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Molecular phylogeny of the spiny-surfaced species of the dinoflagellate *Prorocentrum* with the description of *P. Thermophilum* sp. nov. and *P. criophilum* sp. nov. (Prorocentrales, Dinophyceae)[1]

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ABSTRACT

Spiny-surfaced species of *Prorocentrum* forms harmful algal blooms, and its taxonomic identity is obscure due to the size and shape variability. Molecular phylogenies reveal two major clades: one for *P. cordatum* with sequences mainly retrieved as *P. minimum*, and other for *P. shikokuense* with sequences also retrieved as *P. dentatum* and *P. donghaiense*. Several closely related clades still need to be characterized. Here, we provide SSU- and LSU rRNA, and ITS gene sequences of the strain CCMP3122 isolated from Florida (initially named *P. donghaiense*) and strains *Prorocentrum* sp. RCC6871–2 from the Ross Sea, Antarctica. We describe *Prorocentrum thermophilum* sp. nov. based on the strain CCMP3122, a species also distributed in the open waters of Gulf of Mexico, New Zealand and the Arabian Gulf; and *Prorocentrum criophilum* sp. nov. based on the strain RCC6872, which is distributed in the Antarctic Ocean and the Arctic Sea. *Prorocentrum thermophilum* is roundish (~14 μm long, ~12 μm wide), with an inconspicuous anterior spine-like prolongation under light microscopy, valves with tiny, short knobs (5–7 per μm^2), and several (<7) large trichocyst pores (~0.3 μm) in the right valve, as well as smaller pores (~0.15 μm). *Prorocentrum criophilum* is round in valve view (~11 μm long, 10 μm wide) and asymmetrically roundish in lateral view, the periflagellar area was not discernible under light microscopy, valves with very tiny, short knobs (6–10 per μm^2), and at least twelve large pores in the right valve. Other potentially undescribed species of spiny-surfaced *Prorocentrum* are discussed

Key index words: Dinophyta; HABs; harmful algae blooms; molecular phylogenetics; new species; Prorocentraceae; taxonomy

Abbreviations: BP, bootstrap probability value; CCMP, The Provasoli-Guillard National Center for Culture of Marine Phytoplankton; ML, Maximum Likelihood; NCBI, National Center for Biotechnology Information; NCMA, National Center for Marine Algae and Microbiota; RCC, Roscoff Culture Collection.

INTRODUCTION

Prorocentrum is a common dinoflagellate genus distributed worldwide. It contains benthic species able to produce toxins, and some planktonic species that are responsible of massive blooms in estuarine and neritic waters. Two of these potentially harmful planktonic species, *Prorocentrum cordatum* and *P. shikokuense*, have received considerable attention (Faust et al. 1999, Hajdu et al. 2000, Lu and Goebel 2001, Pertola et al. 2003, Hajdu et al. 2005, Heil et al. 2005, Lu et al. 2005, Takano and Matsuoka 2011, Roselli et al. 2019, Shin et al. 2019, Gómez et al. 2022).

The earlier electron microscopic observations showed the valves of *Prorocentrum cordatum* (as *P. balticum*) covered by tiny spines (Braarud et al. 1958). Dodge (1975) placed the species *P. cordatum*, *P. minimum* and *P. balticum* as member of the spiny-surfaced group of *Prorocentrum*. The taxonomical story is complex implying potential synonymy of the species names *P. cordatum* (Ostenfeld 1901) J.D. Dodge 1975 described from the Caspian Sea, *P. balticum* (Lohmann 1908) A.R. Loeblich 1970 from the Baltic Sea, *P. minimum* (Pavillard 1916) J. Schiller 1931 from the Mediterranean Sea, *P. triangulatum* G.W. Martin 1929 from Atlantic coast of U.S.A., and *P. mariae-lebouriae* (Parke and Ballantine 1957) A.R. Loeblich 1970, *P. pomoideum* Bursa 1959, and *P. cordiforme* Bursa 1959 described from Plymouth, U.K. The considerable confusion that has existed is in large part due to its variability in size

(10–25 μm long), shape (rotund, oval, heart-shaped, triangular), and the presence of a small anterior spine which is not always recognizable under light microscopy (Hulburt 1965, Faust 1974, Hajdu et al. 2000, Pertola et al. 2003, Monti et al. 2010). Velikova and Larsen (1999) considered that *P. cordatum* and *P. minimum* were synonyms, while other authors considered them two distinct species (Pertola et al. 2003, Heil et al. 2005). Authors have regarded *P. minimum* and *P. balticum* as independent species (Steidinger and Tangen 1997, Faust et al. 1999, Heil et al. 2005).

Prorocentrum shikokuense has received much attention, and it is not free of considerable taxonomical debate. Dodge (1975) considered *Prorocentrum obtusidens* as a junior synonym of *P. dentatum* and placed it in the group of the spiny-surfaced *Prorocentrum* together with *P. cordatum*. However, as *P. dentatum* Dodge (1975) illustrated cells of a culture that differed in size and shape from the original descriptions of *P. dentatum* and *P. obtusidens*. That year, Hada (1975) described *P. shikokuense* from the coasts of Japan, with a size and shape similar to the cells illustrated as *P. dentatum* sensu Dodge (1975). Hada's description of *P. shikokuense* only based on line drawings was considered insufficient, and further records in Japan were identified as *P. dentatum* (Fukuyo et al. 1990). The progressive eutrophication of the estuary of the Yangtze River, China, resulted in massive blooms of a species that was named *P. donghaiense* Lu, described with more robust appearance than the original illustrations of *P. shikokuense* (Lu and Goebel 2001). Further morphological and molecular data confirmed *P. shikokuense* and *P. donghaiense* as synonyms (Takano and Matsuoka 2011). Shin et al. (2019) proposed that *P. shikokuense* (=*P. donghaiense*) is a junior synonym of *P. obtusidens*. However, without their own observations of *P. obtusidens*, these authors omitted the important differences in size and shape of *P. shikokuense* and *P. obtusidens*. Gómez et al. (2022) investigated *P. obtusidens* from the type locality,

Adriatic Sea, questioning the synonymy because the true *P. obtusidens* is distinct in shape and size from *P. shikokuense*. The DNA sequences of *P. shikokuense* clustered together with sequences previously retrieved as *P. dentatum* or *P. donghaiense*, evidencing a widespread single species (Gómez et al. 2022).

In addition to these two major clades of *Prorocentrum cordatum* and *P. shikokuense*, the molecular phylogenies showed other closely related clades. These included sequences identified as *P. balticum* from isolates of warm seas (strains CCMP1260 and CCMP1787, with GenBank accession numbers EU927547 and EU927548, respectively) as well as the strain *P.* cf. *balticum* UTSPH3D3 (MW024115) that diverged from the main clades of *P. cordatum* and *P. shikokuense* (Shin et al. 2019, Larsson et al. 2022), as well as the sequences of *P. donghaiense* strain CCMP3122, and the Antarctic isolates *Prorocentrum* sp. strain RCC6871–2 (MN824021–2) and *Prorocentrum* sp. (MT831988).

In this study, we obtained new molecular data on the SSU and LSU rRNA, and ITS gene sequences of two of these clades: one from warm waters based on the strain *Prorocentrum shikokuense* CCMP3122 that is revealed conspecific with the strains *P. balticum* CCMP1260 and CCMP1787, and other from Antarctic waters based on the strains *Prorocentrum* sp. RCC6871 and RCC6872, conspecific with *Prorocentrum* sp. (MT831988). We described two new species for these two clades, and re-visited the synonymy and geographical distribution of *P. cordatum* and *P. shikokuense* based on the existing molecular data, and other potentially undescribed species of spiny-surfaced *Prorocentrum*.

MATERIAL AND METHODS

Cultures and isolates.

The strain CCMP3122 was obtained as *Prorocentrum donghaiense* from the Provasoli-Guillard National Center for Marine Algae and Microbiota (NCMA), https://ncma.bigelow.org/CCMP3122 . The strain was isolated by J. Winshell at New Pass Bridge, Sarasota, Florida, USA (27.333° N, 82.583° W) in 2008. Cells were grown in filtered autoclaved seawater (salinity 31) amended with L1 medium nutrients. Illumination was provided by cool-white fluorescent light bulbs in a 12:12 h light:dark cycle with 100 µmol photons · m^{-2} · s^{-1}, and temperature set at 20ºC.

The strains RCC6871 and RCC6872 were retrieved as *Prorocentrum* sp. from the Roscoff Culture Collection, https://roscoff-culture-collection.org/rcc-strain-details/6872. Seawater was collected during the TAN1901 Antarctic voyage by A. Gutiérrez-Rodríguez from the sampling station at 75.13º S, 176.04º W, depth 80 m, in the Ross Sea in January 2019. Strains were isolated by pipetting by P. Gourvil. Other strain was isolated also in the Ross Sea in the austral summer of 2021 during the "Ross Sea Life in a Changing Climate (ReLICC) 2021 Voyage" (65.162º S, 176.339º E, depth 65 m). The cells were cultured in filtered autoclaved seawater (salinity 32) amended with f/2 medium nutrients. Irradiance at 80 µmol photons · m^{-2} · s^{-1} was provided by LED light in a 12:12 h light:dark cycle.

To compare with these strains, we included scanning electron micrographs of *Prorocentrum cordatum* and *P. shikokuense*. The strain PmK of *P. cordatum* was isolated from the Gulf of Trieste, and provided by the Collection of Sea Microoganisms, CoSMi, of the National Institute of Oceanography and Applied Geophysics, Trieste, Italy. The morphology, physiology and molecular characterization (MH976699) of the

strain are reported in Monti et al. (2010) and Monti-Birkenmeier et al. (2019). Wild cells of *Prorocentrum shikokuense* (MZ593907) were obtained from the Port of Brindisi in September 2018 as described in Gómez et al. (2022).

Holotype specimens are deposited at the Herbarium of the Faculty of Marine Sciences, Campus Universitario de Tafira, Las Palmas de Gran Canaria, Canary Islands 35017, Spain. BCM; herbarium acronyms follow Thiers (2022 continuously updated).

Light observations.

The cell sizes of the strain CCMP3122 were measured and photographed with a BX51 microscope (Olympus, Tokyo, Japan) equipped with an Olympus DP74 camera. In order to characterize the nucleus, cells were stained with DAPI (4′,6-diamidino-2-phenylindole dihydrochloride) (Sigma-Aldrich, St. Luis, MO, USA) and SYBR™ Green DNA stain (Thermo Fisher Scientific, Waltham, MA, USA) and observed with epifluorescence microscopy. Live cells of the strain RCC6872 were measured and photographed with a microscope BX51 equipped with an Infinity Lumenera 3 (Lumenera, Ottawa, Canada). Acid Lugol's solution preserved cells were also measured with an inverted microscope (Eclipse Ti-S, Nikon, Tokyo, Japan) connected with a digital camera.

Scanning electron microscopy.

