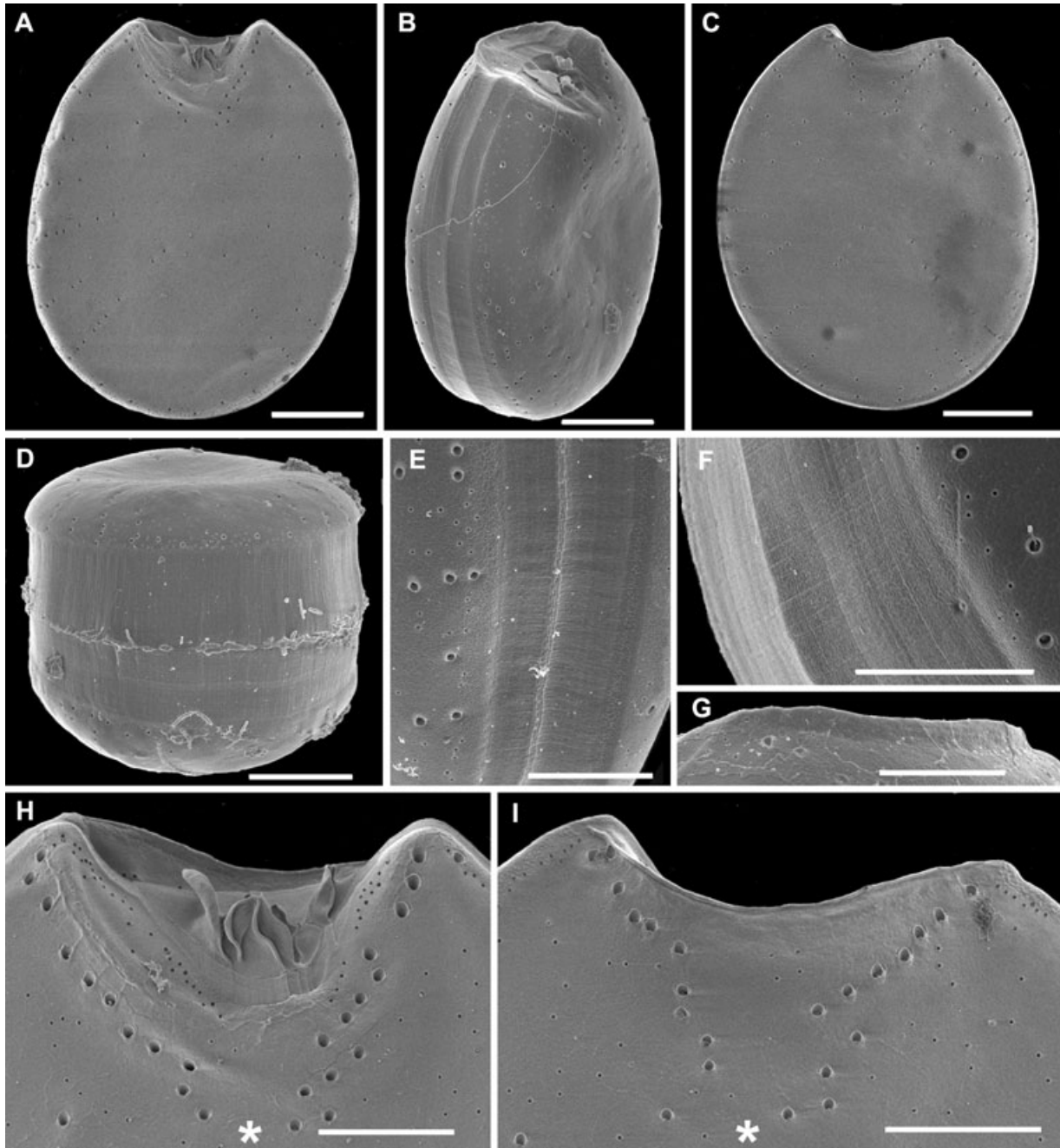


FIG. 2. Scanning electron micrographs of *Prorocentrum tsawwassenense* sp. nov. showing general cell surface features. (A) Right-valve view showing the wide arc-shaped (U-shaped) excavation of the periflagellar area, the projections in the center of the area, and the pattern of pores. (B) Oblique cell view showing the intercalary band, the concave right thecal plate, and the periflagellar area. (C) Left-valve view showing the nearly straight apical end and the pattern of pores. (D) Posterior view showing broad intercalary bands and the shape of the two thecal plates. (E) High magnification view of the cell margin showing the striated intercalary bands and the two pore sizes. (F) High magnification view showing the faint horizontal striations on the intercalary bands. (G) High magnification view of the apical ridge on the left thecal plate. (H) Right valve view of the periflagellar area showing the pattern of pores on the right thecal plate (the asterisk marks the incomplete end of the "V" formed by the large pores), the collar-shaped spine, and the projections in the area and the ridge of the left thecal plate. (I) Left-valve view of the apical area of the left thecal plate showing the pore pattern (the asterisk marks the incomplete end of the "V" formed by the large pores) and the narrow smooth ridge. Scale bars, 10 μm in (A–D) and 5 μm in (E–I).

Patterns of thecal plates and pores. The thecal plates are smooth with round pores of two different sizes (Figs. 2, A–I, and 3, A–C). Small pores (88–174 nm

in diameter) are scattered randomly over the two thecal plates and are densely arranged in rows near the periflagellar area (Fig. 2, A–C, E, F, H, I). The

larger pores (294–490 nm in diameter) are arranged in radial rows of varying length and are absent in the center of each plate (Figs. 2, A–C, H, I; 3C; and 4, A and B). These rows of pores are evenly spaced around the plate periphery, with the antapical rows being relatively short. Irregular rows (or double rows) of large pores form an incomplete “V” at the apical end of the plates (Figs. 2, A–C, H, I; 3C; and 4, A and B). The intercalary band is transversely and also faint horizontally striated (Fig. 2, B, D–F). The right thecal plate is concave, and the left thecal plate is convex (Fig. 2, B and D). The apical margin of the left thecal plate forms a raised ridge (Figs. 2, A–C, G–I; and 3, A and C). The periflagellar area consists of seven to nine platelets (Fig. 3, A–D; the platelet naming follows Taylor 1980 and Fensome et al. 1993). Two platelets tend to divide in some specimens (compare platelets “e” and “g” in Fig. 3, B–D), making the

number of platelets in the periflagellar area a variable feature. Five or six of these platelets form characteristic curved projections (Figs. 2H and 3, A–D). The collar-like projection of platelet “a” is the largest (Fig. 3, B and D). Platelets “b,” “c,” “e,” and “h” have projections as well, which are different in size and also vary in length from cell to cell (Fig. 3, B–D). When visible under the light microscope, these projections appear as spines (see above). Only one large pore was observed in the periflagellar area (Fig. 3, C and D). It is not clear whether an additional pore (e.g., flagellar pore or accessory pore) was hidden behind the projections.

