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New investigation of the cyst-motile relationship for *Votadinium spinosum* reveals a *Protoperidinium claudicans* species complex (Dinophyceae, Peridiniales)

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Abstract :

Protoperidinium claudicans is a planktonic, heterotrophic, bioluminescent dinoflagellate species commonly found in neritic waters. It has long been considered to display phenotypic variation in its second anterior intercalary plate, which could vary between quadra, penta and hexa. The equivalent spinose, cordate cyst goes under the name of Votadinium spinosum. Here we perform cyst incubation experiments from France, Canada, China and Japan, which demonstrate that P. claudicans forms a species complex, with at least two ribotypes with a penta configuration (P. claudicans) and one with a quadra configuration (P. carriae sp. nov.). A fossil-based cyst, V. multispinosum sp. nov., is described as the equivalent of P. carriae. Molecular phylogenetics using Large Subunit ribosomal DNA supports these observations. The cyst-theca relationship for Votadinium psilodora and another, undescribed, cordate, spineless Votadinium species are also reported from China. Macromolecular analyses of the cyst wall of V. multispinosum reveal it is comprised of a protein-rich carbohydrate compound. We show that this compound is not uncommon in dinoflagellate and ciliate cysts and that it is unlikely to preserve very well upon sedimentation and burial.

Keywords : Cysts, cordate, dinoflagellates, pinose, heterotrophic, Protoperidinium carriae, LSU rDNA, molecular phylogenetics, species complex

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1. Introduction

Protoperidinium is a large dinoflagellate genus that currently encompasses 311 heterotrophic species (Guiry in Guiry & Guiry 2023). This genus has been subdivided into several sections based on the structure of the first apical plate (1′) and the second anterior intercalary plate 2a (e.g., Gribble and Anderson 2006). One of these sections is called the *Oceanica* section, whichand includes all species with an ortho 1′ and quadra 2a. Among the species that belong to this section, there are several elongate, slender species such as *P. claudicans, P. oceanicum, P. claudicans, P. paraoblongum*, *P. quadrioblongum, P. steidingerae,* and *P. venustum* (Lebour 1925; Balech 1974; Sarai et al. 2013).

One of such elongate, slender species, *Protoperidinium claudicans*, was first described morphologically as *Peridinium claudicans* by Paulsen (1907, reproduced in Paulsen, 1908)

from the port of Frederikshavn on the east coast of Jutland (Denmark) (see also Matsuoka and Head, 2013 for a historical review of this species). His description did not include details of the tabulation. It was Barrows (1918) who first provided a typical protoperidinioid tabulation for this species that he observed on specimens from Sausalito, CA, U.S.A, showing a quadra~~ngular~~ 2a but also a penta 2a, the latter he considered aberrant due to ecological stress. A penta~~gonal~~ 2a has later been observed by Lebour (1923) at Plymouth, U.K. (reproduced in Lebour 1925) and later by Akselman (1987). However, other 2a shapes have been reported for this species: hexa (Lindemann 1924), hexa and quadra (Paulsen 1931), penta and quadra (Balech 1951; Phan-Tan et al. 2017), quadra (Balech 1988). Review papers have considered all three configurations possible (Dodge, 1982, Hoppenrath et al. 2009). This challenges the classification of *P. claudicans* in the section *Oceanica*, which only contains species with a quadra 2a. *P. claudicans* is considered a widespread species commonly occurring in plankton samples (e.g., Dodge 1982, 1985; Hoppenrath et al. 2009), that is known to prey on diatoms (Jacobson and Anderson 1986) and can be bioluminescent (Kelly 1968).

Wall & Dale (1966, 1967, 1968) related the morphology of cordate, spiny cysts from Woods Hole and Bermuda waters to *P. claudicans* with a penta~~gonal~~ 2a. Later hatching experiments by Dobell (1978), Akselman (1987), and Sonneman and Hill (1997) all observed a penta~~gonal~~ 2a. Reid (1977) related *P. claudicans* to a new cyst-defined species, *Votadinium spinosum*, described from surface sediments from Galway (Ireland) by Reid (1977, re-illustrated in Gurdebeke et al. 2020). *V. spinosum* is considered a widely distributed species (e.g., Zonneveld et al. 2013). *Votadinium* is a fossil genus including eight cordate species without processes ~~(*V. calvum, V. concavum, V. elongatum, V. nanhaiense, V. pontifossatum, V. psilodora, V. reidii, V. rhomboideum*)~~ but only one species with~~*V. spinosum* bears~~ processes (e.g., Gurdebeke et al. 2020; Table 1).

Later studies have enabled to link the morphology to genetic sequences. Molecular data for *Protoperidinium claudicans* was first provided by Yamaguchi et al. (2006) from Otaru, Japan, and demonstrated that it belonged to the *Oceanica* clade, together with *Protoperidinium depressum*. Later studies showed that other species related to cysts attributed to *Votadinium* also grouped in the same clade, and that it included species with a quadra, penta and hexa 2a (Sarai et al. 2013; Gurdebeke et al. 2020; Table 1).

~~*P. claudicans* is considered a widespread species commonly occurring in plankton samples (e.g., Dodge 1982, 1985; Hoppenrath et al. 2009), that is known to prey on diatoms (Jacobson and Anderson 1986) and can be bioluminescent (Kelly 1968).~~

Here we pursue research on *Votadinium* reinitiated most recently by Gurdebeke et al. (2020), and unveil the hidden diversity within the *P. claudicans* association through hatching experiments of cordate spinose cysts from France, Canada, China and Japan, in combination with single-cell PCR of the hatchlings. We demonstrate that *P. claudicans* forms a species complex and describe one new species and its equivalent cyst. We targeted cysts from France, which is not too far from the type localities of both the cyst- and motile-defined species (Ireland and Denmark respectively). Using infrared spectroscopy, we also compare the macromolecular composition of the wall of this new cyst ~~to~~ with those of resting stages of other phylogenetically closely related or spectrochemically similar taxa.

2. Material and methods

2.1. Germination experiments

We collected spinose, cordate cysts for incubation studies from surface sediment samples at several locations in four countries: (1) Patricia Bay in Saanich Inlet, Canada, (2) France, (3) China and (4) Japan (Figure 1 and Table 1). All samples were stored in plastic bags and

refrigerated at 4 °C. In situ sea-surface salinities (SSSs) and sea-surface temperatures (SSTs) were measured when collecting the samples at a few sites (Table 2).

Approximately 0.5–1.0 cm^3 of wet sediment was immersed in filtered seawater after which it was ultrasonicated in a bath (60 s) and rinsed through a 20 µm nylon mesh using filtered seawater. The cyst fraction was separated from this residue using heavy liquid sodium polytungstate (SPT; density = 1.3 g cm^{-1}) (Bolch 1997). Single cysts were then transferred to Nunclon 0.5 ml microwells subjected to an irradiance of 100 µmol photons m^{-2} s^{-1} and 24 h light, and filled with L1 medium. The wells were kept at ~~room temperature~~16°C. For Japanese samples, the sediment sample was ultrasonicated in a bath for 30 s and then sieved through 100 µm and retained on 20 µm sieves using filtered seawater. The cysts were isolated with a micropipette and individually placed in a multiple well plate (TPP, Trasadingen, Switzerland) each containing 1 ml of autoclaved seawater. The plates were ~~kept~~ stored in a culture cabinet at 20 °C with a photon flux density of ~~about~~ approximately 50 µmol photons m^{-2} s^{-1} under a 16 h: 8 h light: dark regime. Cysts were regularly checked for germination, and observations were performed under a Leitz DM inverted light microscope and a BX-50 light microscope (Olympus, Tokyo, Japan). Encysted and excysted cysts and~~as well as~~ motile cells were photographed and measured using a Leica DM 5000B light microscope equipped with a Leica DFC 490 camera with 100x oil immersion objectives. For each motile cell, the length, width, depth, distance between the tips of the antapical horns, and width of the cingulum were measured, where possible. For each cyst, the same parameters were measured; additionally, the length of three randomly chosen spines per cyst were measured. All measurements in the species descriptions cite, in order: the minimum, average (in parentheses) and maximum values (in µm). The standard deviation (SD) is also provided where appropriate. Incubation experiments were done by PM and took place at Concarneau (French and Canadian samples)

and by HG (Chinese samples) and by AY (Japanese samples). All measurements were done by KNM.

2.2. Single-cell PCR amplification and sequencing of the hatchlings.

Surface sediment samples containing spinose, cordate cyst were used from France and Canada (Figure 1 and Table 2). Cysts from France were isolated from the sediment using the heavy liquid separation described above. Hatched motile cells identified through light microscopy were rinsed several times in sterilized distilled water, and then transferred into a PCR tube. The single cell was used as the template to amplify approximately 3300 bp of the nuclear-encoded SSU-ITS-LSU rDNA, using the primers 18SFW (Grzebyk et al. 1998) and D3B (Nunn et al. 1996). A 20µl PCR cocktail containing 0.5 µM primers, 0.8U of KOD Hot Start Master mix (Novagen, Darmstadt, Germany) was subjected to 35 cycles using a thermocycler PCR Biometra TOne (Analytic Jena, Germany). The PCR protocol was: initial denaturation for 2 min at 95 ºC, followed by 35 cycles of 20 s denaturation at 95 ºC, 50 s annealing at 62 ºC, and 1 min extension at 70 ºC. From the PCR product obtained previously, 1µl is taken to carry out a new PCR. The PCR Master Mix kit (Promega, Madison, USA) is used according to the manufacturer recommendations. Different pairs of primers are used, D1R-D3B to obtain the LSU, ITSFW-364R for the ITS and 18SFW-18SRV for the SSU.The amplified products were run on a 1% agarose gel. Positive amplicons were purified using a DNA extraction kit ExoSAP-IT Cleanup (Affymetrix, Cleveland, Ohio, USA) and sequenced in both directions using the BigDye Terminator v3.1 technique (Applied Biosystems, Foster City, California, USA), according to the manufacturer recommendations.

For Japanese samples, cells were isolated using a micropipette from plankton samples or hatched from cysts using a CK-40 inverted light microscope (Olympus, Tokyo, Japan). The cells were identified and photographed following the method of Yamaguchi et al. (2006).

After the photography, the cell was transferred to a drop of autoclaved and filtered seawater on the clean glass slide several times and then it was transferred to a drop of sterile distilled water and broken by a sharp glass stick. The broken cell was transferred into a PCR tube that contains PCR reaction mixture for the first PCR reaction. The protocols for PCR and sequencing are the same as described by Yamaguchi et al. (2006). LSU rDNA sequences have previously been published (Yamaguchi et al. 2006), but their morphology is treated in more detail here.

Cysts from China were isolated from the sediment using the heavy liquid separation described above. ~~Germinated motile cell~~Hatchlings identified through light microscopy were rinsed several times in sterilized distilled water, and then transferred into a PCR tube. The single cell was used as the template to amplify about 1200 bp of the nuclear-encoded LSU rDNA, using the primers D1R (Scholin et al. 1994) and 28-1483R (Daugbjerg et al. 2000). A 50 µl PCR cocktail containing 0.2 µM primers, PCR buffer, 50 µM dNTP mixture, 1U of Ex Taq DNA polymerase (Takara, Dalian, China) was subjected to 35 cycles using a Mastercycler PCR (Eppendorf, Hamburg, Germany). The PCR protocol was: initial denaturation for 3.5 min at 94 ºC, followed by 35 cycles of 50 s denaturation at 94 ºC, 50 s annealing at 45 ºC, and 80 s extension at 72 ºC, plus a final extension of 10 min at 72 ºC. The amplified products were run on a 1% agarose gel. Positive bands were excised and purified using a DNA extraction kit (Sangon, Shanghai, China) and sequenced in both directions using the ABI Big-Dye dye-terminator technique (Applied Biosystems, Foster City, California, USA), according to the manufacturer recommendations.

Single-cell PCR was done by PM ~~and took place at~~in Concarneau for ~~(~~French and Canadian samples~~)~~, by HG for ~~(~~Chinese samples~~)~~ and AY for ~~(~~Japanese samples~~)~~.

2.3. Sequence alignments and phylogenetic analyses

Newly obtained sequences were first aligned with those of related species available in GenBank using 'BioEdit' v7.0.0 (Hall 1999), and subsequently using Mafft (Katoh et al. 2005) (http://mafft.cbrc.jp/alignment/server/). *Akashiwo sanguinea* (Hirasaka) G. Hansen & Moestrup was selected as the outgroup. A Bayesian reconstruction of the data matrix was performed with MrBayes 3.0b4 (Ronquist & Huelsenbeck 2003) using a general time reversible model (GTR +G) chosen by JmodelTest (Posada 2008). Four Markov chain Monte Carlo (MCMC) chains ran for two million generations, sampling every 1,000 generations with a burnin of 10%. A majority rule consensus tree was created in order to examine the posterior probabilities of each clade. Maximum likelihood-based analyses were conducted with RaxML v7.2.6 (Stamatakis 2006) on the T-REX web server (Boc et al. 2012) using the above model. Bootstrap values were determined with 1,000 replicates.