The cultured cells were fixed with acid Lugol's iodine solution, and filtered through a 3 µm pore size polycarbonate membrane (Millipore Ltd., Middlesex, U.K.). The filter was rinsed three times in Milli-Q water, dehydrated through graded ethanol series (30%, 50%, 70%, 80%, 90%, 95%, and two steps in 100%) and finally the filters were

immersed in hexamethyldisilazane, HMDS (Molekula Limited, Newcastle, U.K.) for 30 min (twice). HMDS was evaporated by placing the sample overnight under the fume hood. Filters were mounted on an aluminum stub, sputter-coated with Pd (Polaron SC7620, Quorum Technologies, Ashford, U.K.) and observed at 15 kV with a SEM LEO 438 VP and Zeiss Sigma 300 VP (Carl Zeiss, Oberkochen, Germany). Images were presented on a black background using Adobe Photoshop CS3 (Adobe Systems, San Jose, CA, USA). In order to facilitate the visualization, the thecal pores on the left and right valves surface were labelled using uppercase and lowercase letters of the Latin alphabet for the large and small pores, respectively. The density of knobs on the valve surface was estimated after the counting of the number of knobs inside of a 1 µm-side grid using Adobe Photoshop CS3. The counting field was a 1 µm-side grid as an empty square placed in ten distinct positions for each valve. Ten valves of the strains CCMP3122 and RCC6872 were used for counting. The nomenclature of the structure of the periflagellar area used the terminology in previous studies on *Prorocentrum cordatum* following Pertola et al. (2003) and Monti et al. (2010). Our results and previous SEM observations of *P. cordatum* (Velikova and Larsen 1999, Pertola et al. 2003, Monti et al. 2010) did not report the eight platelets that can be easily observed in larger species such as *P. micans* (Tillmann et al. 2019).

DNA extraction.

For the culture of the strain CCMP3122, cells were collected during the exponential growth phase by centrifugation at 4,500*g* for 10 min at 4ºC. The cell pellets were suspended in DNA lysis buffer (10 mM Tris pH 8.0; 100 mM EDTA pH 8.0; 0.5% SDS; 100 mg · mL^{-1} proteinase K) and incubated at 55ºC for 2 d. The DNA extraction was conducted using a CTAB protocol coupled with Zymo DNA Clean & concentration

kit (Zymo Research Corp, Irvine, CA, USA), as previously reported (Yuan et al. 2015). For the DNA extraction of the strains RCC6871–2, the cells were collected by centrifugation. Two mL of the culture during the exponential growth phase in addition of 2 µL of Poloxamer 188 solution (Sigma-Aldrich, St. Quentin Fallavier, France) were centrifuged at 10,000*g* for 10 min at 4ºC. DNA was extracted from the cell pellets using the NucleoSpin Tissue, Mini kit for DNA from cells and tissue, following the instructions of the manufacturer (Macherey-Nalgel, Düren, Germany).

PCR amplification and sequencing.

For the strain CCMP3122, the extracted DNA was used as the template to amplify the SSU and LSU rRNA and ITS gene by using the primer pairs reported in Table 1 using the Hot-Start Ex Taq DNA polymerase (Takara, Shiga, Japan). Conditions were as follows: 94ºC for 5 min followed by 30 cycles of 94°C 1 min, 55ºC for 38 s, 72°C for 90 s and a final step of extension at 72ºC for 5 min. The PCR products with the expected size were purified using Universal DNA Purification Kit (TransGen Biotech, Beijing, China) and directly sequenced (Shenggong, Xiamen, China). The obtained sequences were submitted to National Center for Biotechnology Information (NCBI) with GenBank accession numbers ON229905 and ON229906. For the strains RCC6871–2, the extracted DNA was used as the template to amplify the gene fragment of SSU- and LSU rRNA and ITS gene by using primer pairs reported in Table 1 with the Hot-Star Taq Plus Master Mix Kit (Quiagen, Hilden, Germany). PCR conditions were as follows for SSU rDNA: 95°C for 5 min followed by 35 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 60 s and a final step of extension at 72°C for 10 min; for ITS: 95°C for 5 min followed by 35 cycles of 95°C for 30s, 60°C for 60 s, 72°C for 90 s and a final step of extension at 72°C for 10 min; and LSU rDNA: 95°C for 5 min followed

by 35 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 90 s and a final step of extension at 72°C for 10 min. The PCR products with the expected size were purified using ExoSAP-It DNA Purification Kit (Applied Biosystems, Foster City, CA, USA) and directly sequenced (Macrogen, Amsterdam, The Netherlands). The obtained sequences were submitted to NCBI with GenBank accession numbers ON426969–ON426970 and ON426997–ON427000.

Phylogenetic analyses.

The small and large subunit rRNA and internal transcriber spacers gene sequences of the strains CCMP3122 and RCC6871–2 were analyzed using Basic Local Search Tool (BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi) against the GenBank database. BLAST hit sequences containing distinct groups of dinoflagellates were downloaded and aligned with sequences of CCMP3122 and RCC6871–2 using ClustalW (Larkin et al. 2007). Phylogenetic trees were inferred using the Maximum Likelihood method based on the General Time Reversible model with Gamma distributed rates in MEGA7 software (Kumar et al. 2016). The final trees were built with representative sequences of the Prorocentraceae. Bootstrap values were obtained after 1000 replications. The sequences of *Scrippsiella acuminata* were used for rooting the phylogenetic trees.

RESULTS

Molecular phylogeny

We obtained new SSU- and LSU rRNA and ITS gene sequences of the strain CCMP3122 from Florida identified as *Prorocentrum shikokuense*, the strains RCC6871 and RCC6872 from the Ross Sea isolated in 2019, identified as *Prorocentrum* sp., and a SSU rRNA gene sequence of another isolate from the Ross Sea isolated in 2021. In the

SSU rRNA gene phylogeny, the sequences of the spiny-surfaced *Prorocentrum* species clustered together with high support (ML bootstrap probability, BP 95%) branching with *P. redfieldii* as a sister group. The clade of the spiny-surfaced *Prorocentrum* species is divided into three groups: one for sequences of *P. shikokuense* (BP 95%), another clade for *P. cordatum* (BP 85%), and the other group with two subclades, one for the sequence of the strain CCMP3122 from Florida, and the other subclade for the sequences of the strains RCC6871 and RCC6872 from the Ross Sea isolated in 2019, another isolate from 2021, and the other isolate *Prorocentrum* sp. voucher DINO:2 (MT830911) collected from the Ross Sea in 2017, as well as an environmental sequence from the Arctic Sea near the North Pole (HQ438148; Fig. 1). The sequences of the strains RCC6871 and RCC6872 were identical (100%) to each other and only differed from *Prorocentrum* sp. voucher DINO:2 (MT830911) in one missing nucleotide. The environmental sequence from the North Pole differed in three nucleotides from the sequences of the Antarctic strains RCC6871–2. Consequently, based on the available SSU rRNA gene sequences, the spiny-surfaced species of *Prorocentrum* contained at least four species: *P. cordatum* and *P. shikokuense* with widespread distributions, and two other species with more restricted distributions, one known from the warm waters of the Gulf of Mexico and the other species known from polar waters (Fig. 1).

In the LSU rRNA gene phylogeny, the sequences of the spiny-surfaced *Prorocentrum* clustered together with strong support (BP 97%), and divided into three major clades: One clade for *P. cordatum*, the second clade for *P. shikokuense* with a sequence retrieved as *Prorocentrum* sp. isolate QUCCCM8 from the Arabian Gulf as an earlier branching lineage (Fig. 2), and the third clade with high support (BP 91%) contains 3 subclades: an early-branching lineage for the sequence retrieved as *Prorocentrum* cf. *balticum* strain UTSPH3D3 (MW024115) from Australia, and two

sister subclades: one subclade with the new sequence of *P. shikokuense* strain CCMP3122, *Prorocentrum* sp. FIU22 (EU165281) and *Prorocentrum* sp. isolate QUCCCM SS1–12 and QUCCCM SS1–13 from the Arabian Gulf (KX853185–6). It should be noted that the strain *Prorocentrum* sp. FIU22 was renamed as *P. shikokuense* strain CCMP3122 when deposited at the Provasoli-Guillard National Center for Marine Algae and Microbiota. The sequences of *Prorocentrum* sp. FIU22 and the new sequence of *P. shikokuense* CCMP3122 were identical (100%), both of which only differed in one nucleotide from *Prorocentrum* sp. isolate QUCCCM SS1. The other subclade with high support (BP 95%) contained sequences of isolates from the Antarctic Ocean: the two strains *Prorocentrum* sp. RCC6871–2, and *Prorocentrum* sp. DINO:1 (MT831988), the latter only differed in one nucleotide. Consequently, based on the available LSU rRNA gene sequences, the spiny-surfaced *Prorocentrum* contained at least six species: 1) *Prorocentrum cordatum*, 2) *P. shikokuense*, 3) *Prorocentrum* sp. isolate QUCCCM8 (KX853176) as an early-branching lineage of *P. shikokuense*, 4) the strain CCMP3122, also present in the Arabian Gulf, 5) an unidentified species from the Antarctic Ocean, and 6) *P.* cf. *balticum* strain UTSPH3D3 from Australia (Fig. 2).

In the ITS gene phylogeny, the sequences of the spiny-surfaced *Prorocentrum* clustered with high support (BP 96%) as a sister clade of the sequences of *P. redfieldii*. The sequences of *P. cordatum* constituted a high supported major clade (BP 92%) with strains from the warm waters of Florida to the cold waters of Norway, and other ocean regions. The other major clade contained the sequences of *P. shikokuense* (Fig. 3). The sequence of the strain *Prorocentrum* sp. isolate QUCCCM86 also branched closely related to the clade of *P. shikokuense*, and the lineage is represented by more sequences in this case of the ITS rRNA gene phylogeny. The sequence of *Prorocentrum* sp. isolate QUCCCM86 from the Arabian Gulf and *P. minimum* CCMP1529 from the Eastern

Pacific were identical (100%). Other two sequences diverging by six nucleotides were *P. minimum* RCC922 from the Eastern Pacific and a strain identified as *P. rostratum* isolated from the coastal NE Atlantic at Vigo, Spain. There are other three lineages, one represented by the single sequence of *P.* cf. *balticum* strain UTSPH3D3 (MW024115), another lineage for the Antarctic strains of *Prorocentrum* sp. RCC6871–2 and *Prorocentrum* sp. (MT831988) that were identical (100%), and another lineage contained the sequences of the strains *P. shikokuense* CCMP3122 from Florida, *P. balticum* strain CCMP1260 from the Gulf of Mexico, *P. balticum* strain CCMP1787 from New Zealand, and *Prorocentrum* sp. isolate QUCCCM SS1–13 from the Arabian Gulf (Fig. 3). The sequences of the strains *P. shikokuense* CCMP3122 and *P. balticum* CCMP1260 were identical (100%), and only differed in one base pair from the *P. balticum* CCMP1787, and four nucleotides from an incomplete sequence retrieved as *Prorocentrum* sp. isolate QUCCCM SS1–13.