Organelles: plastids, mitochondria, and nucleus. This species has all of the ultrastructural features that are commonly found in prorocentroids. The cells are covered with thick plates (Fig. 5, A–C), surrounded by the plasma membrane (Figs. 5B and 6A). The flagellar (or accessory?) pore is surrounded by thecal

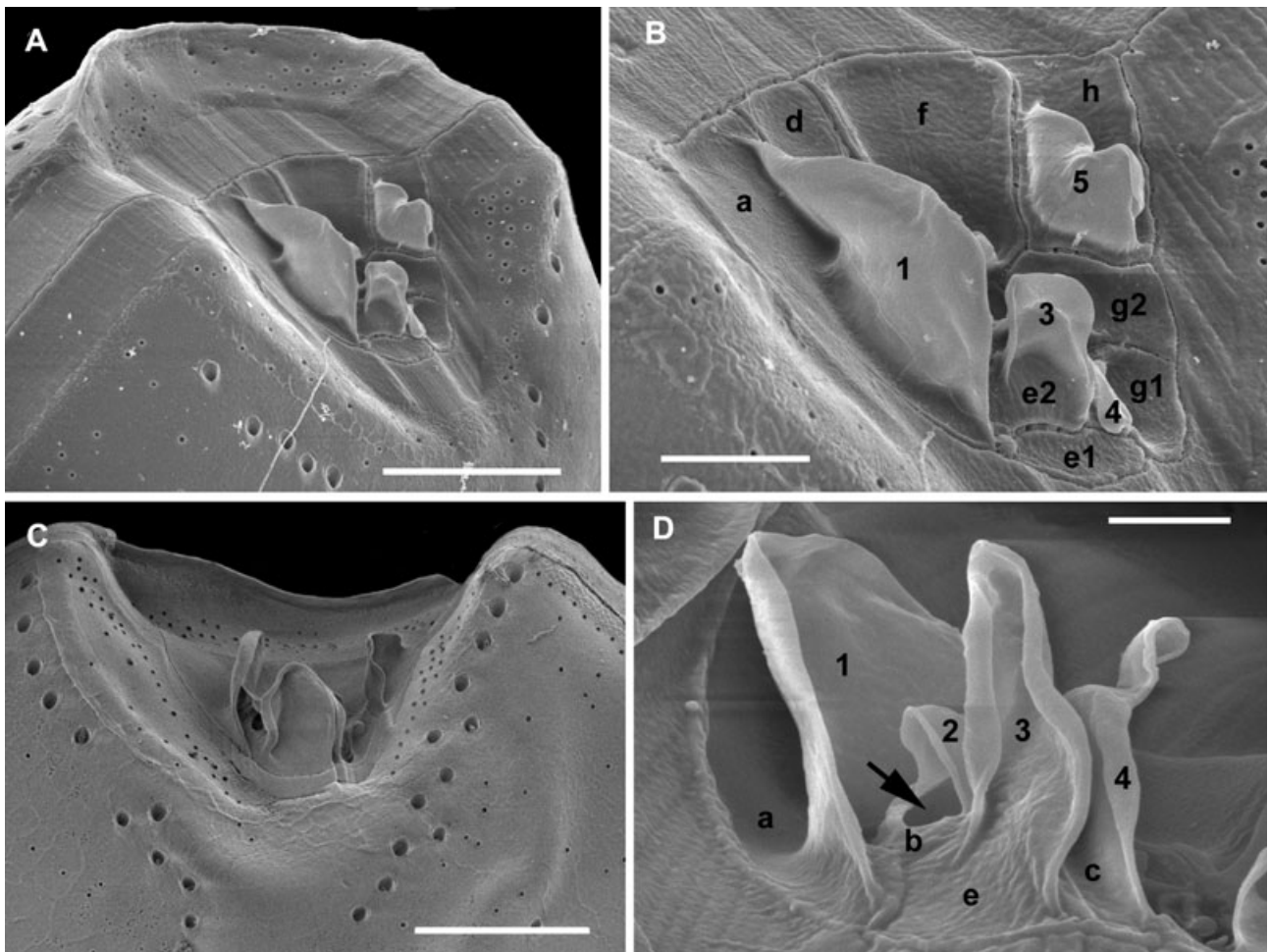


FIG. 3. Scanning electron micrographs of *Prorocentrum tsawwassenense* sp. nov. showing the periflagellar area. (A) Oblique view showing the projections (syn. spines) on the periflagellar platelets that are embedded within the excavation. The periflagellar area is bordered by a ridge on the left thecal plate. (B) High magnification view of the labeled platelets (the platelet naming follows Taylor 1980 and Fensome et al. 1993) and numbered projections shown in (A). (C) Right valve view showing the pattern of pores and the apical projections. (D) High magnification view of the center of the periflagellar area with relatively large projections (labeled with numbers) and a visible flagellar pore (arrow). Scale bars: 5 μm in (A, C), 2 μm in (B), and 1 μm in (D).

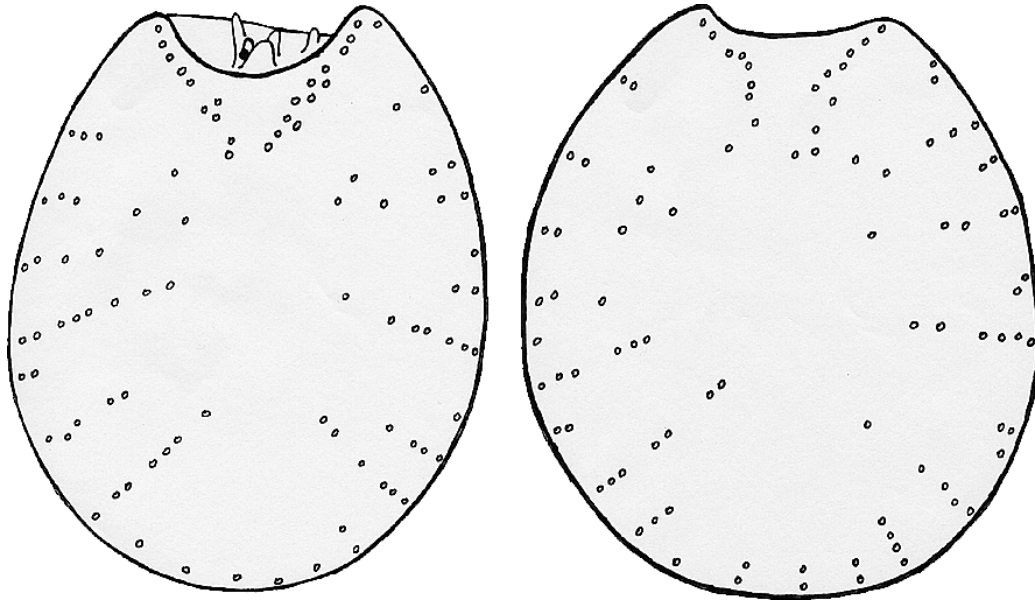


FIG. 4. Line drawings of *Prorocentrum tsawwassenense* sp. nov. showing the pattern of large pores. (A) Right thecal plate. (B) Left thecal plate.