2.4. Light microscope and scanning electron microscope~~Palynological~~ study of palynologically prepared cysts from Canadian and French sediments

For light microscopy of type material from Canada, Holotype and paratype cyst specimens were extracted from surface sediments collected at Site 5 (UVic 2008-5) in Esquimalt Harbour (BC, Canada~~)~~, 44.855°N; -123.44295°W, water depth 8 m) by using a standardized palynological method (see details in Krepakevich and Pospelova 2010). These were photographed and measured using a Leica DM 5000B light microscope equipped with a Leica DFC 490 camera with 100x oil immersion objectives.

For scanning electron microscopy of material from Canada and France, samples from Canada (Saanich Inlet, Station A of Mertens et al. 2012) and France (Vilaine Bay, sample BV5 of Mertens et al. (2009) and Estuaire de la Vie, Station 10 of Liu et al. (2015)) were treated palynologically as in Mertens et al. (2012). Specimens were isolated with a micropipette using an IX51 (Olympus) inverted microscope and transferred onto

polycarbonate membrane filters (Millipore, Billerica, Massachusetts, USA; GTTP Isopore, 0.22 µm pore size), following a method proposed by Chomérat & Couté (2008). After 12–24 h of air-drying, the filters were affixed to aluminum stubs with adhesive tabs (Electron Microscopy Sciences, Hatfield, Pennsylvania, USA). Subsequently, the mounted filters were sputter coated with gold using a Cressington Sputter coater 108auto. The samples were observed at the Station of Marine Biology of Concarneau with a Sigma 300 (Zeiss) field-emission SEM equipped with a conventional Everhart-Thornley and in-lens detectors of secondary electrons at 1.5 and 5 kV. Digital images were saved in Tiff format (2048 × 1768 pixels). Adobe- Photoshop™ Creative Suite 5 (CS5) software was used to remove the background while maintaining the integrity of the original image.

2.5. Macromolecular characterization of the cyst wall

Several visually empty and clean dinoflagellate cysts were isolated onto an Au-coated mirror from filtered (fraction between 150–20 µm) and density-separated (using SPT; 2.1 g cm^{-1}) residues of surface sediment samples (Table 3), this for the macromolecular analysis of their cyst walls via Attenuated Total Reflectance microscope Fourier-transform infrared (ATR micro-FTIR) spectroscopy. For a more detailed description of this isolation procedure see Meyvisch et al. (2022). The collection method of the spectra was analogous to that used in Meyvisch et al. (2023). The resulting spectral dataset was further supplemented with previously acquired (in a similar fashion) spectra of other dinoflagellate cysts and one ciliate cyst *Halodinium verrucatum* (Gurdebeke et al. 2018). The spectra in this final dataset were processed in the open-source software Quasar (version 1.7.0; Toplak et al. 2021) using the Preprocess Spectra widget from the Spectroscopy add-on (version 0.6.8) and included (in that order): cutting out the region between 4000–600 cm^{-1}, Rubberband Baseline Correction,

Savitzky-Golay filtering (Window = 9, Polynomial Order = 2, Derivative Order = 0), and Vector Normalization.

3. Results

3.1. Results of germination experiments from Canada

Undescribed motile cells, here assigned to *Protoperidinium carriae* ~~n.~~ sp. nov., emerged from cordate, spinose cysts isolated from surface sediments from Patricia Bay, Saanich Inlet, BC, Canada (six specimens identified) (Figure 1 and Table 2). These motile cells germinated from the cysts after one or two days of incubation. Often, cells died a few days after germination and never divided. The cells and the corresponding cysts from where they hatched are described below as *Protoperidinium carriae*. The equivalent cyst is described as *Votadinium multispinosum* ~~n.~~ sp. nov.

Division DINOFLAGELLATA (Bütschli 1885) Fensome et al. 1993, emend. Adl et al. 2005

Class DINOPHYCEAE Pascher 1914

Subclass PERIDINIPHYCIDAE Fensome et al. 1993

Order PERIDINIALES Haeckel 1894

Family PROTOPERIDINIACEAE Balech 1988 nom. cons.

Subfamily PROTOPERIDINIOIDEAE (Autonym)

Genus ***Protoperidinium*** Bergh 1881

Protoperidinium carriae ~~n.~~ sp. nov.

Plate 1, figures 1–15, Plate 2, figures 1–6

Holotype. Plate 1, figures 4–9. An illustration is selected because of technical difficulties of specimen preservation (see article 40.5 of the ICN; Turland et al. 2018).

Type locality. Patricia Bay (48° 38.975′N, 123° 28.845′W), Saanich Inlet, B.C., Canada.

Diagnosis. A species of relatively large size of the genus *Protoperidinium* with the tabulation formula Po, X, 4′, 3a, 7″, 3c+t, ?s, 5‴, 2⁗. The motile cell is slender and dorsoventrally flattened, with a long apical horn and two long antapical horns. The epitheca is as long as the hypotheca, and both bear strongly concave sides. Plate 1′ is ortho-type, 1a and 3a are hexa~~gonal~~, and 2a is quadra~~ngular~~ and isodeltaform planate. Thecal plates display polygonal reticulations. The cyst is cordate and light brown in color, with purplish cell contents ~~as well~~. The epicyst bears a pronounced apex with slightly concave to straight sides and the hypocyst has slightly concave to slightly convex sides with two pronounced antapical lobes, separated by a pronounced antapical depression. The cyst surface appears smooth using a light microscope~~under a LM~~, bearing numerous nontabular, short, solid, erect or slight curved, and non-branching processes with acuminate tips all over the cyst surface. The archeopyle corresponds to the second anterior intercalary plate and is isodeltaform planate, rounded and saphopylic.

Derivation of name. The specific epithet is in recognition of the artist Emily Carr (1871–1945), who lived and worked on Vancouver Island.

Gene sequence. The LSU rDNA gene sequence of the motile stages hatched from the cysts—GenBank Accession No. OR879798–OR879800~~XXXXXX~~ (LSU).

Description. *Description of motile cell of* Protoperidinium carriae *(Plate 1, figures 4–9, 12–15; Plate 2, figures 4–6).* The excysted motile cells (six observed and not preserved) were slender and dorsoventrally flattened with a long apical horn and two long antapical horns; the

left one is somewhat shorter than the right one (Plate 1, figures 4–6, Plate 2, figure 4). The epitheca is as long as the hypotheca, and both bear strongly concave sides. The cell contents were purplish. The thecal plates displayed polygonal reticulations (Plate 1, figure 12).

The plate arrangement on the epitheca was bilaterally symmetrical. The oval apical pore plate (Po) was surrounded by a low apical collar formed by the raised edges of the apical plates (Plate 2, figure 5). The canal plate (X) was elongate (Plate 2, figure 5). The first apical plate (1′) was wide and rhombic (ortho-type) and the sides of plate 1′ contacting plates 2′ and 4′ are longer than those contacting plates 1″ and 7″ (Plate 1, figure 9). Plates 2′ and 4′ were elongated and subpentagonal, whereas 3′ was pentagonal (Plate 1, figure 13–14). The first and third anterior intercalary plates (1a, 3a) were hexagonal and equal in size (Plate 1, figure 14). The second anterior intercalary plate (2a) was quadrangular, isodeltaform planate (Plate 1, figure 14). The precingular series consisted of seven plates. Plate 1″, 4″ and 7″ were quadrangular (Plate 1, figures 9, 13), and 2″, 3″, 5″ and 6″ pentagonal (Plate 1, figures 13–14). The cingulum was slightly left-handed (descending), lined with narrow lists and comprising three cingular plates plus a transitional plate (Plate 1, figure 9). The transitional plate (t) was small. Plate 1c reached the end of plate 1″ and 2‴ (Plate 2, figure 5). Plate 2c was the longest of the series and reached ended before the 6″/7″ boundary and the 4‴/5‴ boundary (Plate 1, figure 13). Plate 3c was wider in size thano Plate 1c.

We were unable to dissect and observe the sulcal plates. Sa intrudes into the epitheca (Plate 1, figure 9).

The plate arrangement of the hypotheca was also symmetrical, featuring five postcingular plates. Plate 5‴ was wider than plate 1‴. Plates 1‴, 3‴, and 5‴ were pentagonal, and 2‴ and 4‴ were quadrangular (Plate 1, figures 7, 15). The antapical series comprised two plates, 1⁗ and 2⁗, which formed the antapical horns (Plate 1, figure 8).

The plate formula is thus Po, X, 4′, 3a, 7″, 3c+t, ?s, 5‴, 2⁗.

Description of cyst of Protoperidinium carriae *(Plate 1, figures 1–3, 10–11; Plate 2, figures 1–3).* The peridinioid cysts were cordate, light brown in color, and bearing numerous short solid spines. Living cysts contained abundant purplish and transparent granules (Plate 1, figure 1). The epicyst bears a pronounced apex with slightly concave to straight sides and the hypocyst has slightly concave to slightly convex sides with two pronounced antapical lobes, separated by a pronounced antapical depression. The central body wall was thin (>0.3 µm), with an apparent smooth outer and inner surface using a light microscope ~~under LM~~ (Plate 2, figures 1–2). The processes were numerous, nontabular, short, solid, erect or slightly curved, and non-branching processes with acuminate tips (Plate 1, figures 1–3, 10–11). The process length was fairly constant for individual specimens, except around antapical horns where they became longer (Plate 1, figure 10). The paracingulum was not visible. The parasulcus was beset with processes and was indented, flagellar scars were not visible. The archeopyle was rounded, intercalary and saphopylic, involving the release of plate 2a, isodeltaform planate. The description is based on cysts used in the incubation experiments.

Dimensions. Incubated motile cells: length, 97.0 (109.6) 131.0 µm (SD = 12.6, n=6); width, 62.0 (53.6) 90.0 µm (SD = 9.7, n=6); distance between the tips of the antapical horns, 25.7 (36.9) 46.0 µm (SD = 18.1, n=4).

Cysts germinated to give identifiable thecae: length, 58.0 (64.0) 69.4 µm (SD = 4.7, n=4); width, 62.6 (67.3) 70.7 µm (SD = 3.4, n=4); distance between the tips of the antapical horns, 29 (31.1) 32.8 µm (SD = 1.6, n=4); average length of three spines per cyst, 3.5 (4.9) 5.9 µm (SD = 1.0, n=4); average number of processes per 10x10 µm, 19.0 (22.8) 26.7 (SD = 4.4, n=2).

Genus ***Votadinium*** Reid 1977, emend. Gurdebeke et al. 2020

Votadinium multispinosum ~~n.~~ sp. nov.

Plate 2, figures 7–12

Synonyms.

2004 cyst of *Protoperidinium claudicans*: Orlova et al., 2004, figs. 44–45 (not 46).

2010 *Votadinium spinosum*: Krepakevich and Pospelova pl. 3f.

2010 *Votadinium spinosum*: Pospelova et al. 2010, pl. IV, Fig. 4

2011 *Votadinium spinosum*: Price and Pospelova, pl. IV, fig. 5.

2016 *Votadinium spinosum*: Bringué et al., plate III, fig. 6.

2020 *Votadinium spinosum*: Gurdebeke et al. 2020, pl. 7, figs. 5–12.

2020 *Votadinium spinosum*: Van Nieuwenhove et al., pl. 26, figs. 4~~,~~–5, 7~~,~~–8.

Motile stage equivalent. *Protoperidinium carriae* ~~n.~~ sp. nov., this study.

Holotype. Surface sediment sample from Esquimalt Harbour (BC, Canada), Site 5 (44.855°N; -123.44295°W), water depth 8 m, palynological residue mount UVic ID 2008-5, slide 1, England Finder reference P 56-0 (label on the left); Plate 2, figures 7–9. Kept at the Centre of Excellence for Dinophyte Taxonomy (CEDiT), Herbarium Senckenbergianum Wilhelmshaven, Germany with designation CEDiT2023H168 (Otte et al. 2011).

Paratype. Surface sediment sample from Esquimalt Harbour (British Columbia, Canada), Site 5 (44.855°N, -123.44295°W), water depth 8 m, palynological residue mount UVic ID 2008-5, slide 1, England Finder reference Q 41-2/0 (label on the left); Plate 2, figures 10–12. Kept at the Centre of Excellence for Dinophyte Taxonomy (CEDiT), Herbarium Senckenbergianum Wilhelmshaven, Germany with designation CEDiT2023P169 (Otte et al. 2011).

Type locality. Esquimalt Harbour (BC, Canada), Site 5 (44.855°N; -123.44295°W), water depth 8 m, on Vancouver Island, B.C., Canada.

Diagnosis. The cyst is cordate and light to medium brown in color. The epicyst bears a pronounced apex with slightly concave to straight sides and the hypocyst has slightly concave to slightly convex sides with two pronounced antapical lobes, separated by a pronounced antapical depression. The cyst surface appears smooth ~~under a LM~~using a light microscope, bearing numerous nontabular, short, solid, erect or slight curved, and non-branching processes with acuminate tips all over the cyst surface. The archeopyle is isodeltaform planate, rounded and saphopylic.

Derivation of name. The specific epithet is in reference to the high number of processes all over the cyst surface.