The LSU rRNA and ITS gene phylogenies suggest that there are at least six species of spiny-surfaced *Prorocentrum* (Figs. 2, 3). Most of the sequences corresponded to *P. cordatum* and *P. shikokuense.* There is a group of sequences that clustered as an early-branching of *P. shikokuense*. There are slight divergences among the sequences, and it is not easy to determinate if these four ITS rRNA gene sequences correspond to one or more species. The sequence retrieved as *P.* cf. *balticum* UTSPH3D3 (MW024115) constitutes an independent lineage. The other two lineages are represented by several sequences from distinct collection sites. One lineage exemplified by the strain *P. shikokuense* CCMP3122 represents a species distributed in the warm waters of the Atlantic, Indian and Pacific Oceans. The other lineage exemplified by the strain *Prorocentrum* sp. RCC6872 represents a species isolated in three distinct research cruises in the Antarctic Ocean, and is also distributed in the

Arctic Ocean based on an environmental sequence (HQ438148). We propose new species for these last two lineages.

Species descriptions

Prorocentrum thermophilum F. Gómez, Tangcheng Li, Hu. Zhang & Senjie Lin, sp. nov.

DESCRIPTION. Photosynthetic prorocentroid dinoflagellate with almost round shape in valve view. Cultured cells of average length of 14 μm, and average width of 12 μm. The periflagellar area appeared as a slight indentation in the cell contour, and in some individuals were observed an inconspicuous anterior spine-like prolongation. The valve surface is ornamented with short spines or knobs with a density of 5–7 · μm^{-2} joined by ridges. The right valve showed a row of three large trichocyst or ejectosome pores as round openings of equal diameter (~0.30 μm in diameter) located in the anterior right side, other two large pores located near the right side of the posterior end, and variable number (10–15) of smaller pores (~0.15 μm in diameter) located near the periphery of the valve. The left valve showed 12–15 pores ranging from 0.15 to 0.3 μm in diameter located near the periphery of the valve. A posterior nucleus and two lateral golden-brown chloroplasts.

HOLOTYPE: SEM stub #BCM 8028.

TYPE LOCALITY: New Pass Bridge, Sarasota, Florida, USA (27.333° N, 82.583° W).

HABITAT: Marine, planktonic.

ETYMOLOGY: Ancient Greek θερμός, 'thermos' means warm, and Ancient Greek φιλία, 'philía' means fondness, love. The species has a preferential distribution in warm seas.

MOLECULAR CHARACTERIZATION: The strain CCMP3122 is barcoded in GenBank by the sequences of SSU rDNA, ITS1–5.8S–ITS2, and D1–D3 of LSU rDNA under the accession numbers ON229905 and ON229906.

MORPHOLOGY: Photosynthetic prorocentroid dinoflagellate with almost round shape in valve view. Dimensions of live cells were 11.8 to 16.6 μm long (average 13.9 μm) and 9.8 to 15.2 μm wide (average 12.3 μm; n = 50). The size variation was related to the age of the cell (Fig. 4, A–F), where the smaller cells corresponded to the recently divided cells (Fig. 4G). In valve view, the periflagellar area appeared as a slight indentation in the cell contour (Fig. 4, A and E), and in some individuals an inconspicuous anterior spine-like prolongation was observed (Fig. 4C). The nucleus was located in the posterior region, with a more or less rotund shape (Fig. 4, B and D). The nuclei were ellipsoidal in the larger cells, probably prior to division (Fig. 4, F and M). About 40 chromosomes were counted (Fig. 4, H and I). In valve view, there are two laterally located chloroplasts at both sides of the nucleus (Fig. 4, J-L).

The variation in cell thickness is not discernible in light microscopy because the cells settle with one valve facing up preventing clear sight of the intercalary zone between the two valves. Under SEM, it is possible to observe individuals with the left or right valve almost hemispherical. The width of the cells in lateral view ranged from 8 to 13 μm. Most of the cells showed a thick intercalary band that was thicker towards the posterior end, except for some individuals probably recently divided (Fig. 5G). The valve surface showed a density of 5–7 short knobs per 1 μm^2 (average value of 5.88 knobs per 1 μm^2, n=100 counting fields). In the anterior right valve, the knobs were joined by ridges that were concentric to the periflagellar area (Fig. 5B), while in other valve regions from each knob radiated six ridges to the surrounding knobs describing a hexagonal pattern. The right valve showed a row of three large pores as round openings

of equal diameter (~0.30 μm in diameter) located in the anterior right side adjacent to the flagellar pore (Fig. 5, B–G). There was a pair of large pores located near the right side of the posterior end (Fig. 5D). There was a variable number (10–15) of smaller pores (~0.15 μm in diameter) located near the periphery of the valve. A pore labelled as 'a' was located above the row of three large pores, and a second one, labelled as 'b', was in parallel to the row of three large pores (Fig. 5, A–D). The left valve showed 10–11 large pores and a few small pores located near the periphery of the valve (Fig. 5, H–L). In the side of the auxiliary pore (opposite to the row of three large pores of the right valve), the left valve showed a common pattern with a small pore 'a' followed by a pair of two large pores (Fig. 5, E, F, H, L). The other larger pores were solitary and located near the periphery of the valve (Fig. 5L).

A complex flagellar pore region was located in a V-shaped depression at the anterior end of the cell on the right valve (Fig. 6). The periflagellar area appeared as two circular holes, the flagellar and auxiliary pores, surrounded by folded structures. The flagellar pore is larger than the auxiliary pore, and it is surrounded in the posterior margin by a semicircular collar more or less prominent that can be obtuse (Fig. 6, A and B) or slightly pointed (Fig. 6C) denoted as the single tooth. The anterior margin of the flagellar pore adjacent to the intercalary band is less prominent and showed a semicircular platelet denoted as ridged edge (Fig. 6, B–F). The flagellar and auxiliary pores were split by a double-layer structure that can be pointed as a spine (Fig. 6C) or obtuse (Fig. 6D) denoted as small tooth. The auxiliary pore is surrounded in the posterior end by double-layer structure, resembling the valves of a bivalve mollusk (Fig. 6, A and B), denoted as apical collar. The anterior end of the auxiliary pore showed a tiny platelet that is denoted by the symbol '*' (Fig. 6, D, E, H). There is a triangular area delimited between the apical collar, the auxiliary pore and the intercalary band

denoted as 't' (Fig. 6, D–F). The small size of the periflagellar area and the numerous folds of the structures make it difficult to determine the complete number of periflagellar platelets.

Prorocentrum criophilum Gourvil & Gutiérrez-Rodríguez, sp. nov.

DESCRIPTION. Photosynthetic prorocentroid dinoflagellate with almost round to slight asymmetric round shape in valve view, and almost spherical in lateral view. The periflagellar area was not discernible under light microscopy. Average cell dimensions were ~11 μm long, and ~10 μm wide. The valve surface covered of small dot-like structures evenly distributed with a density of 6–10 per 1 μm^2. The right valve showed at least twelve large trichocyst pores (~0.3 μm in diameter) preferentially located near the valve edge. Three large pores in the anterior right formed a row surrounded by four small pores (~0.15 μm in diameter). The other large pores were arranged in pairs in the right and posterior periphery of the valve. Beyond the small four pores in the right anterior side, only a few (<3) small pores were observed near the periphery of the right valve. At least nine large pores were observed in the left valve. They formed pairs in the periphery of the valve. Small pores were dispersed or forming groups.

HOLOTYPE: SEM stub #BCM 8029.

TYPE LOCALITY: Ross Sea, Antarctic Ocean at 75.13º S, 176.04º W.

HABITAT: Marine, planktonic.

ETYMOLOGY: Ancient Greek κρύος, krúos, 'crio' means cold, and Ancient Greek φιλία, 'philía' means fondness, love. The species has a preferential distribution in cold seas.

MOLECULAR CHARACTERIZATION: The type strain RCC6872 is barcoded in GenBank by the sequences of SSU rDNA, ITS1–5.8S–ITS2, and D1–D3 of LSU rDNA under the accession numbers ON426969–ON426970 and ON426997–ON427000.

MORPHOLOGY: Photosynthetic prorocentroid dinoflagellate with round to slight ellipsoidal shape in valve view with two lateral chloroplasts surrounding the nucleus (Fig. 7, A–D), and in lateral view the cell shape was round or asymmetrical roundish with slight protuberance and the chloroplast covering the cell (Fig. 7, E–H). The settling of the cells in both valve and lateral views revealed that the cells were almost spherical. Under LM, the periflagellar area was not discernible. The dimensions of live cells were 8.9–12.1 μm long (average 10.2 μm) and 8.0–11.6 μm wide (average 9.7 μm; n =38) and Lugol's solution preserved cells were 8.8–13.4 μm long (average 10.9 μm) and 8.0–12.0 μm wide (average 10.1 μm; n=100).

Under SEM, the valves can be almost spherical. The valve surface showed very tiny knobs, like dots, with a density of 6–10 per 1 μm^2 (average value of 7.48 knobs per 1 μm^2, n=100 counting fields), missing ridges joining the knobs. The right valve showed at least twelve large pores (~0.3 μm in diameter) preferentially located near the valve edge. A row of three large pores in the anterior right were surrounded by four small pores (Fig. 8A). The other large pores were arranged in pairs in the right and posterior periphery of the valve (Fig. 8, A–E). Beyond the small four pores in the right anterior side, only a few (<4) smaller pores were observed near the periphery of the right valve (Fig. 8, B and E). At least nine large pores were observed in the left valve. They formed pairs in the periphery of the valve, with one pore closer to the valve edge than the other one (Fig. 8F). Dispersed and small groups of small pores were also observed (Fig. 8D). It was even difficult under SEM to note the periflagellar area in valve view because there was not a clear notch or protuberance (anterior spine; Fig. 8C).

The description of the periflagellar area is incomplete, restricted to few individuals, and a pattern cannot be established. Holes in the flagellar and auxiliary pores were not observed (Fig. 8, G and H). There was a triangular prominent structure identified as the single tooth (Fig. 8, G and H).

Prorocentrum cordatum and P. shikokuense

There are numerous morphological studies of *Prorocentrum cordatum* and *P. shikokuense*. SEM images are provided here to facilitate the comparisons with the new species. *Prorocentrum thermophilum* sp. nov. and *P. criophilum* sp. nov. are rotund in shape (Fig. 9, A and B), and under LM observations can be easily misidentified as *P. cordatum* (Fig. 10, A and B). In contrast, there is no confusion with *P. shikokuense* that is elongated and never rotund (Fig. 10, C and D). *Prorocentrum cordatum* is more similar to *P. thermophilum* than to *P. criophilum*. *Prorocentrum cordatum* is in the size range of *P. thermophilum*, and usually larger than *P. criophilum* (~10 μm). The anterior spine of *P. cordatum* or *P. thermophilum* is sometimes visible under LM, while it is difficult to observe in *P. criophilum*. In lateral view, the cells of *P. criophilum* are almost spherical, while *P. cordatum* or *P. thermophilum* show a slight valve compression. The valve of *P. cordatum* contains numerous trichocyst pores (>20), while *P. thermophilum* and *P. criophilum* contain less than 20 pores per valve. *Prorocentrum thermophilum* and *P. criophilum* showed a row of three large pores adjacent to the flagellar pore that are missing in *P. cordatum*. *Prorocentrum cordatum* showed a density of knobs of ~4 per 1 μm^2, while the new species *P. thermophilum* (5–7 per μm^2) and *P. criophilum* (6–10 $\cdot \mu m^{-2}$) showed a higher density. These differences are only noted by examining SEM pictures, and consequently the distinction of these species under routine LM observations is difficult.