platelets and hidden behind a platelet protrusion (Fig. 6A). The conspicuous nucleus contains distinctly banded condensed chromosomes and a nucleolus (Figs. 5, A and C, and 6B) and occupies a large portion of the posterior half of the cell. Chloroplast(s) are located directly beneath the thecal plates at the cell periphery (Figs. 5, A–C, and 6C). Our data suggest that only two multilobed (reticular) chloroplasts are present—like those described for other *Prorocentrum* species—however, this was not definitively demonstrated by serial sectioning. The ultrastructure of the chloroplasts is consistent with other typical peridinin-containing dinoflagellates: the envelope consists of three membranes, and the thylakoids are organized in stacks of three (Fig. 6C). However, a compound intrachloroplast pyrenoid is located near the transverse midline of the cell (Figs. 5A and 6D). Stacks of two thylakoids traverse the pyrenoid matrix and are more widely spaced to one another than the stacks of three thylakoids positioned outside of the pyrenoid (Fig. 6, D and E). Starch granules and lipid droplets are distributed in the cytoplasm (Fig. 5, A and B). The mitochondria have tubular cristae (Fig. 6F).

Extrusomes. Three types of extrusomes are present: normal dinoflagellate trichocysts with a square shape in transverse section and two types of mucocysts (Fig. 7, A–F). The first type of mucocyst accumulates beneath the apical periflagellar area (Fig. 5A). These mucocysts are vermiform and contain diffuse fibrous material, which is more densely packed in the mucocyst core (Fig. 7, C and D). Although it was difficult to discern, these mucocysts

appear to be surrounded by a single membrane (Fig. 7D). The second type of mucocyst is always positioned beneath the large thecal pores (Fig. 7, E and F). These mucocysts are single membrane-bound, flask-shaped vesicles with a densely stained plug at the base of a pore (Fig. 7E). A spherical vesicle resides above the plug and within the pore (Fig. 7, E and F).

Occurrence. This species was recorded in samples collected in September and October of 2004; in March, April, May, June, July, August, September, and October of 2005; and in January, February, and May of 2006. Although sampling was rarely carried out over the winter months, the species appears to be present throughout the year. Highest population abundances were reached in summer and early autumn. The species co-occurred in some samples with two other sand-dwelling *Prorocentrum* species, *P. fukuyoi* (Leander and Hoppenrath 2008, Murray et al. 2007; a species also registered in the Northfrisian Wadden Sea, Germany, and named “*Prorocentrum* spec. 1” in Hoppenrath 2000b) and the so far undescribed species “*Prorocentrum* spec. 2” (Hoppenrath 2000b).

Phylogenetic relationships. Our molecular phylogenetic analyses of SSU rDNA were consistent with previous studies and indicated that *Prorocentrum* species are members of two different clades: “*Prorocentrum* clade 1” and “*Prorocentrum* clade 2.” These two clades formed part of a weakly supported monophyletic group using Bayesian analysis (Fig. 8). However, in ML analyses, the two clades branched separately from a poorly resolved topological backbone. Nonetheless, Bayesian posterior probabilities for each of