Description. The peridinioid cysts were cordate, light to medium brown in color, and ~~bearing~~ bore numerous small solid spines. The epicyst bears a pronounced apex (Plate 1, figure 1) with slightly concave to straight sides and the hypocyst has slightly concave to slightly convex sides with two pronounced antapical lobes, separated by a pronounced antapical depression. The central body wall was thin (>0.3 μm), with an apparent smooth outer and inner surface using a light microscope ~~under LM~~ (Plate 2, figure 7). The processes were numerous, nontabular, short, solid, erect or slight curved, and non-branching processes with acuminate tips all over the cyst surface (Plate 2, figures 7–12). The process length was fairly constant for individual specimens, except around antapical horns where they became longer (Plate 1, figure 10). The paracingulum was not visible. The parasulcus was beset with processes and was indented, flagellar scars were not visible. The archeopyle was rounded,

intercalary and saphopylic, involving the release of plate 2a, isodeltaform planate. The description is based on cysts extracted from the sediments. SEM observations show that the outer surface of the cyst wall consists out of very fine fibrils, whilst the inner surface is smooth (Plate 8, figure 1–2).

Dimensions. Cysts from palynological preparations: length, 58.5 (64.7) 71.1 µm (SD = 4.1, n=14); width, 56.1 (64.7) 75.1 µm (SD = 6.3, n=14); distance between the tips of the antapical horns, 25.6 (31.4) 37.5 µm (SD = 3.5, n=14); average length of three spines per cyst, 2.8 (4.0) 7.7 µm (SD = 1.2, n=14); average number of processes per 10x10 µm, 16.7 (22.9) 38.0 (SD = 7.6, n=4).

Comments. The geological preservability of the cysts was demonstrated by their ability to withstand palynological treatment and presence in sediments at least as old as ~41 kyr on the California margin (Pospelova et al.~~,~~ 2015). In the northeastern Pacific Ocean, this species is only occasionally found in the pre-Holocene samples from the California margin, the Santa Barbara Basin, or the Gulf of California (Pospelova et al.~~,~~ 2006, 2015; Price et al.~~,~~ 2013) where it rarely contributes >1% of the cyst assemblages. *Votadinium multispinosum* ~~n.~~ sp. nov. has not been reported from the Last Interglacial in the Santa Barbara Basin (MIS5 & 6; Over and Pospelova 2022) or the entire Middle Pleistocene to Holocene from the Gulf of Alaska (Marret et al.~~,~~ 2001). However, it has been found as far as Alaska, but only in a few recent sediment samples from estuarine waters of Prince William Sound (Pospelova, personal ~~communication~~observations~~?~~). The highest abundances of *V. multispinosum* ~~n.~~ sp. nov. (~5-8%) were documented in surface sediment samples from Esquimalt Harbour on Vancouver Island where water depth is ~10 m, sea-surface temperature ranges from ~7°C in February to ~12°C in August, sea surface salinity at ~30 psu, and waters can be characterised as nutrient-

rich. The precise geographical distribution of *V. multispinosum* ~~n.~~ sp. nov. is not clear at this stage, as it has been grouped with other similar spiny taxa (e.g., *V. spinosum*) in all previous publications, but based on those publications that include good cyst illustrations (see in the list of synonyms above) or when the authors of this paper can verify in their palynological slides, we can conclude that this species could be found in coastal waters of the northern Pacific, especially in highly productive estuarine environments (e.g., Pospelova et al. 2008; Radi et al. 2007). The type localities of *P. carriae* and its equivalent *V. multispinosum* are not identical but are both from around Vancouver Island, where both are considered widely distributed.

3.2. Results of germination experiments from France, China and Japan

Undescribed motile cells, here assigned to *Protoperidinium claudicans*, emerged from cordate, spinose cysts isolated from surface sediments from Maresclé, Morbihan, France (six specimens identified). Additionally, ~~,~~ one cyst each was obtained from Fangchenggang, Guangxi, China and ~~one cyst~~ from Quanzhou, Taiwan Strait, China and one cyst. Furthermore, ~~,~~ one motile cell hatched from a cyst and one motile cell isolated from plankton were collected in~~from~~ Hokkaido, Japan (Figure 1 and Table 2). These motile cells germinated from the cysts after one or two days of incubation. Often, cells died a few days after germination and never divided. The cells and the corresponding cysts from where they hatched are described below as *Protoperidinium claudicans*. Two other *Votadinium*-like cysts were hatched from surface sediments from Fangchenggang, Guangxi, China and sequenced and are described ~~below~~ and illustrated here (*Votadinium psilodora* and *Votadinium* sp. 1).

Genus ***Protoperidinium*** Bergh 1881

Protoperidinium claudicans (Paulsen) Balech 1974

Plate 3, figures 1–12, Plate 4, figures 1–15 (French specimens)

Plate 5, Figs. 1–9 (Asian specimens)

Basionym. Paulsen 1907, p. 16, fig. 22.

Type locality. Port of Frederikshavn, east coast of Jutland (Paulsen 1907).

Diagnosis. A species of relatively large size of the genus *Protoperidinium* with the tabulation formula Po, X, 4′, 3a, 7″, 3c+t, ?s, 5‴, 2⁗. The motile cell is slender and dorsoventrally flattened, with a short apical horn and two short antapical horns. The epitheca is as long as the hypotheca, and both bear slightly convex sides. Plate 1′ is of the ortho-type and asymmetrical, 1a and 2a are pentagonal, and 3a is hexagonal. 2a is dextrocamerate. Plates display polygonal reticulations. The cyst is cordate and light brown in color, with orange or transparent cell contents as well. The epicyst bears an apex with convex sides and the hypocyst has convex sides with two antapical lobes, separated by an antapical depression. The cyst surface appears smooth using a light microscopeunder a LM, bearing nontabular, short, solid, erect, or slightly curved, and non-branching processes with acuminate tips. The sulcal area can have a reduced number of processes. The archeopyle is dextrocamerate, rounded and saphopylic.

Derivation of name. Although not specified by Paulsen (1907), it is likely that the epithet is derived from Latin *claudicans,* meaning limping, because of the unequal length of the antapical horns.

Gene sequence. The LSU rDNA gene sequence of the motile stages hatched from the cysts from France (ribotype 1) —GenBank Accession No. OR879790–OR879792XXXXXX (LSU) and from Asia (ribotype 2) —GenBank Accession No. OR879793–OR879795XXXXXX (LSU).

Description. *Description of motile cell of* Protoperidinium claudicans *(Plate 3, figures 4–6, 10–12, Plate 4, figures 5–9, 12–15).* The excysted motile cells (six observed and not preserved) were slender and dorsoventrally flattened with a short apical horn and two short antapical horns; the left one is somewhat shorter than the right one (Plate 3, Figure 3, Plate 4, Figure 5). The epitheca is as long as the hypotheca, and both bear slightly convex sides. The thecal plates displayed polygonal reticulations. The cell contents were orange or transparent in French specimens (Plate 3, figure 3; Plate 4, figure 5), transparent or reddish in Chinese and Japanese specimens (Plate 5, figures 1, 3).

The plate arrangement on the epitheca was bilaterally symmetrical, except for the shape of the anterior intercalary plates and the first apical plate. The oval apical pore plate (Po) was surrounded by a low apical collar formed by the raised edges of the apical plates. The canal plate (X) was elongate. The first apical plate (1′) was wide and rhombic (ortho-type) and asymmetrical and the sides of plate 1′ contacting plates 2′ and 4′ are longer than those contacting plates 1″ and 7″ (Plate 3, figures 5–6). Plates 2′ and 4′ were elongated and subpentagonal, whereas 3′ was pentagonal (Plate 4, figure 7). 1a is pentagonal, and 3a is hexagonal (Plate 3, figure 5–6). The second anterior intercalary plate (2a) was pentagonal and, dextrocamerate (Plate 1, figure 5). The precingular series consisted of seven plates. Plate 1″, 5″ and 7″ were quadrangular (Plate 3, figure 4, Plate 4, figure 14), and 2″, 3″, 4″ and 6″ pentagonal (Plate 3, figures 5, 6). The cingulum was slightly left-handed (descending), lined with narrow lists and comprising three cingular plates plus a transitional plate. The transitional plate (t) was small (Plate 4, figure 8). Plate 1c reached before the end of plate 1″ (Plate 4, figure 7). Plate 2c was the longest of the series and reached ended at the 6″/7″ boundary and the 4‴/5‴ boundary (Plate 4, figure 13). Plate 3c was wider in size thano Plate 1c.

We were unable to dissect and observe the sulcal plates. Sa intrudes into the epitheca (Plate 3, figures 4, 9; Plate 4, figures 8, 13).

The plate arrangement of the hypotheca was symmetrical, featuring five postcingular plates. Plate 5‴ was wider than plate 1‴. Plates 1‴, 3‴, and 5‴ were penta~~gonal~~, and 2‴ and 4‴ were quadra~~ngular~~ (Plate 3, figure 11). The antapical series comprised two plates, 1⁗ and 2⁗, which formed the antapical horns (Plate 3, figure 11).

The plate formula is thus Po, X, 4′, 3a, 7″, 3c+t, ?s, 5‴, 2⁗.

Description of cyst of Protoperidinium claudicans *(Plate 3, figures 1, 7; Plate 4, figures 1–4; 10–11).* The peridinioid cysts were cordate, light brown in color, and bearing small solid spines. Living cysts contained abundant orange and transparent granules. The epicyst bears an apex with convex sides and the hypocyst has convex sides with two antapical lobes, separated by an antapical depression. The central body wall was thin (>0.3 µm), with an apparent smooth outer and inner surface using a light microscope~~under LM~~ (Plate 4, figure 1). The processes were numerous, nontabular, short, solid, erect, or slight curved, and non-branching processes with acuminate tips (Plate 3, figures 1, 7; Plate 4, figures 1–4). The process length was fairly constant for individual specimens, except around antapical horns where they became longer (Plate 4, figure 1). The paracingulum was not visible. The parasulcus can be beset with processes, although often there are less ~~processes~~ present and was indented, flagellar scars were not visible. The archeopyle was rounded and saphopylic, involving the release of plate 2a, dextrocamerate. The description is based on cysts used in the incubation experiments.

Dimensions. Incubated motile cells from France: length, 49.0 (68.1) 82.0 µm (SD = 12.9, n=6); width, 34.3 (50.7) 60.8 µm (SD = 9.5, n=6); distance between the tips of the antapical horns, 16.0 (25.2) 32.1 µm (SD = 11.6, n=5).

Cysts germinated to give identifiable thecae from France: length, 46.7 (51.4) 56.2 µm (SD = 3.2, n=6); width, 47.3 (52.9) 55.1 µm (SD = 2.9, n=6); distance between the tips of the antapical horns, 28.4 (29.9) 31.9 µm (SD = 1.2, n=6); average length of three spines per cyst, 3.9 (5.7) 8.1 µm (SD = 1.8, n=6); average number of processes per 10x10 µm, 7 (11.4) 15.3 (SD = 2.9, n=6).

Incubated motile cells from Asia: length, 71.4 (73.5) 75.6 µm (SD = 2.9, n=2); width, 42.9 (47.5) 52.1 µm (SD = 6.5, n=2); distance between the tips of the antapical horns, 27.6 (28.1) 28.6 µm (SD = 0.7, n=2).

Cysts from Asia: length, 43.2 (46.4) 50.2 µm (SD = 3.6, n=3); width, 40.0 (49.3) 59.4 µm (SD = 9.7, n=3); distance between the tips of the antapical horns, 26.3 (28.1) 31.0 µm (SD = 2.5, n=3); average length of three spines per cyst, 4.4 (5.1) 6.3 µm (SD = 1.0, n=3).

Comments. The geological preservability of the cysts was demonstrated by their ability to withstand palynological treatment. The equivalent cyst is considered to be *Votadinium spinosum.* SEM observations of cysts extracted from palynologically treated sediments show that the outer surface of the cyst wall consists out of very fine fibrils, whilst the inner surface is smooth (Plate 8, figure 3–6). There were only small morphological differences between both ribotypes of *P. claudicans*: the width of the 2a is somewhat smaller in the former than in the latter and the 4'' is somewhat higher in the former than in the latter. Between the corresponding cysts, no clear differences could be observed. The genetics did demonstrate a clear separation of both ribotypes.

Genus *Votadinium* Reid 1977, emend. Gurdebeke et al. 2020

Votadinium psilodora (Benedek 1972) Gurdebeke et al. 2020

Plate 6, figures 1–9

Synonyms.

1972 *Lejeunecysta psilodora*: Benedek, pl. 6, fig. 5.

1981 *Selenopemphix nephroides*, pars: Benedek & Sarjeant, figs 8, 3–4.

1987 *Lejeunecysta psuchra*: Matsuoka, pl. 9, figs 7–8.

1988 *Votadinium calvum* Reid: Bint, fig. 3-J.

1988 *Lejeunecysta psilodora* (Benedek) Artzner & Dorhofer: Gruas-Cavagnetto & Barbin, pl. V, figs 12–13.

1989 Cyst of *Protoperidinium oblongum*: Kojima, fig. 6–2.

2000 *Protoperidinium* sp. 1: Cho, p. 24, pl. 2, fig. 8.

2004 *Lejeunecysta psuchra*: Louwye et al., fig. 10r–s.

2010 Cyst of *Protoperidinium* spp.: Pospelova et al., pl. 5, fig. 5.

2018 Cysts of *Protoperidinum oblongum* Li et al., pl. VI, fig. 11 only.

2020 *Votadinium* ?*pontifossatum* Li et al., pl. III, fig. 11.