DISCUSSION

The molecular phylogenies reveal that most of the available DNA sequences of the spiny-surfaced *Prorocentrum* clustered into two major clades represented by isolates from distinct geographical areas, and at least other four smaller clades with a lower number of sequences and more restricted geographical distributions (Figs. 2–3). The identity and the synonymy of the two major clades is not the aim of this study, but it is important to note that the new species described in this study do not correspond to any of the already described species. The case of the major clade of the species named as *P. shikokuense*, *P. donghaiense*, *P. dentatum* or *P. obtusidens* have been already dealt in Gómez et al. (2022). The conclusion is that *P. shikokuense* (=*P. donghaiense*) is the name for the species of this major clade, and *P. dentatum* and *P. obtusidens* are two independent species that are not yet represented with molecular data. *Prorocentrum shikokuense*, *P. dentatum* and *P. obtusidens* are species with an elongated shape, and unrelated to the roundish and smaller species *P. thermophilum* sp. nov. and *P. criophilum* sp. nov. In the discussion, we comment about several isolates with a roundish morphology that are closely related to the clade of *P. shikokuense* as revealed in the LSU rRNA and ITS gene phylogenies (Figs. 2, 3).

Uncharacterized species closely related to Prorocentrum shikokuense.

The spiny-surfaced *Prorocentrum* species with available molecular data are small and more or less rotund in shape, being difficult to distinguish the species. In contrast, the elongated shape of *P. shikokuense* facilitates the distinction under routine light microscopy observations from the *P. cordatum*-like individuals. Based on the molecular

data, *P. shikokuense* (=*P. donghaiense*) has a widespread distribution, but more restricted than *P. cordatum*. For example, abundant molecular data reveal the presence of *P. cordatum* in the North Atlantic American coasts, while *P. shikokuense* is apparently absent there (Figs. 1–3).

The molecular phylogenies show a group of sequences as an earlier-branching lineage of *Prorocentrum shikokuense*. In the LSU rRNA gene phylogeny, this group is only represented by a sequence identified as *Prorocentrum* sp. QUCCCM8 from Qatar, Arabian Gulf (KX853176; Fig. 2). In the ITS gene phylogeny, the sequence of *Prorocentrum* sp. QUCCCM8 is identical (100%) to that of *P. minimum* strain CCMP1529. The latter strain was isolated off Ecuador and it showed a more ellipsoidal shape than the most typical rotund cells of *P. cordatum* (https://ncma.bigelow.org/CCMP1529). Other two sequences also clustered as early-diverging lineages of *P. shikokuense*, and they diverged by more than six base pairs from *P. minimum* CCMP1529/*Prorocentrum* sp. QUCCCM8. These sequences retrieved as *P. rostratum* strain PR1 from Ria of Vigo, NW Spain (EU244471) and *P. minimum* RCC922 from the open SE Pacific Ocean (FJ823585) differed in three base pairs. It is expected that these genetically closely related strains do not show important morphological differences. The images of *P. minimum* RCC922 (https://roscoff-culture-collection.org/rcc-strain-details/922) showed cells of 14 μm long, with an asymmetric heart-shaped contour and a slight anterior shoulder (Le Gall et al. 2008). As reported in Gómez et al. (2022), it is highly improbable that the sequence EU244471 retrieved as *P. rostratum* strain PR1 belong to that species. Morphological and molecular data are needed to resolve and determine how many species constitute this group of sequences that are clustered as an earlier branch of the clade of *P. shikokuense*.

Another potential uncharacterized species is represented by the sequence identified as *Prorocentrum* cf. *balticum* UTSPH3D3 (MW024115) that constituted an independent lineage closely related to *P. criophilum* sp. nov. and *P. thermophilum* sp. nov. In the ITS gene phylogeny, the sequence of *Prorocentrum* cf. *balticum* UTSPH3D3 is more closely related to *P. criophilum* sp. nov. than to *P. thermophilum* sp. nov. (Fig. 3). The strain was isolated offshore Sydney, Australia (Larsson et al. 2022). The cells were ovate, 13–16 µm long, with visible two anterior projections in the periflagellar area, and a row of three large pores in the anterior right margin of the right valve (Larsson et al. 2022). *Prorocentrum* cf. *balticum* UTSPH3D3 is in the size range of *P. thermophilum*, and it is larger than *P. criophilum*. However, the surface ornamentation of *Prorocentrum* cf. *balticum* UTSPH3D3 is closer to *P. thermophilum* than *P. criophilum*. Larsson et al. (2022) only provided a few SEM pictures of folded thecae that do not allow to determinate the complete arrangement of the trichocyst pores for a comparison. *Prorocentrum* cf. *balticum* UTSPH3D3 constitutes a distinct species of spiny-surfaced *Prorocentrum* needing a complete morphological characterization.

The identity of Prorocentrum thermophilum *sp. nov.*

There are sequences identified as *Prorocentrum balticum* (strain CCMP1260, CCMP1787) or *P. donghaiense* (CCMP3122) that diverged from the clade of sequences of *P. cordatum*. We propose *P. thermophilum* sp. nov. based on sequences from the warm Atlantic, Indian and Pacific Oceans that constitute a well-supported clade, and distinct from the major clades of *P. cordatum* and *P. shikokuense* (Figs. 1–3).

The strains CCMP1260, CCMP1787 and CCMP3122 are publicly available for morphological and molecular studies. The origin of the type strain CCMP3122 was *Prorocentrum* sp. FIU22. It was later identified as *P. shikokuense*. However, the images

of the culture of the strain CCMP3122 corresponded to rotund cells (https://ncma.bigelow.org/CCMP3122), unequivocally differing from the elongated cells of *P. shikokuense* (Fig. 10, C and D). Since 2009, the strain CCMP3122 was cultured at a maintenance temperature of 14ºC. At the type locality, Sarasota at Florida, the average sea water temperature ranged from 17.7°C in January to 30.4°C in August (https://www.seatemperature.org/north-america/united-states/sarasota.htm). According to website of the NCMA, the cells were 12–16 µm long and 10–14 µm wide. In this study, the strain CCMP3122 were cultured at temperature of 20ºC, and the cells showed similar dimensions (11.8–16.6 µm long, 9.8–15.2 µm wide).

The ITS gene sequence of the strain CCMP3122 is identical (100%) to that of *Prorocentrum balticum* CCMP1260, and it only differed in one nucleotide from *P. balticum* CCMP1787. The strain CCMP1260 was isolated from the central Gulf of Mexico (approx. 25° N, 90° W) in 1981 and since then cultured at 20°C. The strain CCMP1787 corresponded to a strain named CAWD38 of the Culture Collection of Micro-algae of the Cawthron Institute in New Zealand. The strain was isolated by Dr. L. Mackenzie in the coasts of Taharoa, New Zealand (38.171º S, 174.701º E) in 1993. Ferrell (2008) obtained the ITS gene sequences of the strains CCMP1269 and CCMP1787. Ferrell (2008, p. 112, pl. 22) reported SEM micrographs of the cells of the strain CCMP1269, but her pictures corresponded to a larger benthic *Prorocentrum* species https://v3.boldsystems.org/index.php/Taxbrowser_Taxonpage?taxid=317175 . The species is also present in the Arabian Gulf at Qatar based on the sequence of isolates QUUCCM SS1–12 (KX853185) and QUUCCM SS1–13 (KX853186, KX853197). Images of these isolates were not available (Al Muftah et al. 2016). The available molecular data show that *P. thermophilum* sp. nov. is present in warm waters of the Atlantic, Indian and Pacific Oceans. However, this character cannot be

unequivocally used to differentiate it from *P. cordatum* because both species may coexist. For example, the strain *P. cordatum* CCMP2811 (EU927543) was isolated at Sarasota, Florida, that is the type locality of *P. thermophilum* sp. nov.

Under routine microscopy analyses, it is not easy to differentiate *Prorocentrum thermophilum* sp. nov. from *P. cordatum*. The proposal of a new name *P. thermophilum* needs to discard other names of species closely related to *P. cordatum*. If *P. cordatum* and *P. balticum* are considered distinct species, according to the literature they can be differentiated by the size (Faust et al. 1999). *Prorocentrum balticum* is small (9–12 μm long), while *P. cordatum* is larger (>15 μm long). The cells of *P. thermophilum* (12–16 μm long) are larger than *P. balticum*.

The Table 2 revised other species of *Prorocentrum* with roundish or oval cells less than 25 μm long. Illustrations of the original descriptions are reported in Figure S1 in the Supporting Information. Schiller (1918, 1928) described several planktonic species of *Prorocentrum* from the Adriatic Sea. The descriptions of *P. rotundatum*, *P. cornutum*, *P. nanum*, *P. sphaeroideum*, *Exuviaella ovum*, *E. pusilla* or *E. pyriformis* do not fit in size and/or shape with *P. thermophilum* sp. nov. (Table 2). Dodge (1975, p. 119) reported for *P. balticum*: "*Exuviaella aequatorialis* would appear to be a slightly compressed form of this species as it has a similar shape and also has spiny plates". Hasle (1960) described *Exuviaella aequatorialis* from the Central Pacific Ocean at 145º W, 0–2º N. She reported a cell of 19 μm in diameter, and the line drawing showed two notches in the periflagellar area. Hasle (1960) reported the periflagellar area as two similar tear-shaped holes that do not correspond to the observations in *P. thermophilum* (Figs. 6, S1). The cultured cells of *P. thermophilum* ranged from 12–16 μm long. There are other strains isolated from the warm Pacific Ocean. *Prorocentrum cordatum* CCMP1529 (https://ncma.bigelow.org/CCMP1529) or *P. minimum* RCC922

(https://roscoff-culture-collection.org/rcc-strain-details/922) are smaller than *E. aequatorialis* and the cell contour were not rotund. Based on the scarce available data, it is not easy to determinate whether *E. aequatorialis* constitutes an early description of *P. thermophilum* sp. nov.