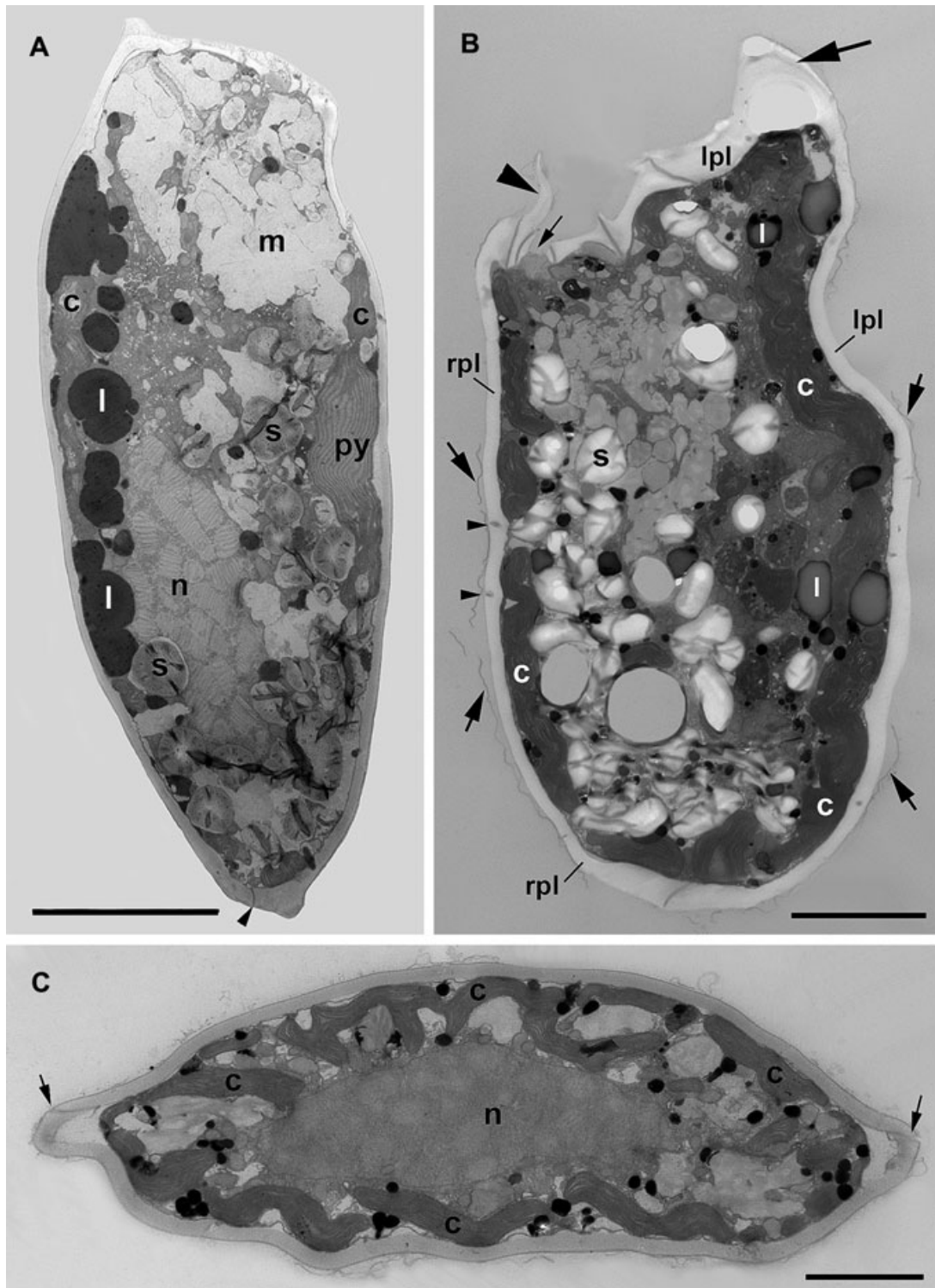


FIG. 5. Transmission electron micrographs of *Prorocentrum tsawwassenense* sp. nov. showing longitudinal and transverse sections through three different cells. (A) Longitudinal micrograph showing the suture between the two large thecal plates (arrowhead), mucocysts (m) packed beneath the periflagellar area, chloroplasts (c) at the cell margin, a centrally located pyrenoid (py), the large nucleus (n) in the posterior half of the cell, starch grains (s), and lipid globules (l). Scale bar, 10 μm . (B) Longitudinal micrograph showing the right theca plate (rpl) and the left theca plate (lpl) with a ridge (large arrow) bordering the periflagellar area. Thecal pores (small arrowheads) and the plasma membrane surrounding the cell are visible in some places (medium arrows). The flagellar pore (small arrow) is visible behind a protrusion of a platelet (large arrowhead). Chloroplasts (c), starch grains (s), and lipid droplets (l) are also shown. Scale bar, 5 μm . (C) Transverse micrograph through the posterior half of the cell containing the nucleus (n). The chloroplasts (c) and the sutures between the two large thecal plates (arrows) are visible at the cell margin. Scale bar, 4 μm .

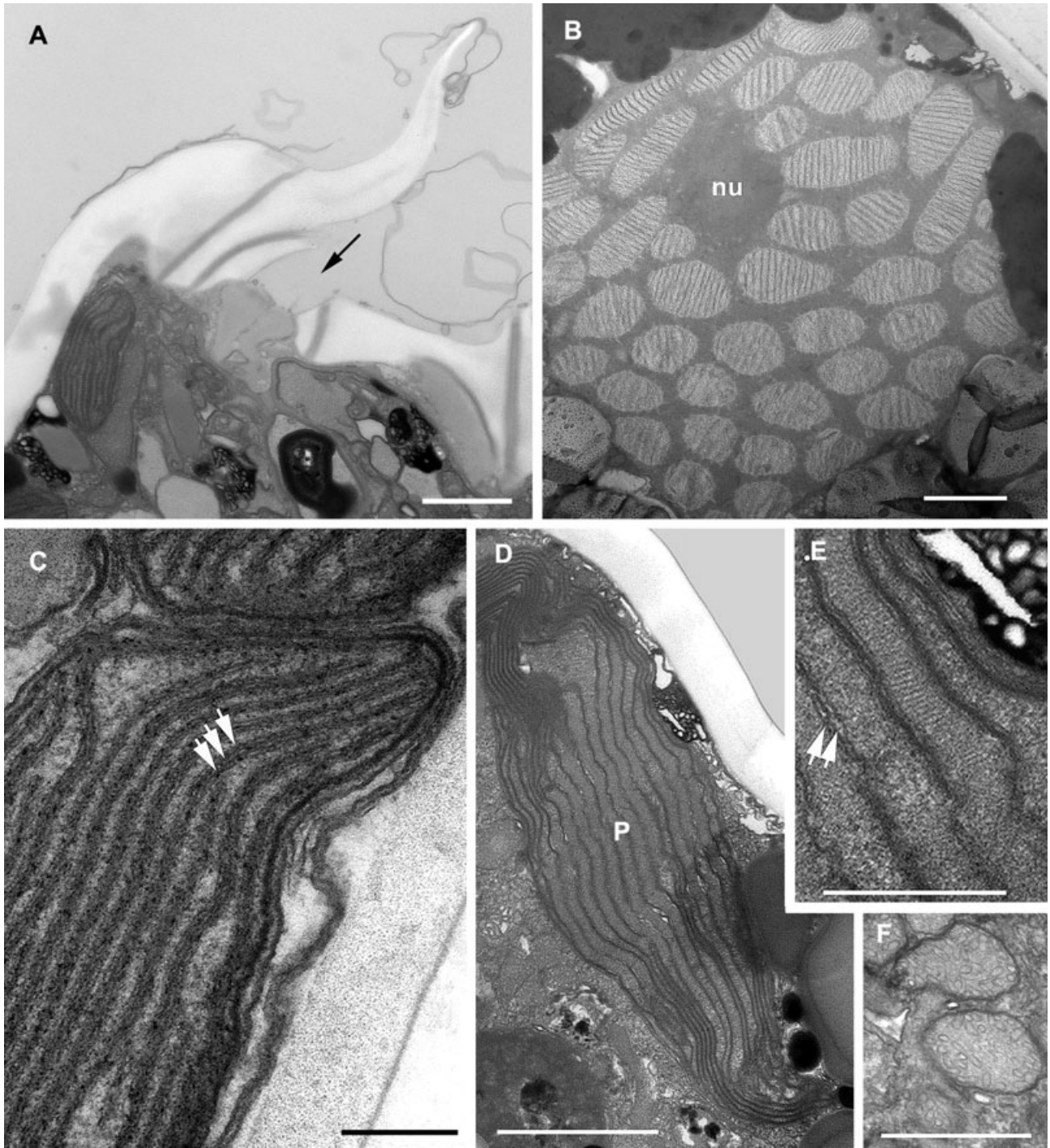


FIG. 6. Transmission electron micrographs of *Prorocentrum tsawwassenense* sp. nov. showing ultrastructural details. (A) The flagellar pore (arrow) is positioned between two platelets beneath a large projection. Scale bar, 1 μ m. (B) The nucleus contains condensed chromosomes and a nucleolus (nu). Scale bar, 2 μ m. (C) High magnification view of the chloroplast showing thylakoids in stacks of three (arrows). Scale bar, 0.25 μ m. (D) High magnification view of the chloroplast showing a compound, interchloroplast pyrenoid (P) without a starch sheath. Note the nearly parallel thylakoids running through the pyrenoid. Scale bar, 2 μ m. (E) High magnification view of the crystalline pyrenoid matrix showing the parallel thylakoids in stacks of two (arrows). Scale bar, 0.5 μ m. (F) Mitochondria with tubular cristae. Scale bar, 1 μ m.

the two *Prorocentrum* clades were very high (1.0); bootstrap support values using ML were also relative high for *Prorocentrum* clade 2 (83) and *Prorocentrum* clade 1,

excluding *P. panamensis* (93) (Fig. 8). *Prorocentrum* clade 2 included the benthic species *P. concavum*, *P. lima*, *P. arenarium*, and *P. maculosum*; *Prorocentrum*