2020 *Votadinium psilodora* Gurdebeke et al., pl. 4, figs. 1–11.

Motile stage equivalent. Unknown *Protoperidinium* sp. with ortho 1′ and quadra~~ngular~~ 2a (Plate 6, figures 7–9).

Description. The peridinioid cysts were penta~~gonal~~ in ambitus, light brown in color. The epicyst bears a pronounced apex with slightly convex sides and the hypocyst has slightly convex sides with two elongated antapical horns, separated by a pronounced antapical depression. The central body wall was thin (>0.3 µm), with an apparent smooth outer and inner surface using a light microscope~~under LM~~. No ornamentation was visible. The paracingulum was slightly visible (Plate 6, figures 2, 6). The parasulcus was indented, flagellar scars were not observed. The archeopyle was rounded, intercalary and saphopylic,

possibly involving the release of plate 2a. The description is based on cysts used in hatching experiments.

Gene sequence. The LSU rDNA gene sequence of the motile stages hatched from the cysts from China —GenBank Accession No. OR879796–OR879797~~XXXXXX~~ (LSU).

Dimensions. Cysts extracted from Chinese sediments: length, 69.0 (71.6) 76 µm (SD = 3.8, n=3); width, 60.0 (60.3) 61.0 µm (SD = 0.6, n=3); depth, 43.0 µm (n=1).
Incubated motile cells from China: length, 80.0 (82.5) 85.0 µm (SD = 3.5, n=2); width, 55.0 (57.5) 60.0 µm (SD = 3.5, n=2); depth, 45.0 (45.0) 45.0 µm (SD = 0.0, n=2).

Comments. Although the cell was hatched from the cyst, we consider that there was not enough information to fully describe the species.

Votadinium sp. 1

Plate 7, figures 1–9

Synonyms. None.

Motile stage equivalent. Unknown *Protoperidinium* sp. with ortho 1′ and quadra~~ngular~~ 2a (Plate 7, figures 4–9).

Description. The cordate cysts were light to medium brown in color. The epicyst is shorter than the hypocyst and bears an apex with slightly convex sides and the hypocyst has slightly convex sides with two pronounced, elongated antapical lobes, separated by an antapical depression. The central body wall was thin (>0.3 µm), with an apparent smooth outer and

inner surface using a light microscope~~under LM~~. No ornamentation was visible. The paracingulum was not visible. The parasulcus was indented, flagellar scars were not observed. The archeopyle was rounded, intercalary, and saphopylic, involving the release of plate 2a, isodeltaform planate. The description is based on cysts used in hatching experiments.

Gene sequence. The LSU rDNA gene sequence of the motile stages hatched from the cysts from China —GenBank Accession No. OR879789~~XXXXXX~~ (LSU).

Dimensions. Cysts extracted from sediments: length, 51.7 (59.2) 75.0 µm (SD = 10.6, n=4); width, 50.0 (60.0) 70.0 µm (SD = 8.8, n=4).

Incubated motile cells from China: length, 77.9 (81.3) 85.0 µm (SD = 2.9, n=4); width, 55.0 (59.1) 65.0 µm (SD = 4.6, n=4).

Comments. Although the cell was hatched from the cyst, we consider that there was not enough information to fully describe the species.

3.3. Phylogenetics

The LSU rDNA based phylogeny (Figure 4) demonstrates that *P. carriae* forms a separate clade, far from the clade containing *P. claudicans* from France, China and Japan. *Votadinium psilodora* is basal to the clade containing *P. carriae*, and *Votadinium* sp. 1 is basal to the clade containing *P. claudicans*. The *P. claudicans* species form two separate clades: one with *P. claudicans* from China and Japan (ribotype 1), and another one with French specimens (ribotype 2).

3.4 Results of macromolecular analyses of cyst walls

A total of 32 processed ATR micro-FTIR spectra of an equal number of individual cysts covering seven taxa were used for comparison (Table 3). Average spectra for each taxon were calculated (Figure 5) and these reveal the presence of a range of functional groups previously identified in the macromolecules comprising dinoflagellate and ciliate cyst walls (Meyvisch et al. 2023; Gurdebeke et al. 2018, Gurdebeke et al. 2023).

4. Discussion

Although for the species descriptions only few specimens were hatched and few cells sequenced, we consider these sufficient to describe the species, since the morphological observations and sequences were reproducible. Sequencing heterotrophic species has a low success rate (e.g. Mertens et al. 2023, p. 16).

4.1. *Comparative morphology of the motile stage of* P. carriae *within the* Oceanica *group*

Among the species that are elongate and have a quadra~~ngular~~ 2a, there is *P. oceanicum, P. quadrioblongum, P. venustum* and *Peridinium oblongum* var. *inaequipes*. *P. oceanicum* is much longer and more elongate, having a size of 129–210 µm, and a width of 68–128 µm (Balech 1988). *Protoperidinium quadrioblongum* has similar size (100–105 µm, width 65–80 µm and thickness 40 µm (Sarai et al. 2013), but *P. carriae* has a more elongate shape and more concave sides. *Protoperidinium venustum* is somewhat longer (110–120 µm) and wider (75–80 µm) and has antapical horns that are more diverging (Matzenauer 1933). *Peridinium oblongum* var. *inaequipes* is most closely similar but has antapical horns that are more widely diverging (Mangin 1930). *P. claudicans* has a penta~~gonal~~ 2a. *P. claudicans* and *P. carriae* can also be separated based on size (Figure 2).

4.2. *Comparative morphology of the cyst of* V. multispinosum *within the* Votadinium *group*

V. spinosum and *V. multispinosum* were both previously identified as *V. spinosum*, but this study demonstrates that they are two different species and can be distinguished using morphological criteria. Next to the obvious difference in body size between the larger *V. multispinosum* and *V. spinosum* (Figure 3), there is a significant difference in process density (~~Figures 2–3~~Plate 8). In addition, there are often ~~much less~~fewer processes on the sulcal area of *V. spinosum*. There is also the difference in archeopyle shape (isodeltaform planate vs. dextrocamerate), but this is much more difficult to observe.

Evitt (1985) expressed doubt about the archeopyle of *P. claudicans* and *P. oblongum* as being 2a, and suggested that they could be apical. Our observations suggest that the archeopyle shape corresponds well to the 2a. Sonneman and Hill (1997, p. 161, figs. 18a–c) displayed a similar penta~~gonal~~ archeopyle for a cyst identified as *V. spinosum* / *P. claudicans*.

The difference in color of cell contents (purple, reddish, orange or transparent) does not seem to be species specific, as within the Asian ribotype of *P. claudicans*, cysts with a reddish and transparent cell content have been observed.

4.3. The identification of P. claudicans *and relation to* Votadinium spinosum

P. claudicans was described by Paulsen (1907) from the port of Frederikshavn, Denmark. Since then, the species has not been redescribed from Danish waters, and has for example not been seen by Hansen and Larsen (1992). Paulsen (1907) did not specify the shape of the 2a in the original description, so uncertainty remains about whether the type specimen did indeed have a penta~~gonal~~ 2a. Later, he describes specimens from the Alboran Sea with hexa and quadra configuration (Paulsen 1931), and in Paulsen (1949) he suggests that quadra, penta and hexa are possible. Throndsen et al. (2007, p. 96) describe *P. claudicans* with both hexa and

penta 2a from the North Sea, whilst Hoppenrath et al. (2009, p. 149) describe *P. claudicans* from the German bight as penta and rarely hexa or quadra. Paulsen's specimen was 96 µm in length, which is significantly longer than specimens observed here (49.0–82.0 µm). Given the lack of further information on specimens from the type locality, and for convenience sake, we have chosen to use Lebour (1923) as species concept for *P. claudicans*; this concept uses a penta 2a, relatively small cells (51–96 µm). This corresponds quite well to the morphology of the cells that we observed, except for a more rectangular 2a in our specimens, which mayight as well be due to an inaccuracy in Lebour's drawing (Lebour 1925, plate XXV, fig. 1).

Protoperidinium claudicans has been related to its cyst equivalent *Votadinium spinosum* by Reid (1977). *Votadinium spinosum* has been described by Reid (1977) from Galway, Ireland. Reid (1977) related his new species to *P. claudicans* based on cyst-theca experiments by Wall and Dale from Woods Hole and Bermuda, who described cells exclusively with a pentagonal 2a. The cysts that we observed correspond very well to *V. spinosum* as described by Reid (1977), which has a similar shape, size (49x51–54x63 µm), similar density and length of processes (3–7 µm) and archeopyle. Similar cysts have been observed by Nehring (1997) from the German Bight. In conclusion, in absence from evidence from the type locality of *P. claudicans*, we identify our taxon as *P. claudicans* with as cyst-equivalent *V. spinosum*.

P. claudicans, as used here, displays some similarities to other *Protoperidinium* species with an ortho-penta configuration. *P. paraoblongum* has similar shapes of the anterior intercalary plates, but is larger and more slender. There is also superficial similarity with *Peridinium oceanicum* var. *inaequipes* L.A.Mangin 1930, but the latter is much larger (165 µm in length).

There were only minor morphological differences between French and Asian specimens, despite that there was a genetic separation between French and Asian specimens

when comparing the LSU rDNA sequences. In our view, the morphological distinctions between them are insufficient to warrant classification as distinct species. ~~In our opinion, there is not enough morphological difference to describe them as different species.~~ Further hatching experiments that include other genes should be done in the future.

4.4. Identification of V. psilodora *within the* Votadinium *group and* Votadinium *sp. 1*

The identified *V. psilodora* corresponds well with *L. psilodora* as described by Benedek (1972) specifically the body shape and archeopyle, although they are somewhat smaller and less rounded than Benedek's specimens (69.0–76 µm as compared to 90–96 µm). The motile stage of *V. psilodora* is similar to *P. larsenii*, but does not have an asymmetrical 1', (Phan-Tan et al. 2017).

Votadinium sp. 1 is closely similar to *V. rhomboideum*, but has a less deeply incised sulcus and no flagellar scar was observed. The motile stage is similar to *P. quadrioblongum* but does not have an elongated apical horn and no antapical horns that are directed outwards.

A detailed description of the corresponding motile stages is ~~foreseen~~ planned in a future study.

4.5. Phylogenetics and implications for the Oceanica *group*

On a species level, the phylogenetic tree (Fig. 4) separates well ~~all~~ the discussed species from each other. Furthermore, *P. claudicans* and *P. carriae* do not cluster together in the phylogenetic diagram, suggesting that cordate, process bearing cysts are not a monophyletic trait. It seems that the presence of processes on the cysts have evolved at different times, and could have led to an evolutionary advantage (e.g., Mertens et al., 2009, p. 67). This implies that the presence or absence of processes can be used to separate species, but not on a higher taxonomic level. The first occurrence of *V. spinosum* in the fossil record is considered to be

the Lower Pleistocene (Bujak and Matsuoka, 1986; Powell et al., 1990), but unfortunately both records do not illustrate nor describe the taxon, which makes the first occurrence for both species currently unknown and in need of restudy. Both *Votadinium psilodora* and *Votadinium* sp. 1 are genetically sufficiently distant from other species in the group.

On a group level, the phylogeny demonstrates also that species that have been assigned to the *Oceanica* group cluster together in a monophyletic group, despite some of them having a penta~~gonal~~ or hexa~~gonal~~ 2a. There does not seem to be any clear logic in the positions of species that have a quadra, penta or hexa configuration. In addition, for each species in this group that has a cyst-based equivalent, it can be assigned to the cyst-based genus *Votadinium*, which is coherent with what ~~is~~ was previously observed by Gurdebeke et al. (2020). The long biostratigraphical range of *V. psilodora* suggests that its phylogenetic position is ancestral for the group or that it has wall that is more resistant to degradation.

4.6. Biogeography and ecology of P. claudicans *and* P. carriae

Biogeographical ranges of *P. claudicans* have previously been recorded for the Atlantic by Dale (1996). The apparent presence of multiple species within this species complex suggests a necessary revision of their distribution ranges.~~The apparent existence of several species in this species complex, suggests a revision is needed.~~ For the time being, we only have verified occurrences of *P. carriae* in B.C. waters (Canada). *P. claudicans* ribotype 1 occurs in European waters and *P. claudicans* ribotype 2 in Japanese and Chinese waters. More sequences are needed to understand their larger distribution.

It is not clear whether *P. claudicans* co-occurs with *P. carriae* in the Canadian Pacific. *Protoperidinium claudicans* with a penta~~gonal~~ 2a was described previously by Wailes (1939, Fig. 109) but the cells of 100–120 µm x 68–85 µm are much larger than the typical *P. claudicans*. The shape of the 2a is also more elongated than the 2a shapes that are recorded

here from European waters. Dobell (1978) ~~also recorded~~hatched smaller, penta~~gonal~~ *P. claudicans* ~~that were somewhat smaller~~ (70–125 µm x 50–70 µm), ~~that she hatched~~ from cordate, spinose cysts. Although we were unable to confirm the presence of *P. claudicans* in ~~these~~ Canadian Pacific waters, these records suggest that the possibility of its presence remains. ~~these records indicate that its presence remains thus possible.~~ Corroborating evidence for this is the large variability in size and shape of cordate, spinose cysts we have noted in these waters.