The identity of Prorocentrum criophilum *sp. nov.*

Prorocentrum criophilum is a species with a polar distribution. It is especially abundant in the Ross Sea where it has been isolated in 2017, 2019 and 2021. The species is associated with the blooms of the haptophyte *Phaeocystis antarctica* (Bolinesi et al. 2020). Due to the small size (9–12 µm), the species is inefficiently sampled by plankton nets, and it appears in non-concentrated water samples. Hasle (1960, p. 30) reported that *Exuviaella baltica* was present in sub-Antarctic samples with high abundance. She illustrated a round cell of about 10–12 µm in diameter (Fig. S1). It should not be confused with *Prorocentrum antarcticum* that is larger in size. *Prorocentrum antarcticum* was first reported as *Exuviaella* sp. with cells 20–23 µm long (Balech and El-Sayed 1965). Hada (1970) described the new species *Exuviaella antarctica* as 15–17 µm long that he considered conspecific *Exuviaella* sp. by Balech and El-Sayed (1965) (Table 2, Fig. S1). Balech (1976) transferred *Exuviaella antarctica* into *Prorocentrum*, and he reported an oval shape with few minute scattered pores. In Antarctic waters, two *Prorocentrum* species have been commonly reported. Estrada and Delgado (1990) reported *Prorocentrum* cf. *antarcticum* and *Prorocentrum* sp. (small). Kopczyńska et al. (1998) cited *P. minimum* and *P. antarcticum*. Scott and Marchant (2005) reported SEM pictures of *P. antarcticum* and *P. balticum*. For *P. antarcticum*, they reported ovate cells of 15–25 µm, with a smooth theca. For *P. balticum*, Scott and Marchant (2005) reported: "round cell in valve view, 9–22 µm long, 14–21 µm diameter, not or barely

compressed in side view; older cells with a large intercalary band. Periflagellar area with a triangular flagellar pore and a smaller accessory pore. Spiny surface". Their images of *P. balticum* fit with *P. criophilum* sp. nov. *Prorocentrum balticum* is small (9–12 μm long, Lohmann 1908, Faust et al. 1999) and in the size range of *P. criophilum*. However, there is no any morphological study with modern methods (SEM) illustrating the differences between *P. balticum* and *P. cordatum* (=*P. minimum*) that can be used to compare it with *P. criophilum*. *Prorocentrum balticum* was described as a bloom-forming species in Kiel, Germany (Lohmann 1908). In the type locality of *P. balticum*, Kimor et al. (1985) reported abundances up to 31 million of cells $\cdot L^{-1}$ with SEM pictures of cells which morphology fit with *P. cordatum*. Currently, all the DNA sequences along the European coasts clustered in the clade of *P. cordatum*, and there are no molecular data evidencing that *P. cordatum* and *P. balticum* are independent species. This suggests that *Prorocentrum balticum* may correspond to a small form of *P. cordatum*. A morphological and molecular comparative study of *P. cordatum* and *P. balticum* is still missing. However, this lack of definition of *P. balticum* should not render the advances in order to characterize the distinct clades belonging to the group of *P. cordatum*. We consider that *P. criophilum*, the abundant widespread species in the Antarctic Ocean, is distinct from the uncharacterized *P. balticum*.

Among the similar species of *Prorocentrum* described from cold waters are *Exuviaella granii* and *E. pacifica*. Gaarder (1938) described *E. granii* near Tromsø, in the Norwegian Arctic coast, as an oval cell with a length of 22–24 μm that does not fit with in size with *P. criophilum* sp. nov. (9–12 μm long). Kuz'mina (1960) described *Exuviaella pacifica* from the sub-arctic waters near the Kuriles Is., NW Pacific Ocean. The cells were 12.5–20 μm long, 17–20 μm wide with an ovate shape, and with a marked notch in the periflagellar area (Kuz'mina 1960; Fig. S1). The shape and size do

not fit with *P. criophilum* sp. nov. that also lacks a marked notch in the periflagellar area. Hasle (1969) cited the name '*Prorocentrum circulare* Gaarder' for a cells collected in the Pacific Southern Ocean. However, this a 'nomen nudum' and there is not a formal description or illustration of the species in order to compare with *P. criophilum* sp. nov.

The molecular phylogenies reveal two clades with a high number of sequences for *Prorocentrum cordatum* and *P. shikokuense*, and potentially other four lineages that may represent four or more species (Figs. 2–3). Here, we characterized two of these species. Other species remains to be characterized. The scarce detail of the old original descriptions, and the lack of distinctive morphological characters in the tiny round cells of *Prorocentrum* makes it difficult to assign it to already described species. In addition, the lack of study that will define unequivocally by modern methods the identity of *P. balticum* is impeding the clarification of the taxonomy of the spiny-surfaced *Prorocentrum*.

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TABLE 1. List of oligonucleotide primers used in PCR assays.

Primer	Primer sequence (5'-3')	Reference
Strain CCMP3122		
18SCOMF:	ACCTGGTTGATCCTGCCAGT	Zhang et al. 2005
18SCOMR:	TCACCTACGGAAACCTTGT	Zhang et al. 2005
18ScomF-3end:	GTCGTAACAAGGTTTCCGTAGGTG	Wang et al. 2014
com28SR1	TCACGCATAGTTCACCATCTTTCG	Wang et al. 2014
Strains RCC6871–2		
EukA-F	AACCTGGTTGATCCTGCCAGT	Medlin et al. 1988
Euk-NSF573	CGCGGTAATTCCAGCTC	Dolven et al. 2007
EukB-R	TGATCCTTCTGCAGGTTCACCTAC	Medlin et al. 1988
S69-R	CCGTCADTTCCTTTRAGDTT	Probert et al. 2014
329-F	GTGAACCTGCRGAAGGATCA	Guillou et al. 2004
D1R-F	ACCCGCTGAATTTAAGCATA	Lenaers et al. 1989
D1R-R	TATGCTTAAATTCAGCGGGT	Lenaers et al. 1989
D2C-R	CCTTGGTCCGTGTTTCAAGA	Scholin et al. 1994

TABLE 2. Comparison of morphological characteristics of *Prorocentrum* species. See Figure S1 for the original illustrations.

Taxon	Shape	Length, width in μm	Type locality	Reference
Exuviaella cordata	oval	22–24, 18–20	Caspian Sea	Ostenfeld 1901
Exuviaella baltica	rotund	9–12	Kiel, Baltic	Lohmann 1908
Exuviaella minima	heart-shaped	18–19, 14–16	Gulf of Lions	Pavillard 1916
Prorocentrum nanum	rotund, irregular	8–12, 8–12	Adriatic Sea	Schiller 1918
P. rotundatum	rotund	16–24, 16–21	Adriatic Sea	Schiller 1918
Exuviaella ovum	oval	14, 10	Adriatic Sea	Schiller 1918
Exuviaella pusilla	oval	8–10, 6.5–7.5	Adriatic Sea	Schiller 1928
Exuviaella granii	oval	22–24	Norway	Gaarder 1938
P. triangulatum	triangular	17–22	NJ, U.S.A.	Martin 1929
E. mariae-lebouriae	round/oval	10–22, 8–20	Plymouth, U.K.	Parke and Ballantine 1957
P. cordiforme	heart-shaped	6–12, 5–9	Plymouth, U.K.	Bursa 1959
P. pomoideum	irregular oval	9–20, 11–16	Plymouth, U.K.	Bursa 1959
E. aequatorialis	rotund	19, 19	Central Pacific	Hasle 1960
Exuviaella pacifica	oval	12.5–20, 17–20	NW Pacific	Kuz'mina 1960
Exuviaella antarctica	oval	15–17, 12–15	Antarctic Ocean	Hada 1970

FIG. 1. Phylogenetic tree based on SSU rRNA gene sequences, showing the position of the sequences of spiny-surfaced *Prorocentrum* species by Maximum Likelihood (ML). New sequences are reported in bold type. Names as retrieved from GenBank and the name of the strains at the culture collections. Numbers near branches denote ML bootstrap probability value (BP). BP <70 is omitted. Scale bar denotes 0.002 substitutions per site.

FIG. 2. Phylogenetic tree based on LSU rRNA gene sequences, showing the position of the sequences of spiny-surfaced *Prorocentrum* species by Maximum Likelihood (ML). New sequences are reported in bold type. Names as retrieved from GenBank and the name of the strains at the culture collections. Numbers near branches denote ML bootstrap probability value (BP). BP <70 is omitted. Scale bar denotes 0.02 substitutions per site.

FIG. 3. Phylogenetic tree based on ITS gene sequences, showing the position of the sequences of spiny-surfaced *Prorocentrum* species by Maximum Likelihood (ML). New sequences are reported in bold type. Names as retrieved from GenBank and the name of the strains at the culture collections. Numbers near branches denote ML bootstrap probability value (BP). BP <70 is omitted. Scale bar denotes 0.1 substitutions per site.

FIG. 4. Light micrographs of *Prorocentrum thermophilum* sp. nov. (strain CCMP3122 of the Provasoli-Guillard National Center for Marine Algae and Microbiota, Bigelow, U.S.A., initially identified as *P. donghaiense*). Bright field (A, C, E, G, J, K) and

epifluorescence (B, D, F, H–I, L–M). The nuclei are stained DAPI-stained with ultraviolet light excitation (B, D, F, I, M) and SYBR™ Green (H). A–F. Note the different sizes. C. The arrow points a tiny anterior spine. G–H. Dividing cell. I. Note the chromosomes. J–L. Note the two lateral chloroplasts. M. DAPI-stained nuclei. Chloroplast (chl), nucleus (nu), periflagellar area (pf). Scale bar = 5 μm.

FIG. 5. Scanning electron micrographs of *Prorocentrum thermophilum* sp. nov. (strain CCMP3122 of the Provasoli-Guillard National Center for Marine Algae and Microbiota, Bigelow, U.S.A., initially identified as *P. donghaiense*). All micrographs correspond to distinct individuals. A–B. Right lateral view. C–D. View of the right valve. E–F. Anterior view. G–I. Left lateral view. J. Anterior view. K. Right lateral view. L. View of the left valve. Uppercase and lowercase letters denote the large and small pores, respectively. The symbol * denotes other additional pores. Auxiliary pore (ap), flagellar pore (fp), intercalary band (IB), left valve (LV), right valve (RV), suture between the two valves (su). Scale bar = 2 μm.

FIG. 6. Scanning electron micrographs of the periflagellar area of *Prorocentrum thermophilum* sp. nov. (strain CCMP3122 of the Provasoli-Guillard National Center for Marine Algae and Microbiota, Bigelow, U.S.A., initially identified as *P. donghaiense*). All micrographs correspond to distinct individuals. Apical collar (ac), auxiliary pore (ap), flagellar pore (fp), forked tooth (ft), intercalary band (IB), left valve (LV), ridged edged (re), right valve (RV), single tooth (st), triangular space between the intercalary band, auxiliary pore and apical collar (t). The symbol * indicates a platelet between the auxiliary pore and the intercalary band. Scale bar = 1 μm.

FIG. 7. Light micrographs of *Prorocentrum criophilum* sp. nov. (strain RCC6872 retrieved as *Prorocentrum* sp. from the Roscoff Culture Collection, Roscoff, France). Scale bar = 5 μm.

FIG. 8. Scanning electron micrographs of *Prorocentrum criophilum* sp. nov. (strain RCC6872 retrieved as *Prorocentrum* sp. from the Roscoff Culture Collection, Roscoff, France). A. Anterior view of the right valve, and right-anterior view of the left valve. Note the row of three large pores in the anterior right valve (denoted as A, B, C). B–C. Valve view of the right valve. Note the pairs of pores in the posterior half of the valve. D. Posterior view. E. Inner right valve. F. View of the left valve. G–H. Detail of the periflagellar area. Uppercase and lowercase letters denote the large and small pores, respectively. The symbol * denotes other small pores. Apical collar (ac), auxiliary pore (ap), flagellar pore (fp), intercalary band (IB), left valve (LV), right valve (RV), single tooth (st). Scale bar = 2 μm (A–F), 1 μm (G–H).