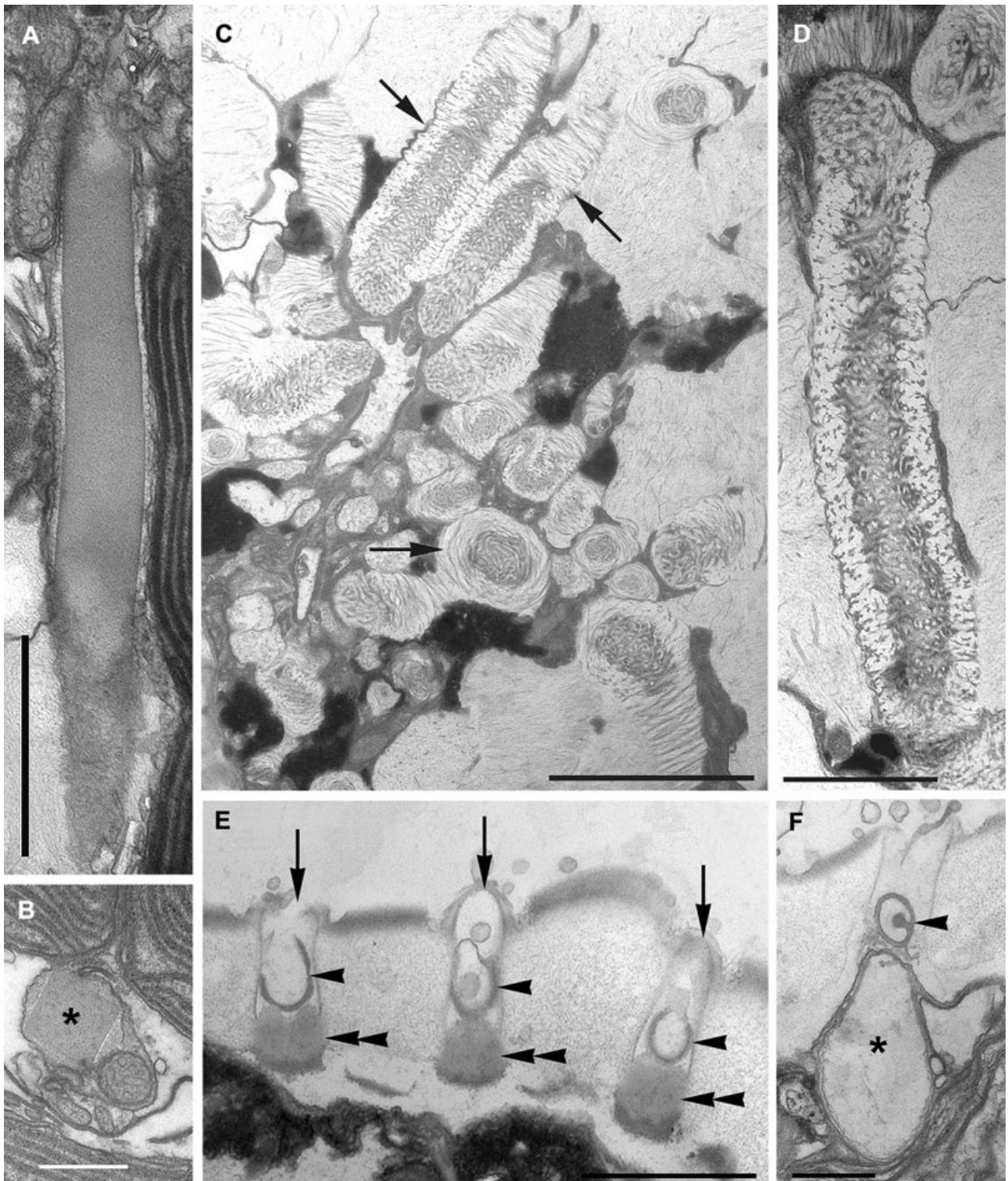


FIG. 7. Transmission electron micrographs of *Prorocentrum tsawwassenense* sp. nov. showing the different extrusome types. (A) Spindle-shaped trichocyst in longitudinal section. Scale bar, 1 μ m. (B) Spindle-shaped trichocyst in transverse section showing the square-shaped profile (asterisk). Scale bar, 0.5 μ m. (C) Type 1 mucocysts (arrows) near the apical cell region, shown in both longitudinal and transverse section. Scale bar, 2.5 μ m. (D) Longitudinal micrograph through a type 1 mucocyst. Scale bar, 1 μ m. (E) Micrograph showing large pores (arrows) in the thecal plates, each subtended by a spherical vesicle (arrowhead) and densely stained plug (double arrowheads). Scale bar, 1 μ m. (F) Micrograph of a type 2 mucocyst showing a spherical vesicle (arrowhead) and a flask-shaped vesicle (asterisk) that subtends a large thecal pore. Scale bar, 0.5 μ m.

clade 1 included benthic and planktonic species, namely, *P. mexicanum*, *P. micans*, *P. gracile*, *P. triestinum*, *P. minimum*, *P. donghaiense*, *P. dentatum*, *P. emarginatum*, and *P. panamensis* (Fig. 8). The new species *P. tsawwassenense* branched with high support within *Prorocentrum* clade 1. The earliest diverging

lineage within *Prorocentrum* clade 1 was *P. panamensis*, followed by *P. tsawwassenense* and *P. emarginatum*. The benthic species *Adenoides eludens* branched with weak support as the nearest sister lineage to *Prorocentrum* clade 1 (Fig. 8). The nearest sister lineages to *Prorocentrum* clade 2 were *Togula britannica* and *Peridinium*