Protoperidinium carriae, with cyst equivalent *V. multispinosum*, occurs in higher abundances in summer in Saanich Inlet, which could be related to a higher diatom production (Price and Pospelova 2011, then identified as *V. spinosum*). Further investigations are needed to determine if this species is confined to temperate waters or exhibits a broader range of tolerance.~~More records should investigate whether this species is restricted to temperate waters or has a wider tolerance.~~

4.7. Macromolecular comparison of the cyst of *V. multispinosum* with other cysts

The macromolecule in the cyst wall of *V. multispinosum* is strongly enriched in proteinaceous moieties as is apparent from the strong intensities in absorption bands related to amides, relative to the intensity of the broad band related to hydrogen bond stretching between 3550–3200 cm^{-1} (Fig. 5). Other than that, it also contains aliphatic moieties and a low amount of carbohydrates. In this way, *V. multispinosum*'s cyst wall is very similar to that of the closely related, smooth walled species *V. calvum*. Slightly stronger carbohydrate bands in the latter species might reflect systematic compositional differences or might originate from small quantities of optically difficult to detect cellular remnants present within the cysts. Interestingly, distantly-related gymnodinialean cysts of *Polykrikos kofoidii* and *P. schwartzii* (both sensu Matsuoka et al. 2009) are also protein-rich, but are more pigmented than those of

Votadinium, which is apparent from the higher intensities of secondary amine absorption bands (notably between 1620–1570 cm^{-1}; Fig. 5) thought to originate from— possibly eumelanin —pigments responsible for the typically brown color ~~present mostly in~~of many cysts produced by heterotrophic dinoflagellates (Meyvisch et al. 2023). The transparent cyst of the ciliate *Halodinium verrucatum* (Gurdebeke et al. 2018) is also comprised of a very proteinaceous and polysaccharide-containing, though non-pigmented (hence the absence of a shoulder between the Amide I and II bands) macromolecule (Fig. 5). Proteins are often quickly depolymerized and further degraded upon sedimentation and burial (de Leeuw and Largeau 1993), which might explain the less frequent occurrence of all the above-mentioned cyst species (and ciliate cysts in general) ~~from~~ in older (sub)fossil samples. In contrast to proteinaceous *Votadinium* cysts, many other protoperidinioid cysts such as *Quinquecuspis concreta* and *Lejeunecysta* sp. are composed of a relatively less protein-rich, more pigmented, sugary and aliphatic macromolecule (Fig. 5). This compound is previously referred to as 'colored dinosporin' (Meyvisch et al. 2023) and is thought to preserve quite well under low-oxidative conditions. Note that in the dataset reported by Meyvisch et al. (2023), protein-rich dinoflagellate cysts belonging to the genera *Polykrikos*, *Qia* and *Votadinium* were included within the 'colored dinosporin' group based on the presence of wall pigments. However, the differences in protein contents and associated preservability which are more explicitly addressed and visualized in this work might advocate for a future erection of an additional spectrochemical group encompassing all such proteinaceous forms.

As a final note, it seems that spectra for *V. multispinosum* reported by Gurdebeke et al. (2020) (as "*V. spinosum*") and measured in micro-FTIR transflection mode, are fairly comparable to those presented here and measured in ATR-mode (Fig. 4). As the cavity of the thin-walled cyst collapses upon drying after picking, greatly reducing geometrical scattering effects reported by Meyvisch et al. (2022).

5. Conclusions

Throughout its history, *P. claudicans* has been recognized for showcasing distinct variations in its second anterior intercalary plate, which can assume forms ranging from quadra to penta and hexa. The outcomes of the incubation experiments from France, Canada, China, and Japan reveal that *P. claudicans* forms a species complex. Within this complex, there exist at least two ribotypes displaying a penta configuration (*P. claudicans*) and one with a quadra configuration (*P. carriae* sp. nov. with equivalent cyst *V. multispinosum* sp. nov.). The validity of these findings is corroborated by molecular phylogenetics. Since a hexa configuration has also been recorded (Matsuoka and Head 2013, Fig. 4e), future sequencing needs to show that this is a third species in this complex. Sequences of two cordate, spineless cysts demonstrate that several other species remain to be described in this complex. Macromolecular analyses of the cyst wall of *V. multispinosum* sp. nov. reveal that it is comprised of a poorly preservable, protein-rich compound which is also quite commonly found in other dinoflagellate and ciliate resting stages.

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Figure captions

Figure 1. Map showing all sampling locations: A. Vancouver Island, BC, Canada (where Patricia Bay in Saanich Inlet and Esquimalt Harbour are located); B. Maresclé, France; C. Fangchenggang, Guangxi, China; D. Quanzhou, Taiwan Strait, China; E. Ishikari, Hokkaido Japan; F. Otaru, Hokkaido, Japan; G. Saroma Lake, Hokkaido, Japan; H. Lianyungang, Jiangshu, China.

Figure 2. Measurements of motile cells (theca) from Canada (squares, *V. multispinosum*), from France~~, China~~ and Japan (triangles and filled circles respectively, *V. spinosum*).

Figure 3. Measurements of cysts from Canada (squares, *V. multispinosum*), from France (triangles, *V. spinosum*) and, China and Japan (~~triangles and~~ filled circles, *V. spinosum*).

Figure 4. ~~Phylogenetic tree based on LSU rDNA sequences.~~ Molecular phylogeny of *Protoperidinium claudicans* and related species inferred from partial Large Subunit rDNA (LSU rDNA) sequences based on Bayesian inference (BI). *Akashiwo sanguinea* was used as outgroup. Numbers at nodes represent Bayesian posterior probabilities and the ML bootstrap values; asterisks indicate the maximal support in BA and ML (1.0 and 100%, respectively). Bootstrap values > 50% and posterior probabilities above 0.7 are shown. Newly obtained sequences were indicated as bold. Scale bar = nucleotide substitutions per site.

Figure 5. Average processed ATR micro-FTIR spectra of selected dinoflagellate and ciliate cyst taxa. Grey rectangles highlight absorption bands discussed in the manuscript. * = sensu Matsuoka et al. (2009).

Plate captions

Plate 1. Two cyst-theca experiments of *Protoperidinium carriae* sp. nov. from Patricia Bay, BC, Canada. 1–9. First incubation experiment. 1. Living cyst with purplish cell contents. 2. High focus of germinated cyst showing operculum. 3. Low focus of germinated cyst with a focus on the archeopyle. ~~4~~3–9. Hatchling from cyst shown in 1–3. 4. ~~, showing p~~Cell showing purplish cell contents. 5~~4~~. Same hatchling showing pusule (Pu). 6. High focus on two antapical horns. 7–9. Fluorescent images showing plate configurations. 7. Focus on third postcingular plate (3‴). 8. Focus on the two antapical horns, positioned on the two antapical plates (1‴′ and 2‴′). 9. High focus on ventral view, showing the apical plate (1′) and contact

with two precingular plates (1″ and 7″). 10–15. Second incubation experiment. 10. Living cyst with purplish cell contents. 11. Hatched cyst with operculum detached. 12–15. Hatchling from cyst shown in 10–11. 12. Cell showing purplish cell contents. 13. Lateral view of cell showing plate configuration. 14. Dorsal view showing quadra 2a and surrounding plates. 15. Ventral view showing sulcus and surrounding postcingular plates (5‴ and 1‴). Scale bar = 10 µm.

Plate 2. 1–6. Third cyst-theca experiment of *Protoperidinium carriae* sp. nov. from Patricia Bay, BC, Canada. 1–3. Hatched cyst. 4–6. Hatched cell. 7–12. Holotype and paratype of *Votadinium multispinosum* from palynologically prepared sediment from Site 5 in Esquimalt Harbour (BC, Canada). 7–9. High to mid focus of holotype (UVic 2008-5-1-CRD). 10–12. High to mid focus of paratype (UVic 2008-5-1-CRD). Scale bar = 10 µm.

Plate 3. Two cyst-theca experiments of *Protoperidinium claudicans* from Maresclé, France ~~(1–6=CON8F1; 7–12: CON8D6)~~. 1–6. First incubation experiment (CON8F1). 1. Hatched c~~C~~yst. 2–6. Hatchling from cyst shown in 1. 2. High focus of ventral view. 3. Mid focus showing cell outline and yellowish lipid bodies. 4–6. Fluorescent images. 4. High focus of ventral view showing first apical plate (1′) and contacting plates. 5. High focus of dorsal view showing penta 2a and contacting plates. 7–12. Second incubation experiment (CON8D6). -7. Hatched ~~C~~cyst. 8–12. Hatchling from cyst shown in 7. 8. Mid focus showing cell outline. 9. Fluorescent image of high focus of ventral view showing first apical plate (1′) and contacting plates. 10. High focus of dorsal view showing penta 2a. 11. Fluorescent image of high focus of antapical plates and third postcingular plate (3‴). Mid focus of lateral view showing cell outline. Scale bar = 10 µm.

Plate 4. Living cysts and ~~T~~two cyst-theca e experiments of *Protoperidinium claudicans* ~~xperiment~~ from Maresclé, France ~~(4–9=CON9E3; 10–15= MARE CON9B2)~~. 1–2. Living cyst from Maresclé. 1. High focus showing process distribution. 2. Mid focus. 3. Living cyst from Concarneau showing yellowish lipid bodies. 4–9. First incubation experiment (CON9E3). ~~4–9. Maresclé.~~4. Hatched cyst. 5–9. Hatchling from cyst shown in 4. 5. Mid focus showing cell outline and yellowish lipid bodies. 6. Mid focus showing different orientation. 7. Fluorescent image of high focus of ventro-lateral side. 8. Fluorescent image of high focus of ventral side. 9. Fluorescent image of high focus of dorsal side showing shape of 2a. 10–15. Second incubation experiment (MARE CON9B2). 10–11. Mid and high focus of hatched ~~C~~cyst. 12–15. Hatchling from cyst shown in 10–11. 12. Mid focus of cell outline. 13. Fluorescent image of high focus of ventral side. 14-15. Fluorescent images of high and mid focus of dorsal side. Scale bar = 10 µm.

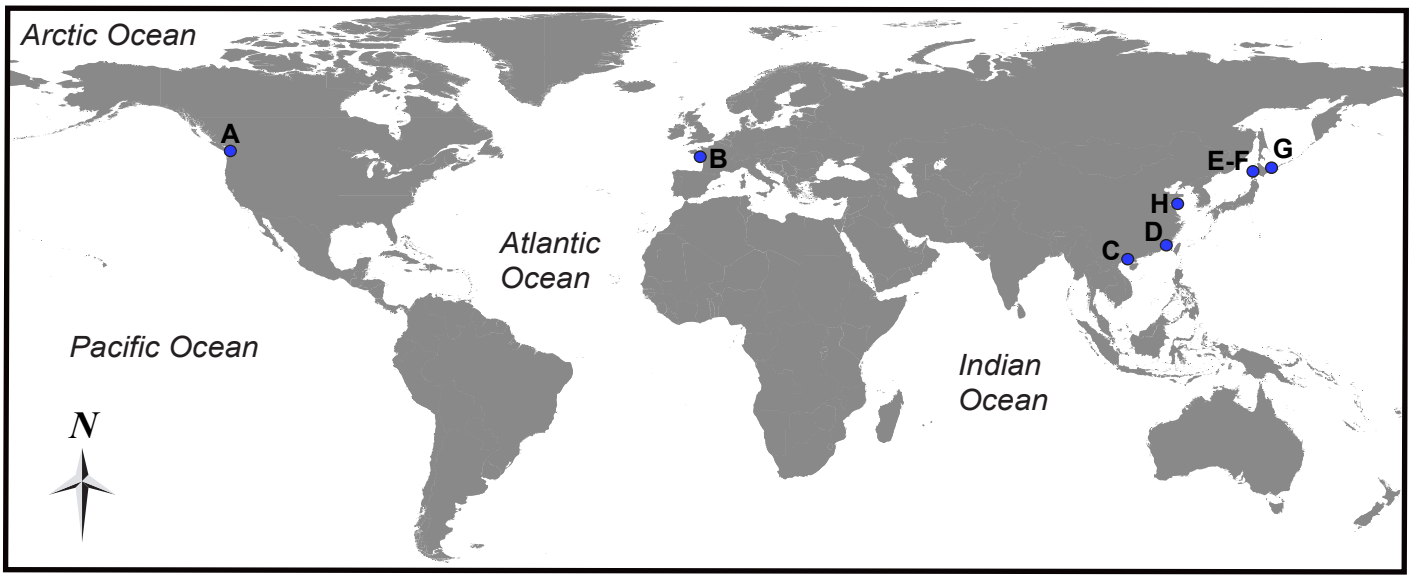
Plate 5. 1. Cyst from surface sediment from Fangchenggang, Guangxi province, China (FC16). 2. Hatched ~~C~~cyst from surface sediment from Quanzhou, Taiwan Strait. 3. Cyst from surface sediment from Ishikari Bay, Hokkaido (GenBank AB255842), showing purplish lipid bodies. 4–7. Motile cell hatched from a similar cyst as shown in 3, from Saroma Lake, Japan showing purplish lipid bodies. 4. High focus of ventral side. 5. High focus of dorsal side. 6. High focus on dorsal cingulum. 7. High focus of lateral view. 8–9. Mid focus of ~~M~~motile cell isolated from Plankton from Otaru, Japan (GenBank AB255840). Scale bar = 10 µm.