FIG. 9. Line drawings of the right valve of the new species of *Prorocentrum*. The empty circles indicate the large and small pores. A. *Prorocentrum thermophilum* sp. nov. The inset shows the pattern of reticulation formed by the ridges joining the knobs. B. *Prorocentrum criophilum* sp. nov. The small points indicate the knobs. Scale bar = 2 μm.

FIG. 10. Scanning electron micrographs of cultured cells of *Prorocentrum cordatum* (A–B) (strain PmK from the Gulf of Trieste, Italy) and wild cells of *P. shikokuense* from the port of Brindisi, Italy (C–D). A. The arrows point the numerous thecal pores in the periphery of the valve. The arrowhead points the periflagellar area. B. Note the numerous trichocyst pores in the inner right valve. C–D. Note the shape variability from individuals collected in the same sample. Scale bar = 2 μm.

Figure S1. Illustrations of *Prorocentrum* from the literature.

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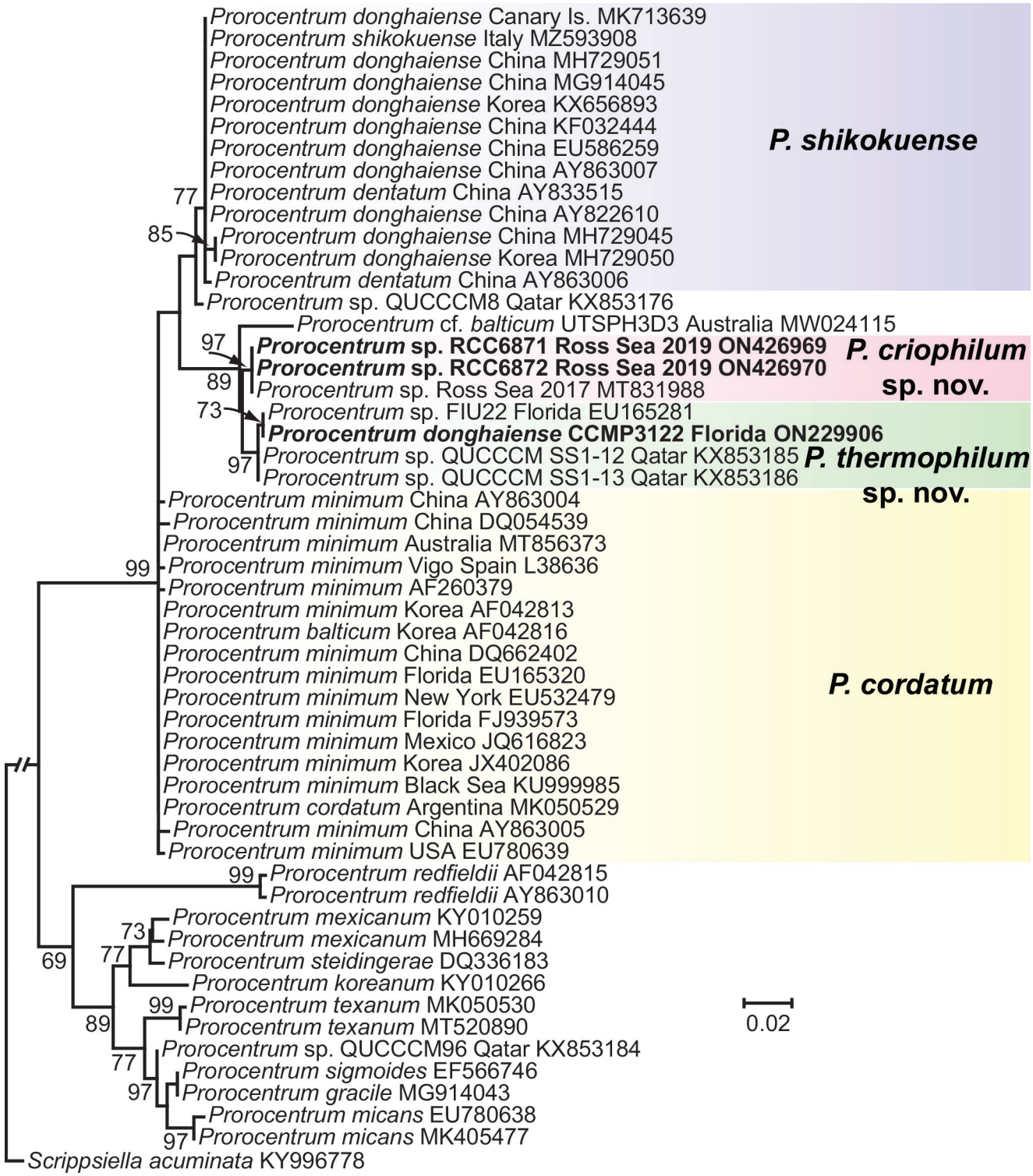

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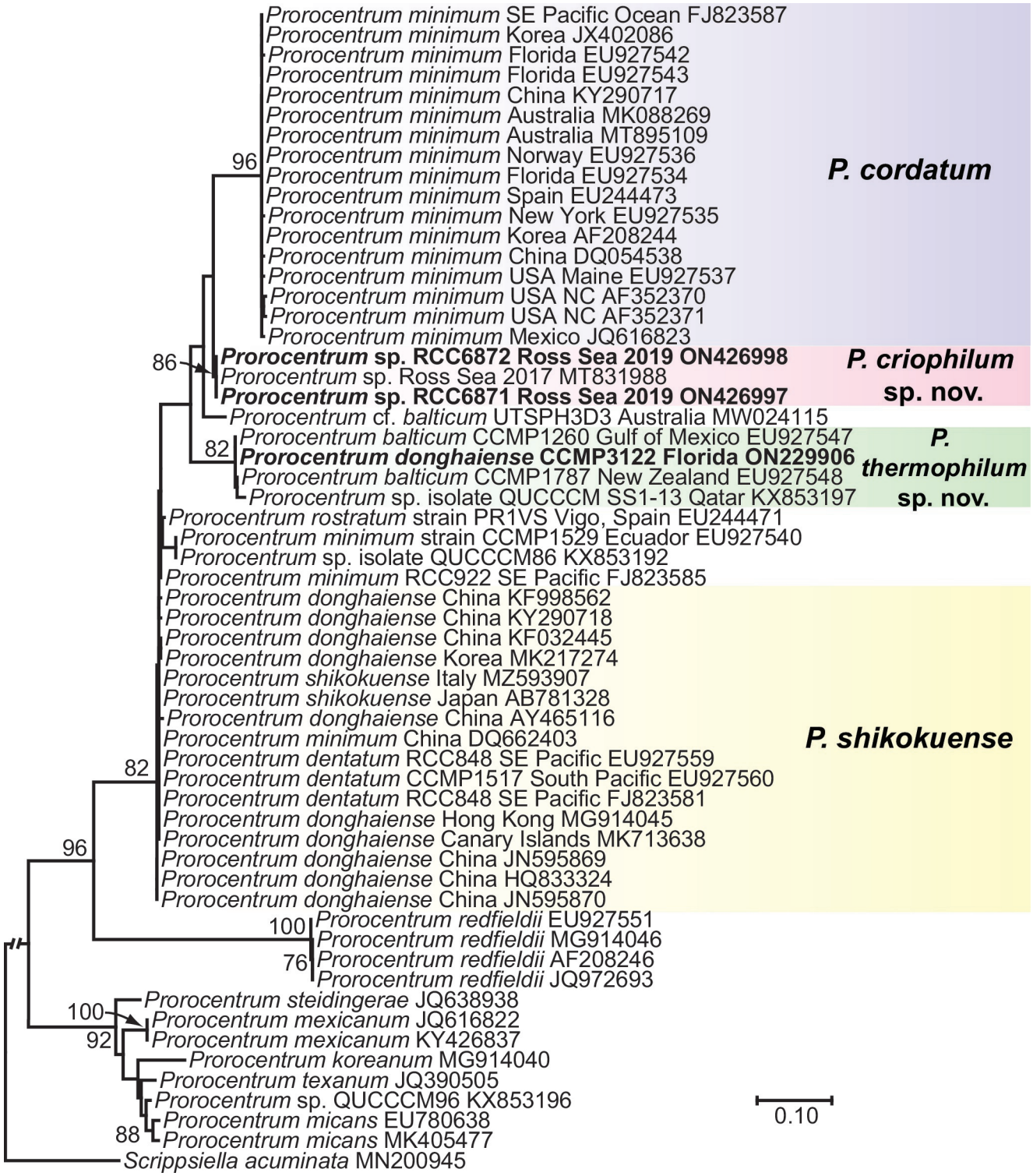

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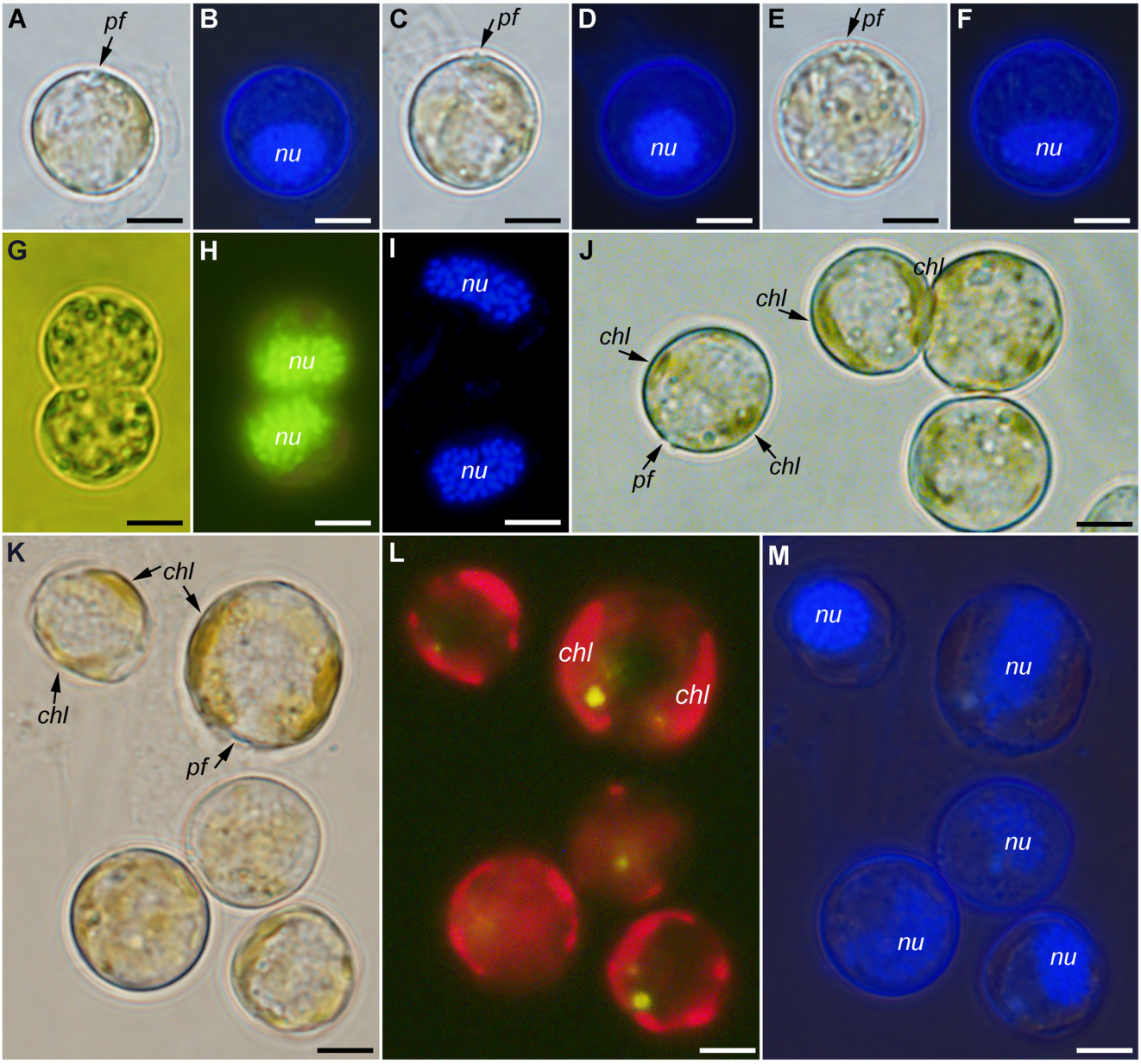