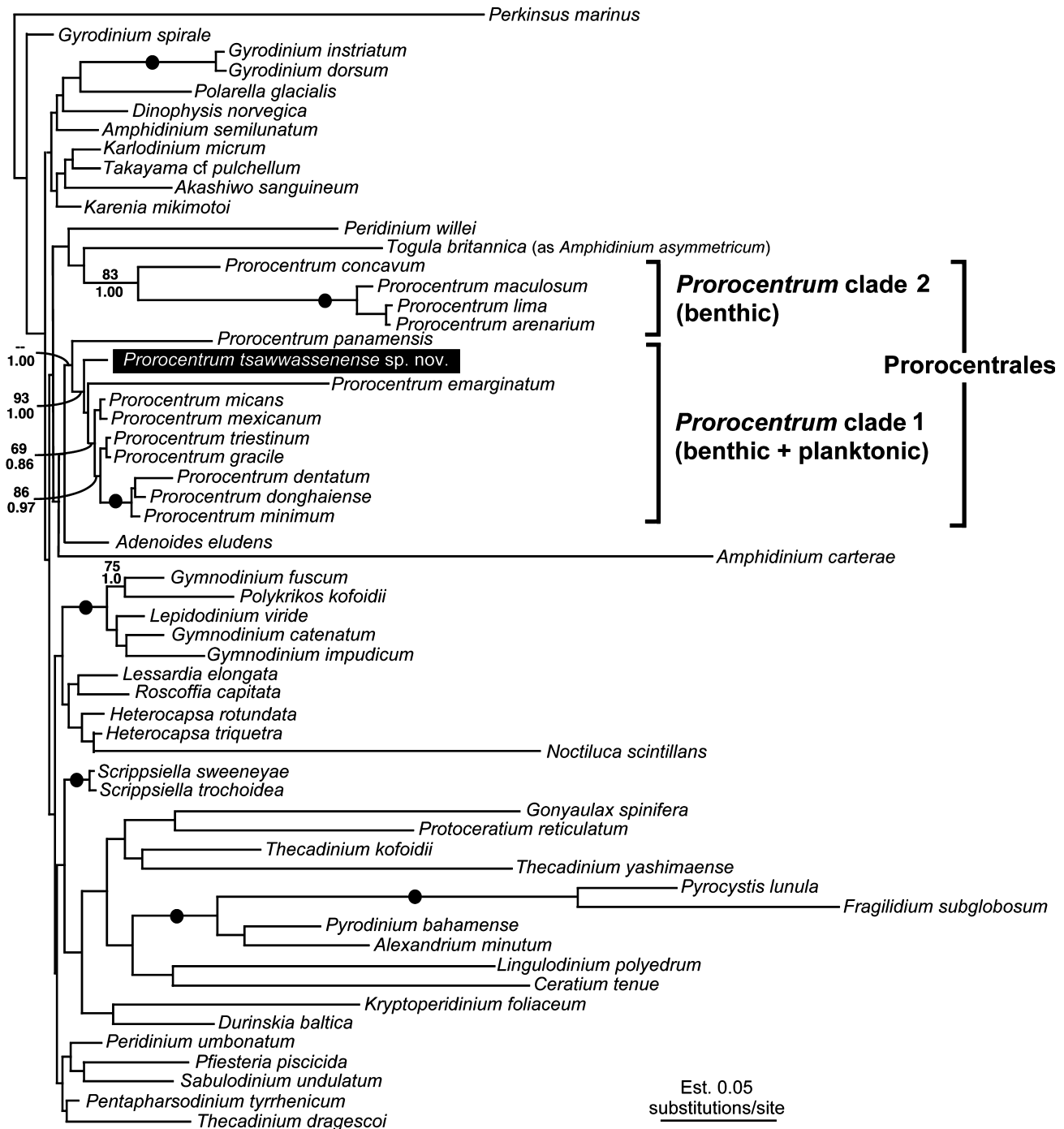


Fig. 8. Phylogenetic tree obtained by Bayesian analysis (model GTR + G + I) on alignment of 58 SSU rDNA sequences and 1,623 unambiguously aligned sites. Numbers at the branches denote bootstrap percentages using maximum likelihood—GTR (top) and Bayesian posterior probabilities (bottom). Black dots on branches denote robust bootstrap percentages and posterior probabilities of 95% or higher. The sequence derived from this study is highlighted in bold. GTR, general time reversible.

willei, but these relationships received very low statistical support values (Fig. 8).

DISCUSSION

Comparative morphology. The classification of *Prorocentrum* species is based on cell shape and size, thecal plate surface morphology, intercalary band morphology, and architectural details of the periflagellar area. Only six benthic *Prorocentrum* species have morphological theca characters in common with *P. tsawwassenense* sp. nov.—namely, *P. clipeus*, *P. caribbaeum*, *P. emarginatum*, *P. fukuyoi*, *P. rhathymum*, and *P. formosum* (Loeblich et al. 1979, Fukuyo 1981, Faust 1990, 1993a,b, Hoppenrath 2000a, Cortés-Altamirano and Sierra-Beltrán 2003, Murray et al. 2007; Table 1). The distinguishing features of *P. tsawwassenense* sp. nov. are (i) the wide U-shaped (syn. arc-shaped) excavation of the periflagellar area in the right thecal plate, (ii) the raised ridge on the left thecal plate near the periflagellar area, (iii) a prominent collar-shaped spine on plate “a,” and (iv) a thecal pore pattern with evenly spaced radial rows of large pores. Moreover, *P. tsawwassenense* sp. nov. is novel in having at least five projections (smaller spines or protrusions) in the periflagellar area and two apical rows of pores on both thecal plates.

None of the other described species of *Prorocentrum* show all of the characteristics listed above (Table 1). Although *P. clipeus* also has a wide U-shaped periflagellar area bordered by a ridge (syn. collar) on the left thecal plate, this species lacks a prominent collar-shaped spine, large pores, and apical rows of pores. Moreover, *P. clipeus* only has one projection in the periflagellar area (Hoppenrath 2000a; Table 1). Like *P. tsawwassenense* sp. nov., *P. caribbaeum*, *P. emarginatum*, and *P. rhathymum* have a prominent collar-shaped apical spine and radial rows of pores on the thecal plates. However, these species possess a V-shaped excavation in the periflagellar area on the right thecal plate rather than a U-shaped excavation and possess only one projection in the periflagellar area rather than several. Also unlike *P. tsawwassenense* sp. nov., these species lack an apical ridge on the left thecal plate (Faust 1990, 1993b, Fukuyo 1981; Table 1). Moreover, *P. caribbaeum* and *P. emarginatum* lack apical rows of pores like those present in *P. tsawwassenense* sp. nov. and *P. rhathymum*. *P. formosum* has a prominent collar-shaped apical spine, a V-shaped periflagellar area on the right thecal plate, no apical ridge on the left thecal plate, only two projections in the periflagellar area, no radial rows of pores, and one apical row of pores on the right valve and probably two apical rows of pores on the left valve (Faust 1993a; Table 1). *P. formosum* is perhaps most distinctive in having the nucleus positioned in the anterior half of the cell (Faust 1993a), a feature shared only with two other benthic *Prorocentrum* species: *P. elegans* and *P. sabulosum* (Faust 1993b, 1994; Table 1). Additionally, species of *Prorocentrum* show differences in

the number of platelets in the periflagellar area, but these details are not known for all species described so far (Tables 1). The marine planktonic species *P. micans* and *P. tsawwassenense* sp. nov. have similar radial rows of large pores. But *P. micans* has only one apical row of pores on the right valve, and it differs in having a large winged apical spine and only one projection in the V-shaped periflagellar area.

There are several more benthic species of *Prorocentrum*, all of which differ from *P. tsawwassenense* sp. nov. in the shape of the periflagellar area, in the absence of radial rows of pores, and in having less than two projections in the periflagellar area. Moreover, each of these species differs in specific combinations of several other morphological characters, such overall cell shape and size, thecal plate ornamentation, intercalary band morphology, and the presence or absence of a pyrenoid in the plastids. The two known freshwater planktonic species of *Prorocentrum*, namely, *P. foveolata* and *P. playfairi*, are most similar to several benthic species (Croome and Tyler 1987), but not to *P. tsawwassenense* sp. nov.