Plate 6. *Votadinium psilodora* from surface sediments from Fangchenggang, Guangxi, China. 1. Living cyst showing yellowish lipid bodies. 2–6. Hatched cysts. 2. High focus of dorsal side, showing archeopyle. 3. High focus on archeopyle. 4–5. Mid focus showing cyst outline. 6. High focus of dorsal side showing operculum in place. 7–9. Hatchling. 7. High focus of

ventral side showing first apical plate and contacting plates. 8. Mid focus showing cell outline. 9. High focus of dorsal side. Scale bar = 10 µm.

Plate 7. *Votadinium* sp. 1 from surface sediments from Fangchenggang, Guangxi, China. 1. Living cyst with transparent lipid bodies. 2–3. Hatched cysts. 2. High focus of dorsal side showing archeopyle and detaching operculum. 3. High focus of archeopyle and operculum. 4–9. Hatchling. 4. High focus of ventral side. 5. Fluorescent image of high focus of ventral side of apex. 6. Fluorescent image of high focus of dorsal side of apex. 7. Fluorescent image of high focus of ventral side of antapex. 8. Fluorescent image of high focus of dorsal side of antapex. 9. Fluorescent image of high focus of lateral view. Scale bar = 10 µm.

Plate 8. SEM micrographs of *Votadinium multispinosum* and *Votadinium spinosum*. 1–2. *V. multispinosum* from Saanich Inlet (Station A). 1. Complete cyst. 2. Zoom on part of cyst shown in 1, showing the fibrillar nature of the wall surface. 3–4. *V. spinosum* from Vilaine Bay (BV5). 4. Complete cyst. 3. Zoom on part of cyst shown in 4, showing the fibrillar nature of the wall surface. 5–6. *V. spinosum* from Estuaire de la Vie (Station 10) showing process distribution. All scale bars = 10 µm, except for 2 and 3 = 1 µm.


Arctic Ocean
A
B
E-F
G
H
D
C
Atlantic
Ocean
Pacific Ocean
Indian
Ocean
N

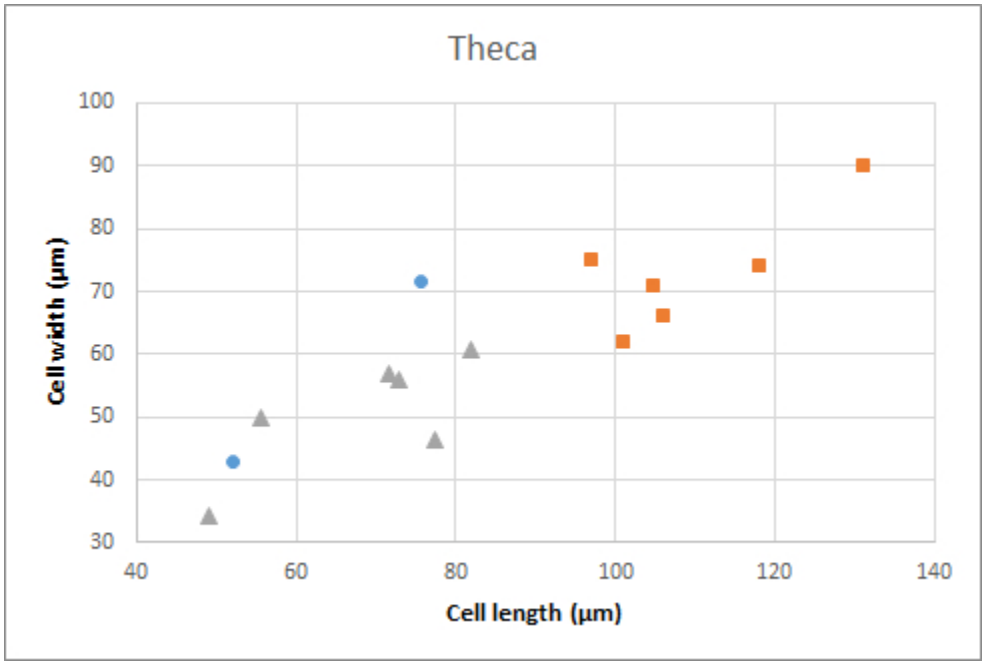


Figure 2. Measurements of motile cells (theca) from Canada (squares, V. multispinosum), from France and Japan (triangles and filled circles respectively, V. spinosum).

172x115mm (72 x 72 DPI)

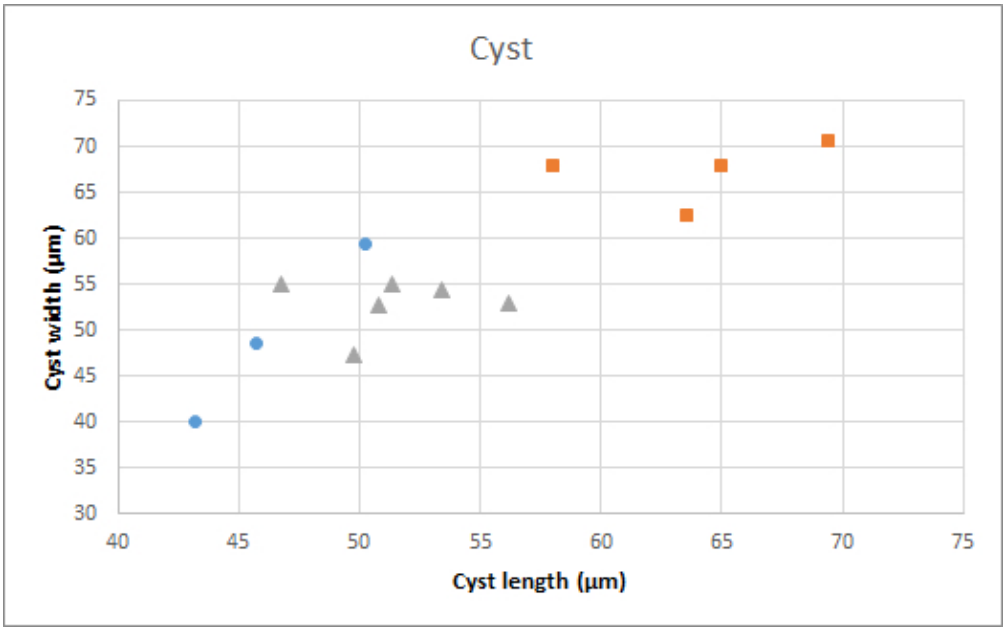
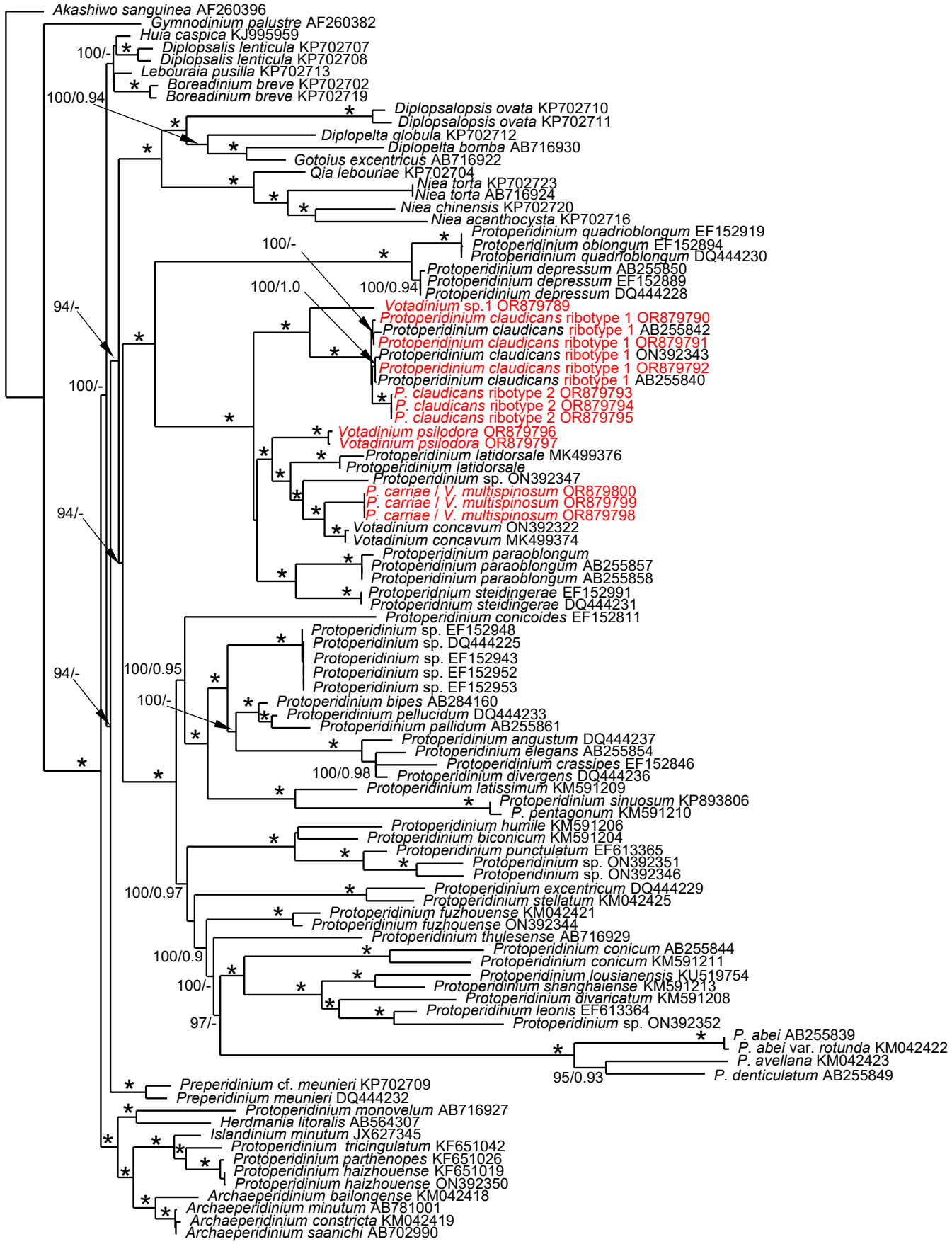


Figure 3. Measurements of cysts from Canada (squares, V. multispinosum), from France (triangles, V. spinosum) and China and Japan (filled circles, V. spinosum).

184x115mm (72 x 72 DPI)


Akashiwo sanguinea AF260396
Gymnodinium palustre AF260382
Huia caspica KJ995959
Diplopsalis lenticula KP702707
Diplopsalis lenticula KP702708
Lebouraia pusilla KP702713
Boreadinium breve KP702702
Boreadinium breve KP702719
Diplopsalopsis ovata KP702710
Diplopsalopsis ovata KP702711
Diplopelta globula KP702712
Diplopelta bomba AB716930
Gotoius excentricus AB716922
Qia lebouriae KP702704
Niea torta KP702723
Niea torta AB716924
Niea chinensis KP702720
Niea acanthocysta KP702716
Protoperidinium quadrioblongum EF152919
Protoperidinium oblongum EF152894
Protoperidinium quadrioblongum DQ444230
Protoperidinium depressum AB255850
Protoperidinium depressum EF152889
Protoperidinium depressum DQ444228
Votadinium sp.1 OR879789
Protoperidinium claudicans ribotype 1 OR879790
Protoperidinium claudicans ribotype 1 AB255842
Protoperidinium claudicans ribotype 1 OR879791
Protoperidinium claudicans ribotype 1 ON392343
Protoperidinium claudicans ribotype 1 OR879792
Protoperidinium claudicans ribotype 1 AB255840
P. claudicans ribotype 2 OR879793
P. claudicans ribotype 2 OR879794
P. claudicans ribotype 2 OR879795
Votadinium psilodora OR879796
Votadinium psilodora OR879797
Protoperidinium latidorsale MK499376
Protoperidinium latidorsale
Protoperidinium sp. ON392347
P. carriae / V. multispinosum OR879800
P. carriae / V. multispinosum OR879799
P. carriae / V. multispinosum OR879798
Votadinium concavum ON392322
Votadinium concavum MK499374
Protoperidinium paraoblongum
Protoperidinium paraoblongum AB255857
Protoperidinium paraoblongum AB255858
Protoperidnium steidingerae EF152991
Protoperidnium steidingerae DQ444231
Protoperidinium conicoides EF152811
Protoperidinium sp. EF152948
Protoperidinium sp. DQ444225
Protoperidinium sp. EF152943
Protoperidinium sp. EF152952
Protoperidinium sp. EF152953
Protoperidinium bipes AB284160
Protoperidinium pellucidum DQ444233
Protoperidinium pallidum AB255861
Protoperidinium angustum DQ444237
Protoperidinium elegans AB255854
Protoperidinium crassipes EF152846
Protoperidinium divergens DQ444236
Protoperidinium latissimum KM591209
Protoperidinium sinuosum KP893806
P. pentagonum KM591210
Protoperidinium humile KM591206
Protoperidinium biconicum KM591204
Protoperidinium punctulatum EF613365
Protoperidinium sp. ON392351
Protoperidinium sp. ON392346
Protoperidinium excentricum DQ444229
Protoperidinium stellatum KM042425
Protoperidinium fuzhouense KM042421
Protoperidinium fuzhouense ON392344
Protoperidinium thulesense AB716929
Protoperidinium conicum AB255844
Protoperidinium conicum KM591211
Protoperidinium lousianensis KU519754
Protoperidinium shanghaiense KM591213
Protoperidinium divaricatum KM591208
Protoperidinium leonis EF613364
Protoperidinium sp. ON392352
P. abei AB255839
P. abei var. rotunda KM042422
P. avellana KM042423
P. denticulatum AB255849
Preperidinium cf. meunieri KP702709
Preperidinium meunieri DQ444232
Protoperidinium monovelum AB716927
Herdmania litoralis AB564307
Islandinium minutum JX627345
Protoperidinium tricingulatum KF651042
Protoperidinium parthenopes KF651026
Protoperidinium haizhouense KF651019
Protoperidinium haizhouense ON392350
Archaeperidinium bailongense KM042418
Archaeperidinium minutum AB781001
Archaeperidinium constricta KM042419
Archaeperidinium saanichi AB702990
100/-
100/0.94
94/-
100/-
94/-
94/-
100/1.0
100/0.94
100/0.95
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100/0.98
100/0.97
100/0.9
100/-
97/-
95/0.93
0.1