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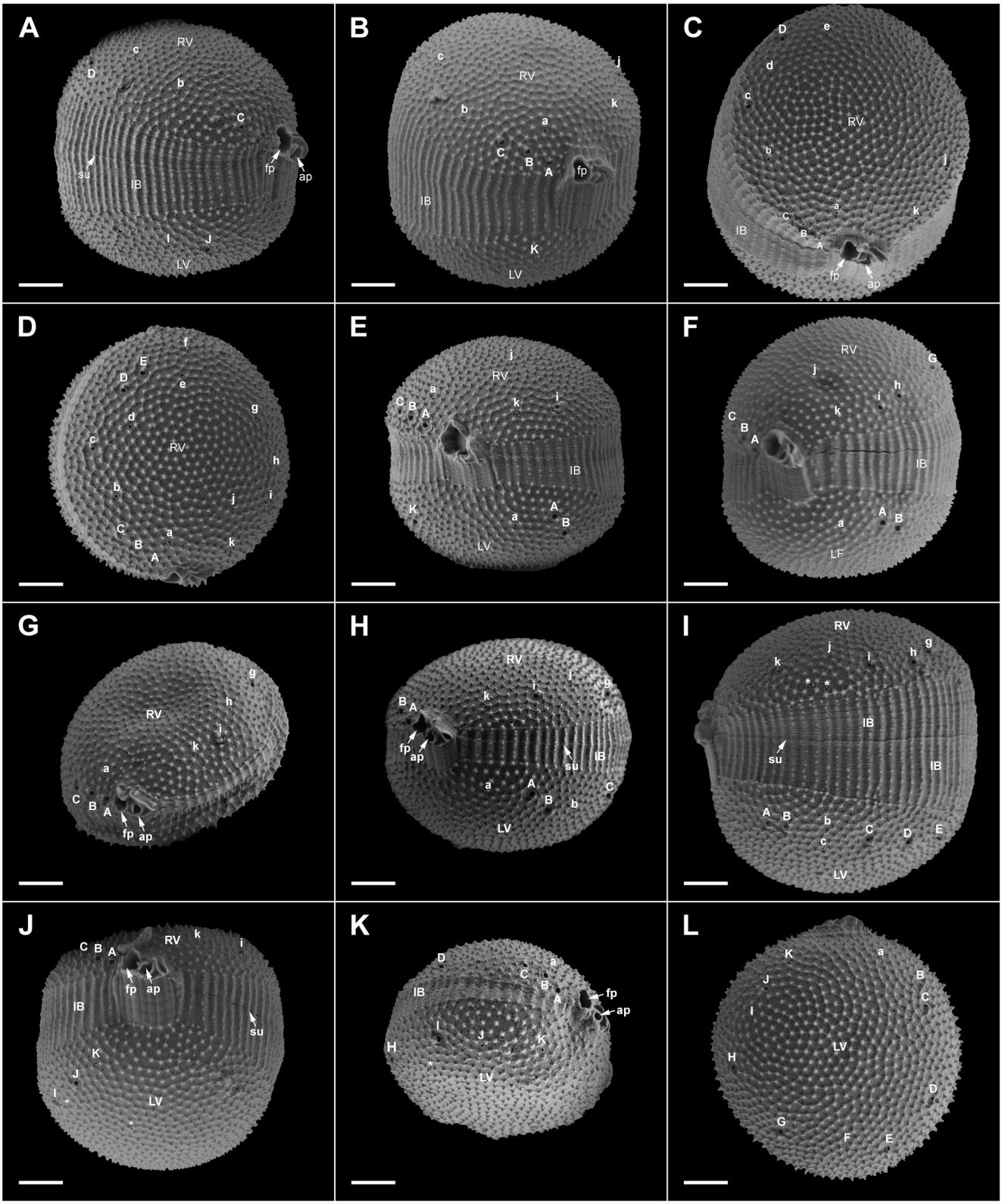

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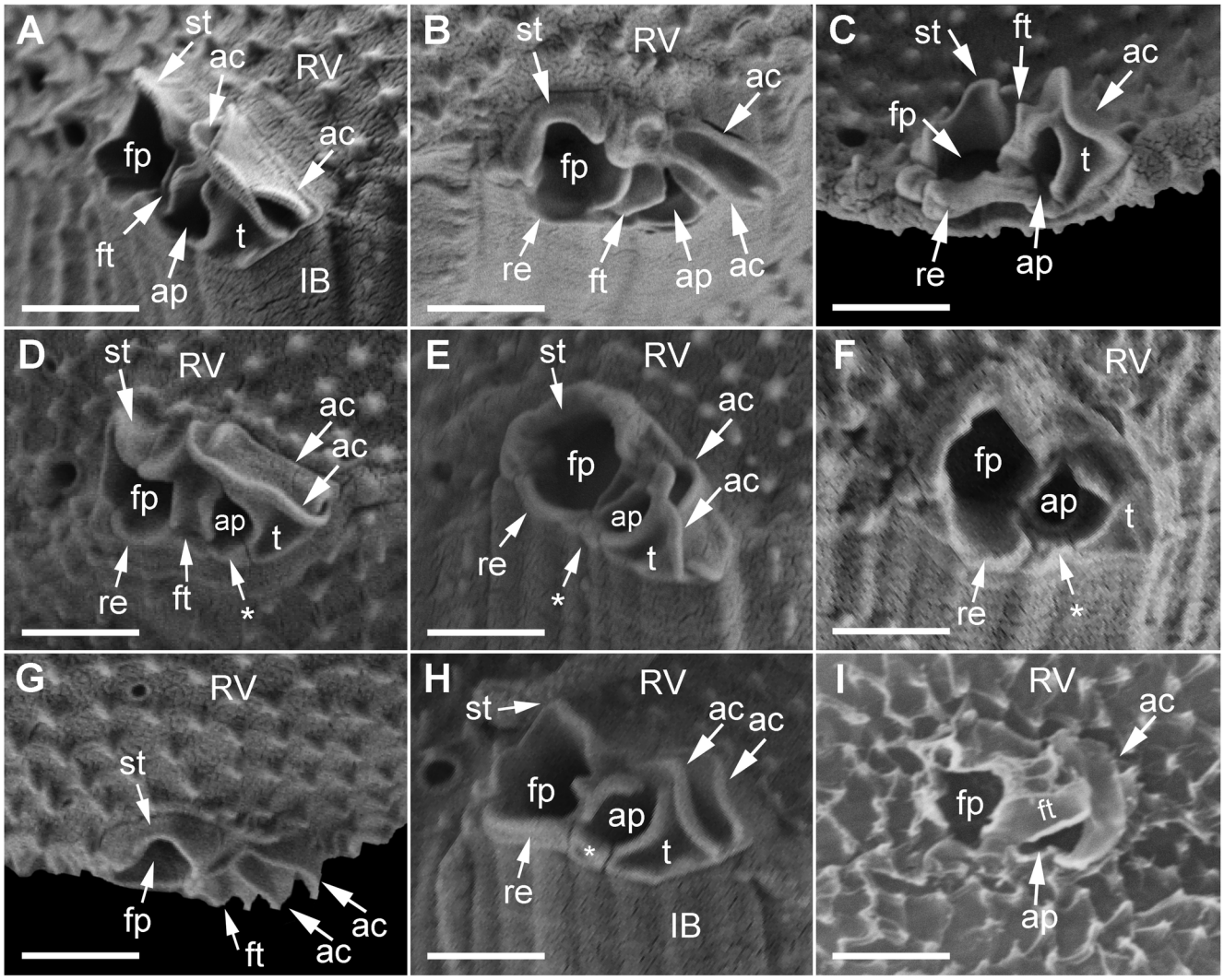

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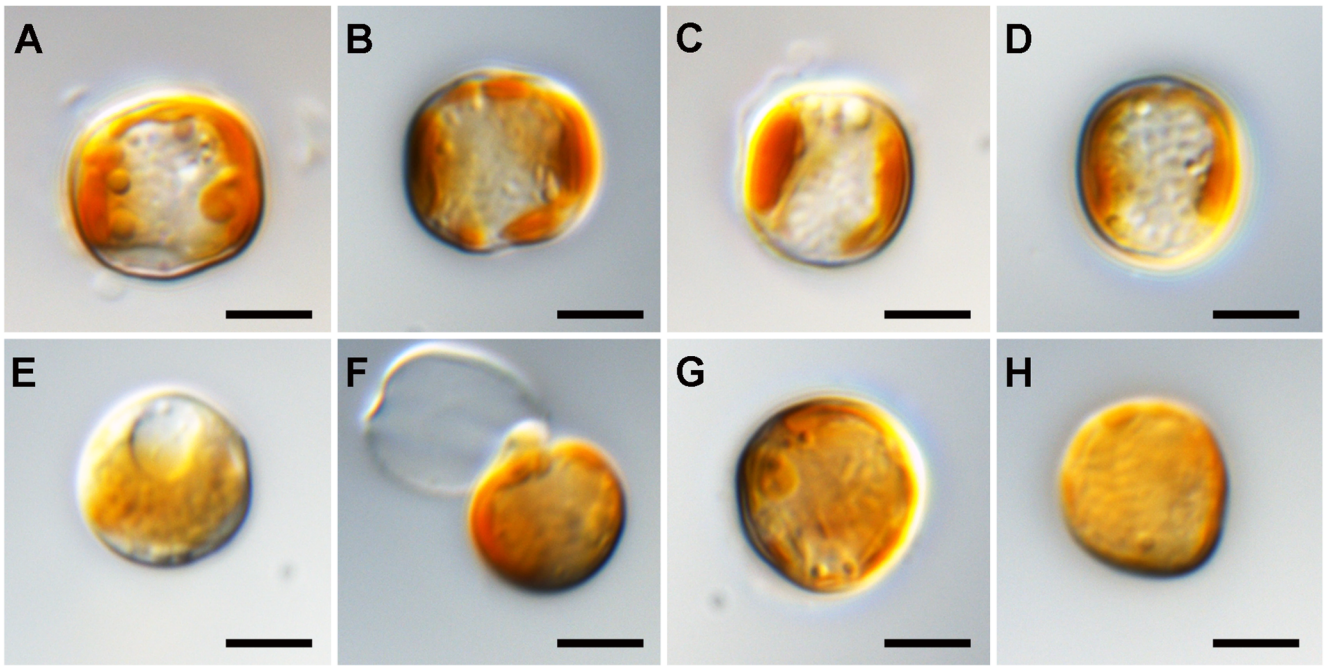