Significant ultrastructural differences between species of *Prorocentrum* include the presence and organization of a pyrenoid in the plastids and the presence or absence of trichocysts and mucocysts (vesicles containing diffuse fibrous material). A compound, intrachloroplast pyrenoid was observed in *P. tsawwassenense* sp. nov. in the central area of the cell. This type of pyrenoid is not visible under the light microscope and is very similar to the one described in *P. micans* (Kowallik 1969). These cryptic pyrenoids can either be conspicuously well organized or rather inconspicuous when viewed with TEM (Dodge and Bibby 1973). In contrast, the presence of pyrenoids in many *Prorocentrum* species is easily detectable using the light microscope, because a conspicuous starch sheath surrounds these pyrenoids. This obvious ring-structure is characteristic of, for example, *P. lima*, *P. hoffmannianum*, and *P. ruetzlerianum* (e.g., Faust et al. 1999). Schnepf and Elbrächter (1999) pointed out that pyrenoids might be useful taxonomic characters. Current data show that 14 *Prorocentrum* species either have no pyrenoid or have a pyrenoid without a starch sheath, and 19 species have a stalked pyrenoid with a starch sheath.

P. tsawwassenense sp. nov. contains trichocysts and two types of mucocysts. Trichocysts have also been observed in some benthic *Prorocentrum* species, but in most cases, these observations were based on SEM data rather than TEM data. Published TEM data for *Prorocentrum* species are rare, and some of these data focus exclusively on the flagellar apparatus (Bouck and Sweeney 1966, Kowallik 1969, Dodge and Crawford 1971, Dodge and Bibby 1973, Honsell and Talarico 1985, Zhou and Fritz 1993, Heimann et al. 1995, Roberts et al. 1995, Schnepf and Elbrächter 1999). Only three benthic species, namely, *P. lima*, *P. maculosum* Faust, and *P. tsawwassenense* sp. nov., have been investigated more

TABLE 1. Comparison of morphological features of benthic *Proocentrum* species with similarities to *P. tsaruassenense* sp. nov.

	<i>P. tsaruassenense</i>	<i>P. clipeus</i>	<i>P. caribbaeum</i>	<i>P. emarginatum</i>	<i>P. rhathymum</i>
Cell shape	Oval	Round	Heart-shaped	Oval	Oval
Cell size					
Length [μm]	40-55	54-55 (37-44)	40-45	35-40	30-38
Width [μm]	30-47.5	50-52.5	30-35	30	20-25
Theca	Smooth	Smooth	Smooth, minute depressions	Smooth	Smooth (radial furrows)
Pyrenoid ornamentation	Yes (TEM)	Probably yes (LM)	No (LM)	?	No (LM)
Nucleus	Round to oval posterior	Kidney-shaped posterior	Kidney-shaped posterior	Kidney-shaped posterior	Round posterior
Periflagellar area					
Shape (rv view)	Wide U-shaped	Wide U-shaped	Wide V-shaped	Narrow V-shaped	Wide V-shaped
Ridge on left valve	Yes	Yes	No	No	No
Collar-shaped spine	Yes	No	Yes	Yes	Yes
Protrusions					
Only one	5	Yes	Yes	No	Yes
More than one	7-9	10	?	No	8
No. platelets	Radial rows	None	Radial rows	Radial rows	Radial rows
Porc pattern	Two apical rows (rv)	(radial rows)*	One apical row (rv)?	?	One apical row (rv)
	Yes	No	No apical row (lv)?	Yes	No apical row (lv)
Marginal pores	Devoid	Devoid?	Yes?	Devoid	Yes
Valve center	0.3-0.5	None	0.3	0.2	Devoid?
Large pores [μm]	0.09-0.17	0.12	0.1	0.1	Yes
Small pores [μm]	Trans. & horiz. str.	Smooth	Trans. striated	Trans. striated	Trans. striated
Intercalary band	Yes (TEM)	?	Yes (SEM)	?	Yes (SEM)
Trichocysts	Yes (TEM)	?	?	?	?
Mucocysts					
	<i>P. formosum</i>	<i>P. elegans</i>	<i>P. arenarium</i>	<i>P. lima</i>	<i>P. jukuyoi</i>
	<i>P. formosum</i>	<i>P. elegans</i>	<i>P. arenarium</i>	<i>P. lima</i>	<i>P. cassubicum</i>
Cell shape	Oval	Oval	Round to slight oval	Ovoid	Oval to oblong
Cell size					
Length [μm]	25-28	15-20	30-32	31-47	22-25
Width [μm]	15-16	10-14	30-32	22-40	18-30
Theca	Smooth	Smooth	Smooth	Smooth	Smooth
Pyrenoid ornamentation	No	No (in LM)	?	Yes, starch sh. (LM)	No (LM)
Nucleus	(LM) anterior	? Anterior	? Posterior	Round posterior	Round to kidney-sh. posterior
Periflagellar area					
Shape (rv view)	Wide V-shaped	Wide V-shaped	Wide V-shaped	Wide V-shaped	Narrow V-shaped
Ridge on left valve	No	No	No	No	No
Collar-shaped spine	Yes	No	No	No	Yes
Protrusions					
Only one	2?, narrow	Ridge?	No	Yes	?
More than one	5-6	No	No	8	?
No. platelets		7	?	8	9

TABLE 1. Continued

	<i>P. formosum</i>	<i>P. elegans</i>	<i>P. arenarium</i>	<i>P. lima</i>	<i>P. fukuyoi</i>	<i>P. cassubicum</i>
Pore pattern	Uneven, one apical row (rv)? Two apical rows (lv)?	Yes, one apical row (rv)? One apical row (lv)?	No, random poroids	No, random pores	No; pore rows?	No
Marginal pores	Yes	Yes?	Yes	Yes	?	?
Valve center	Devoid	Devoid	Devoid	Devoid	Small pores	?
Large pores [µm]	0.2	0.12	?	?	0.3	No
Small pores [µm]	<0.1	0.06?	No?	No	0.1	Yes?
Intercalary band	Trans. striated	Trans. striated	No striation, smooth	Smooth	Horiz. striated	?
Trichocysts	?	?	?	No (TEM)	?	No (TEM)
Mucocysts	?	?	?	Yes (TEM)	?	?

(...)* = data from Murray 2003; ? = no data available; ...? = not mentioned in the text, inferred from images; rv = right valve; lv = left valve.

comprehensively with TEM (Zhou and Fritz 1993, this study). *Prorocentrum lima*, *P. maculosum*, and *P. cassubicum* (Woloszynska) Dodge (as *Exuviaella cassubica* Woloszynska) have been shown (or inferred) to lack trichocysts (Dodge and Bibby 1973, Zhou and Fritz 1993). Taken altogether, it is not clear whether trichocysts are a general feature of *Prorocentrum* species or a potential species-specific character.