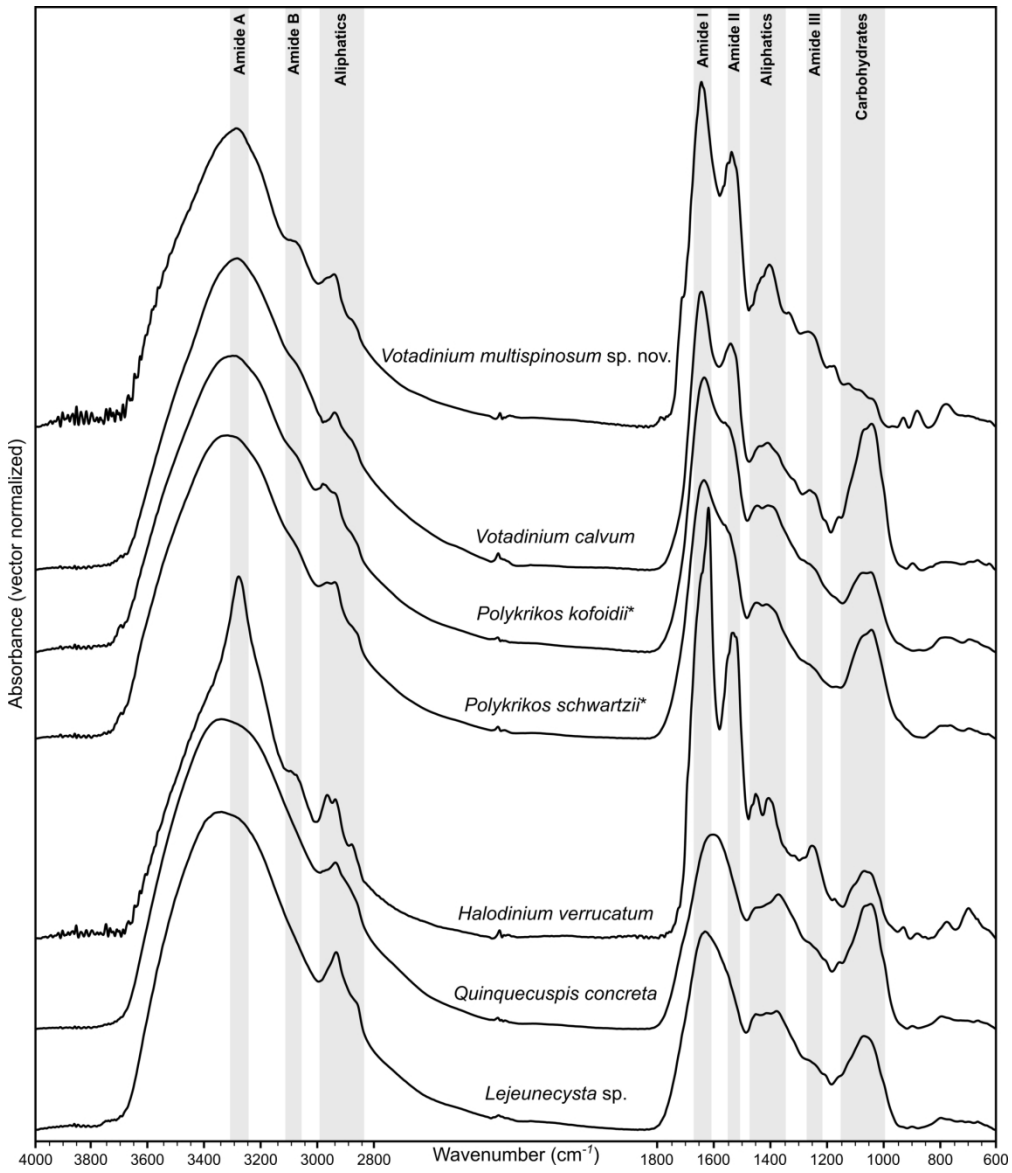
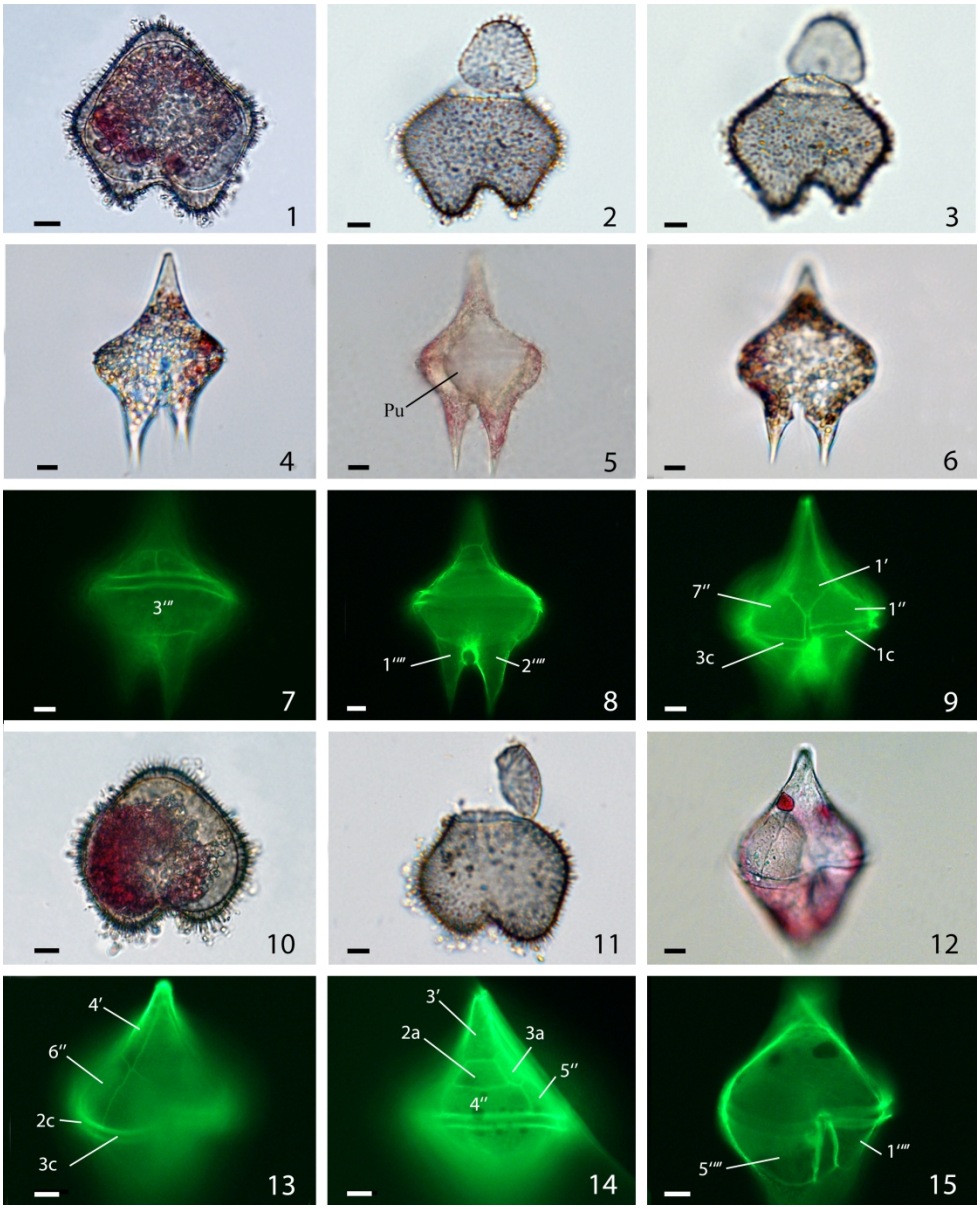


Figure 5. Average processed ATR micro-FTIR spectra of selected dinoflagellate and ciliate cyst taxa. Grey rectangles highlight absorption bands discussed in the manuscript. * = sensu Matsuoka et al. (2009).

185x214mm (300 x 300 DPI)



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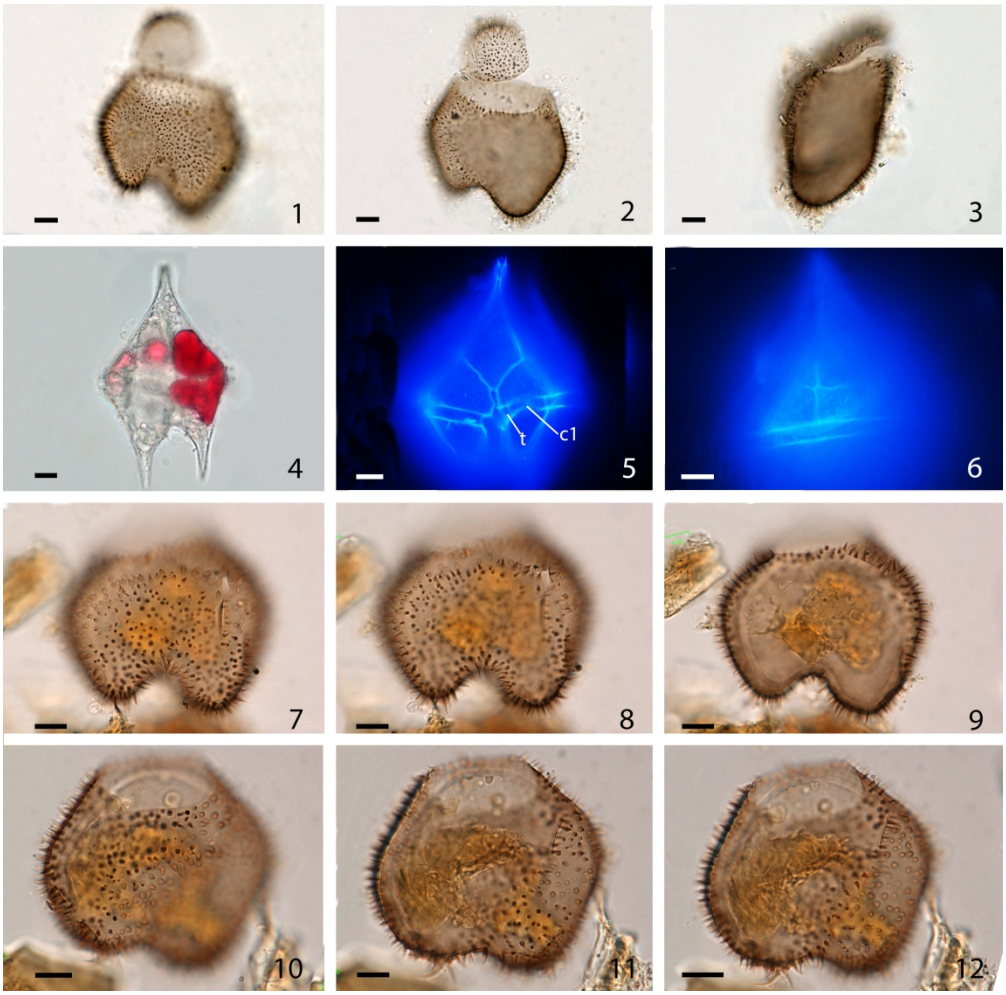