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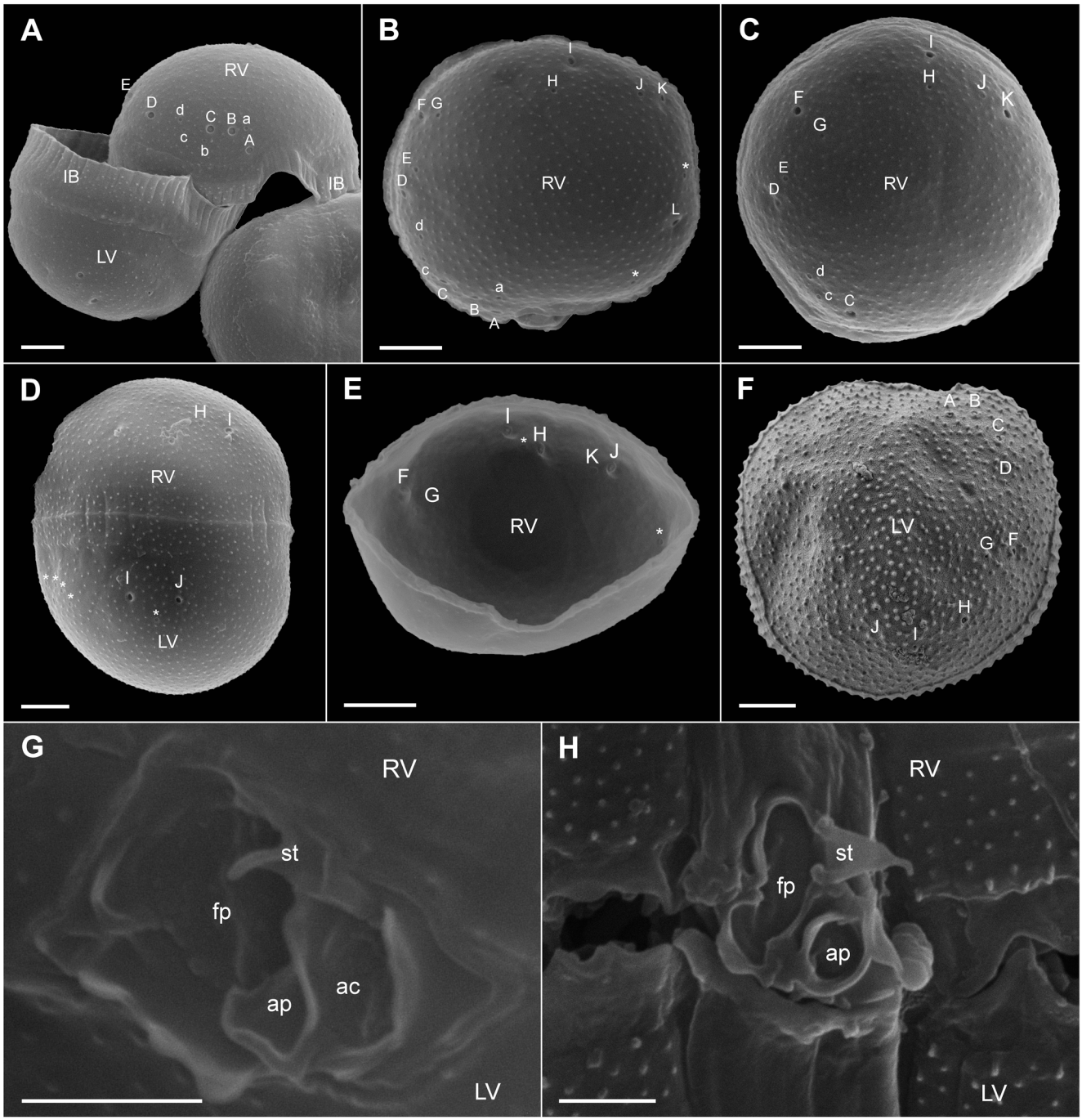

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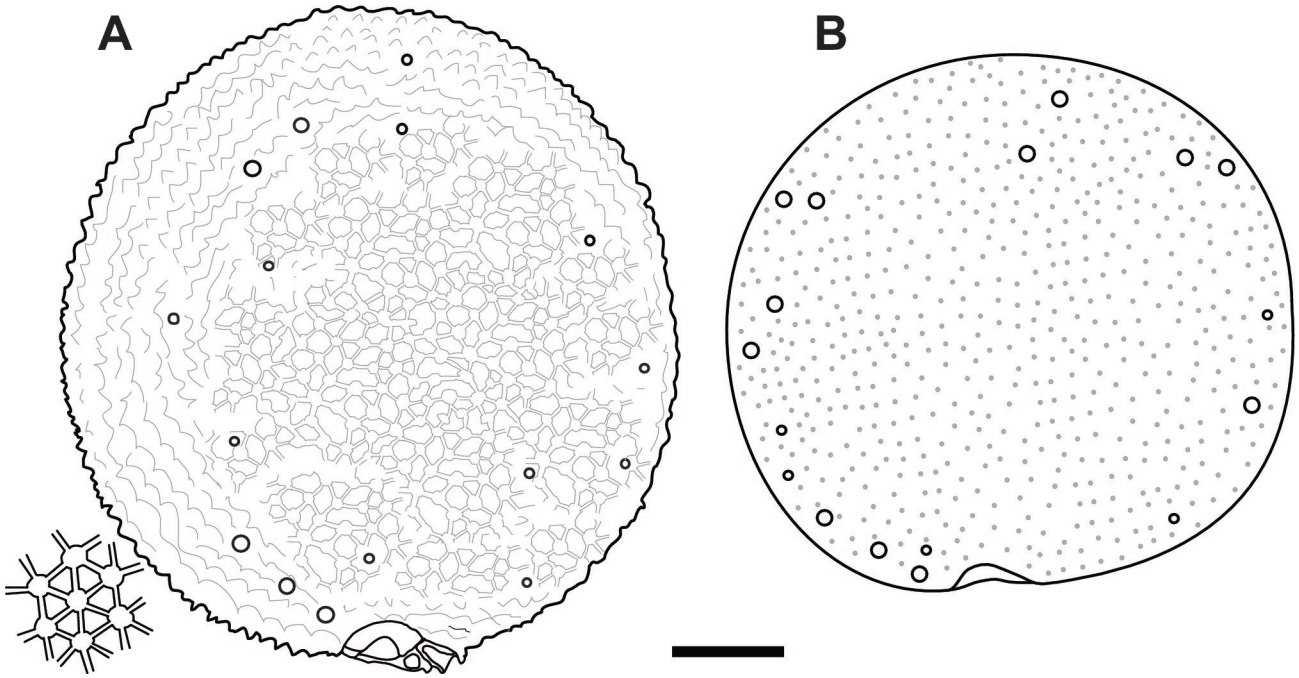

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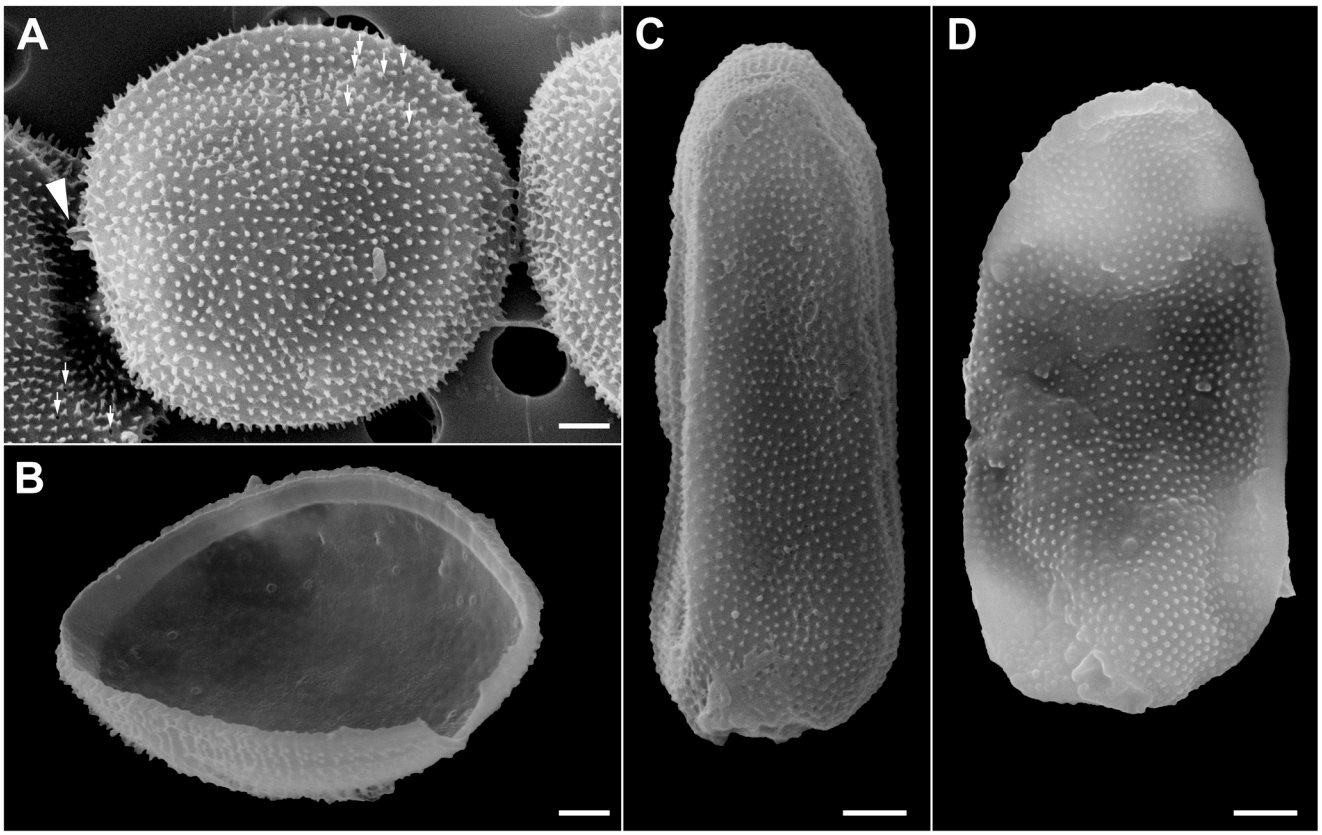

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