The same ambiguity applies to the presence or absence of mucocysts in *Prorocentrum* species. In nearly all *Prorocentrum* species investigated so far, a large number of “vesicles containing diffuse fibrous material” (syn.: mucus vesicles) are present in the apical region of the cell, beneath the periflagellar area (Dodge and Bibby 1973, M. Schweikert pers. comm.). We refer to these vesicles as “type 1 mucocysts.” Type 1 mucocysts have been described in several other dinoflagellate genera as well: *Gymnodinium fuscum* (Hansen et al. 2000), *Gyrodinium spirale* (Hansen and Daugbjerg 2004), and *Polykrikos lebourae* (Hoppenrath and Leander 2007b). The second type of mucocysts present in *P. tsawwassenense* sp. nov., namely, “type 2 mucocysts,” have also been described in *P. lima* and *P. maculosum* (Zhou and Fritz 1993). Type 2 mucocysts are flask-shaped, single membrane-bounded vesicles with a narrow neck and a paracrystalline plug and are positioned immediately beneath larger thecal pores (Fig. 7, E and F).

The presence of trichocysts and mucocysts (type 2) as ejectisomes—associated with thecal pores—in one species/cell is significant and has not been demonstrated before for any other *Prorocentrum* species. McLachlan et al. (1997) used two morphological characters to reinstate the genus *Exuviaella*, the exclusive presence of mucocysts as extrusomes and the absence of valve spines and of a large apical spine or tooth. The genus *Prorocentrum* in turn should be characterized by possessing only trichocysts as extrusomes and by having an apical spine or tooth (McLachlan et al. 1997). Our findings clearly demonstrate that the presence or absence of trichocysts and mucocysts are features suitable only for species characterization and not useful for higher-level delineations. The type species *P. micans* possesses trichocysts and type 1 mucocysts (Dodge and Bibby 1973), as do most of the so far ultrastructurally investigated *Prorocentrum* species. That the presence or absence of an apical spine is not a sufficient distinguishing feature for *Prorocentrum* and *Exuviaella* has been discussed earlier (Abé 1967, Dodge 1975). The separation made by McLachlan et al. (1997) is not verified by morphological characters anymore. Murray et al. (2007) demonstrated that above the level of species, habitat may be a poor character for differentiating groups or defining clades of *Prorocentrum*. So the last feature defining the genus *Exuviaella* sensu McLachlan et al. (1997) is the production of diarrhetic shellfish

poisoning (DSP) toxins. However, toxic and non-toxic strains of the same species have been detected, and this feature cannot be used to separate the taxa appearing in different clades inferred from phylogenetic analyses of SSU rDNA sequences.

Occurrence. *P. tsawwassenense* sp. nov. has not been registered in any previous studies on the biodiversity of marine benthic dinoflagellates. For instance, an intensive taxonomic study of the sand-dwelling dinoflagellates in the northeastern Pacific Ocean (Baillie 1971), which is the type locality of this new species, only recognized *Exuviaella marina*. However, it is highly likely that *P. tsawwassenense* sp. nov. was also present at the time of this study, but was not distinguished from *E. marina*. At low magnifications under the light microscope, both species look very similar. The diagnostic features that distinguish these two species become obvious only through routine observations of the periflagellar area (shape and "spines"). Usually, *P. fukuyoi* is slightly smaller and slightly darker in color.

Phylogenetic relationships. *P. tsawwassenense* sp. nov. branched early within *Prorocentrum* clade 1 with high statistical support (Fig. 8). Among the most closely related species to *P. tsawwassenense* sp. nov. in the Bayesian analyses were *P. emarginatum*, *P. mexicanum* (misidentified *P. rhathymum?*), and *P. micans* (Fig. 8). These three species are also the most similar to *P. tsawwassenense* sp. nov. from a morphological perspective (Table 1). All four species have radial rows of pores on their thecal plates and a prominent, collar-shaped spine in the periflagellar area; however, *P. micans* has further modified this spine into a larger winged structure. Three other species listed in Table 1, namely, *P. clipeus*, *P. caribbaeum*, and *P. formosum*, share several features with *P. tsawwassenense* sp. nov. as well, but SSU rDNA sequences have yet to be generated from these taxa. *P. tsawwassenense* looks slightly asymmetrical, because the convexity of the cell edges is different, and the periflagellar area is not fitting into an isosceles triangle (according to Grzebyk et al. 1998). This seems to be consistent with the phylogenetic analysis, in which *P. tsawwassenense* clusters with other asymmetrical species.

Our analyses confirmed earlier reports that *Prorocentrum* species branch within the GPP complex and form two distinct clades: a clade combining both benthic and planktonic species (*Prorocentrum* clade 1) and a benthic clade (*Prorocentrum* clade 2; Grzebyk et al. 1998, Litaker et al. 1999, Saldarriaga et al. 2004, Murray et al. 2005; Fig. 8). These two clades were well supported in our analyses, and the genus as a whole had a common origin in the Bayesian analysis, albeit with weak statistical support (Fig. 8). A weakly supported sister relationship between the two *Prorocentrum* clades has also been recovered in previous phylogenetic analyses of LSU rDNA sequences (Saldarriaga et al. 2004). Nonetheless, we attribute the tenuous relationship between

Prorocentrum clade 1 and *Prorocentrum* clade 2 in both Bayesian and ML analyses of dinoflagellate SSU and LSU rDNA sequences to a poor phylogenetic signal at deep nodes and associated methodological artifacts (Zardoya et al. 1995, Pearce and Hallegraeff 2004, Saldarriaga et al. 2004, Murray et al. 2005). Taxon sampling within the genus *Prorocentrum*, and within the dinoflagellates in general, is likely an additional factor influencing this poor phylogenetic resolution.

From a comparative morphological perspective, however, members of the order Prorocentrales form a robust monophyletic group that includes both benthic and planktonic species (e.g., Taylor 1980, 2004, Fensome et al. 1993, Saldarriaga et al. 2004). Although molecular phylogenetic data neither support nor refute the monophyly of the order, these data do help provide significant insights into the evolutionary history within *Prorocentrum* clades 1 and 2. For instance, the molecular phylogenetic data suggest that the planktonic species within *Prorocentrum* clade 1 (e.g., *P. micans*) are nested within a stem group of benthic species. In other words, molecular phylogenetic data indicate that planktonic *Prorocentrum* species are evolutionarily derived from benthic prorocentroid ancestors. However, the number of independent transitions from a benthic to a planktonic mode of life (or vice versa) remains obscure, and at present, molecular phylogenetic data using ribosomal gene sequences cannot adequately address hypotheses of morphological character evolution within the group. Answers to these questions will require much improved knowledge of *Prorocentrum* diversity and the exploration of several different molecular phylogenetic markers, such as nucleus encoded protein genes.

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Supplementary Material

The following supplementary material is available for this article:

Appendix S1. GenBank accession numbers.

This material is available as part of the online article from: <http://www.blackwell-synergy.com/doi/abs/10.1111/j.1529-8817.2008.00483.x>.

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