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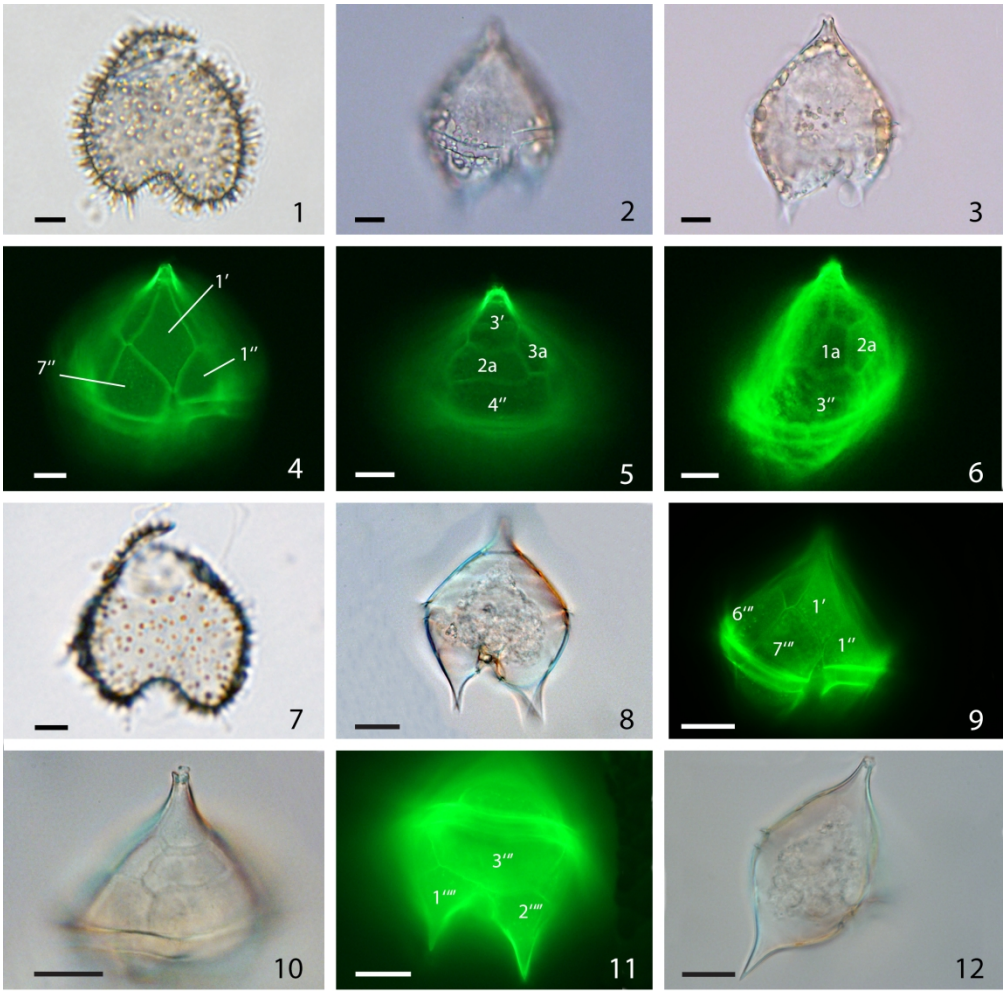

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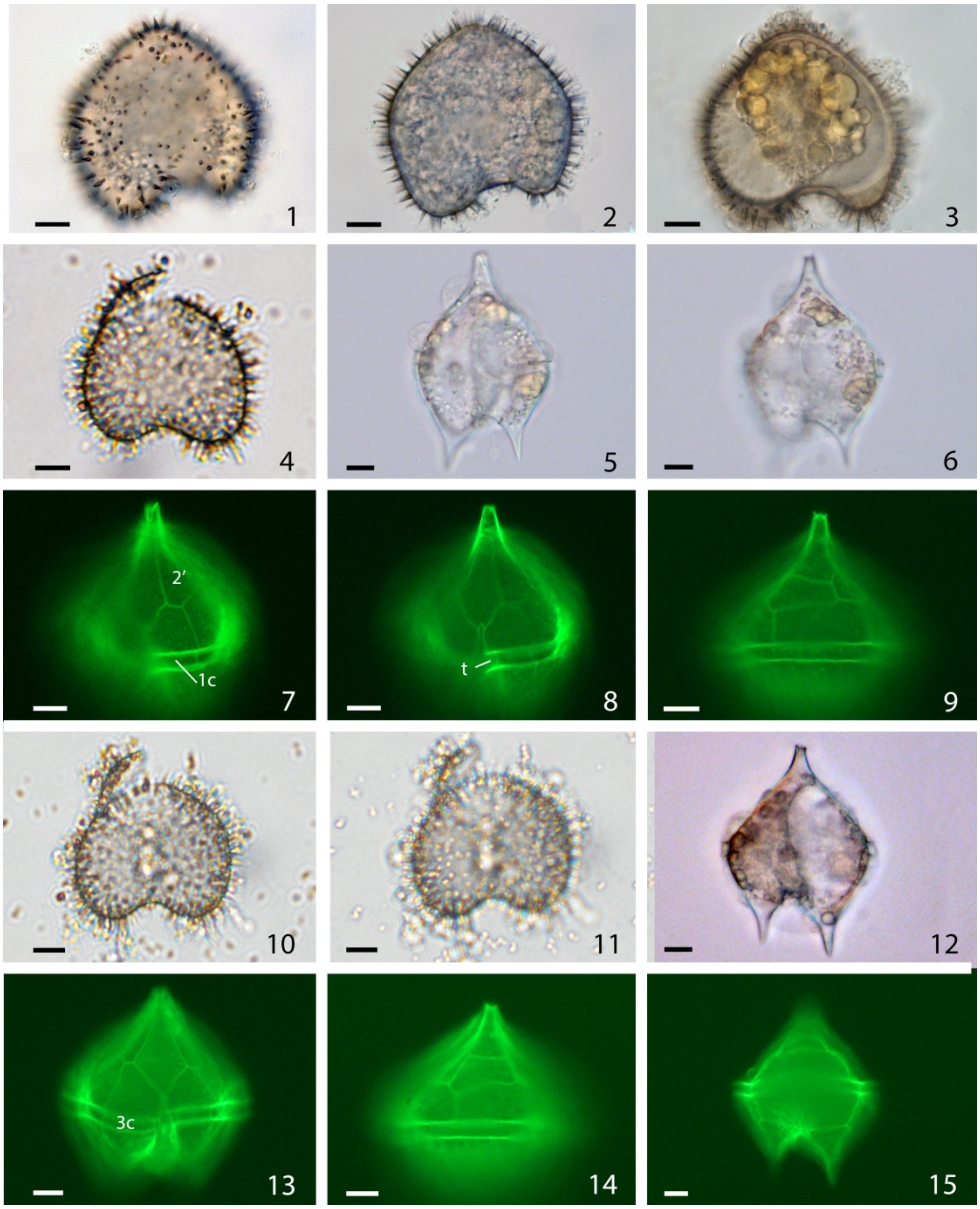

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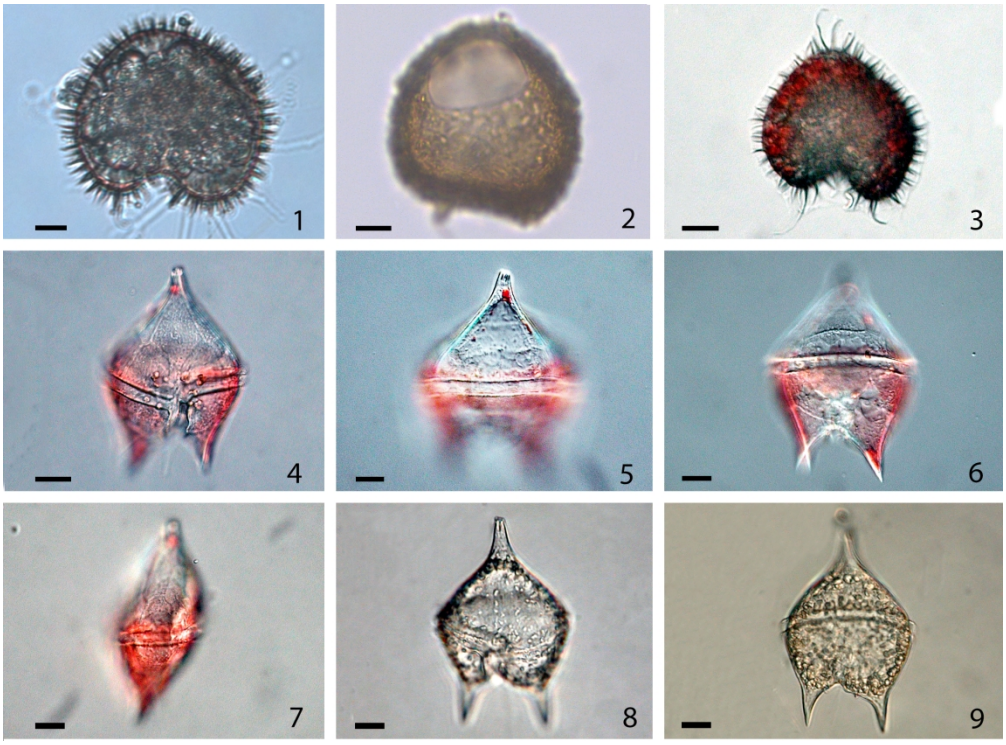

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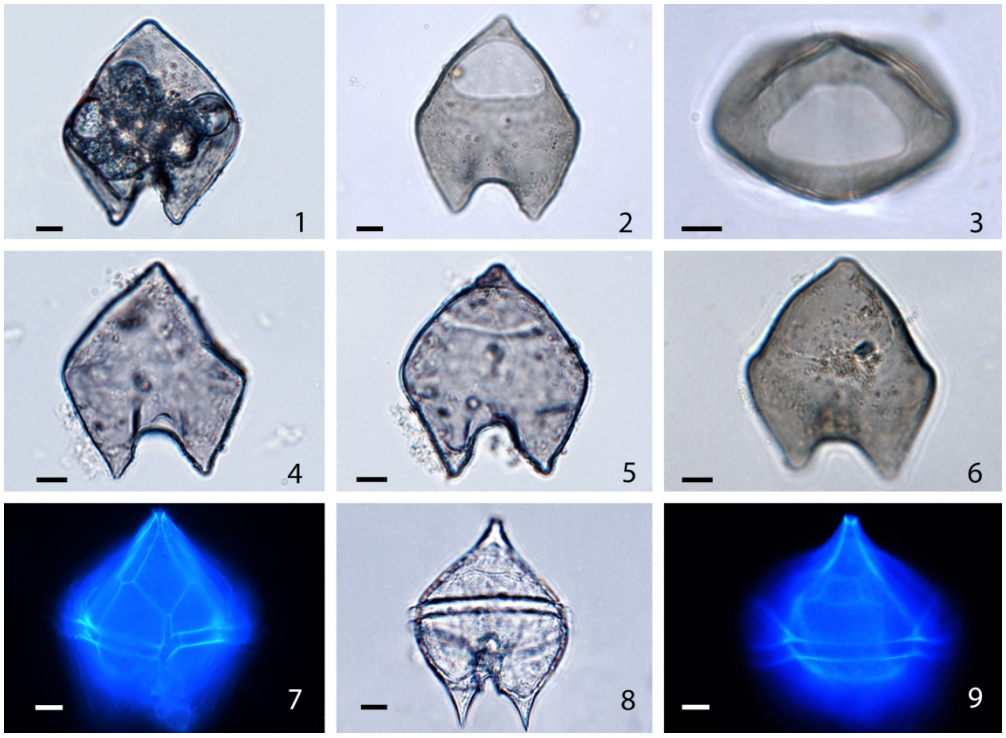

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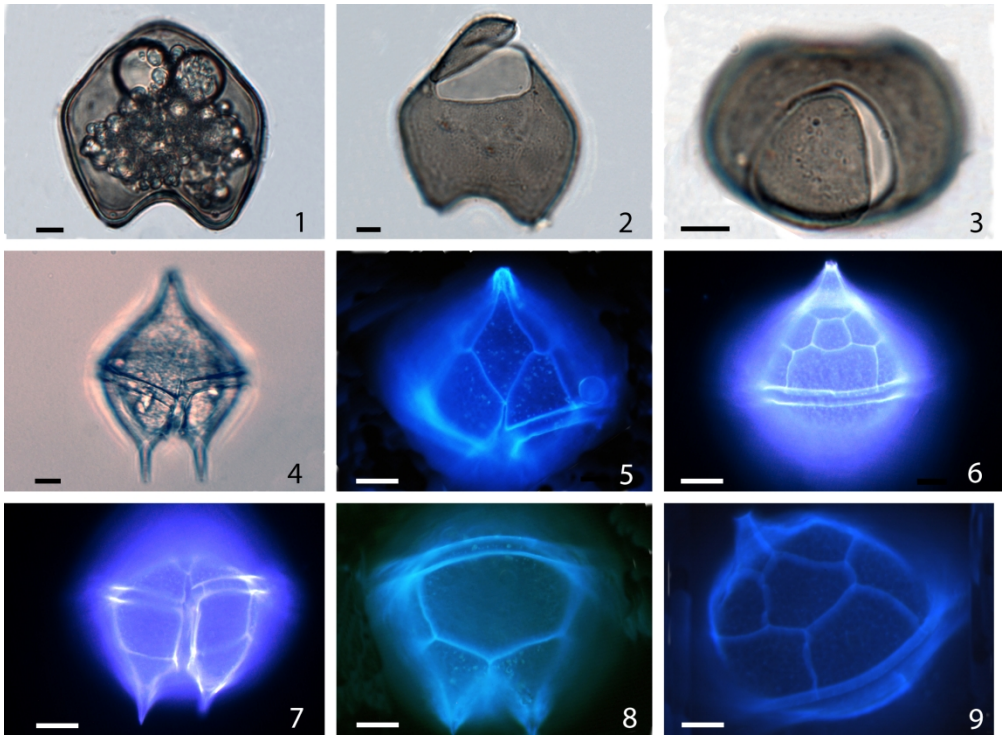

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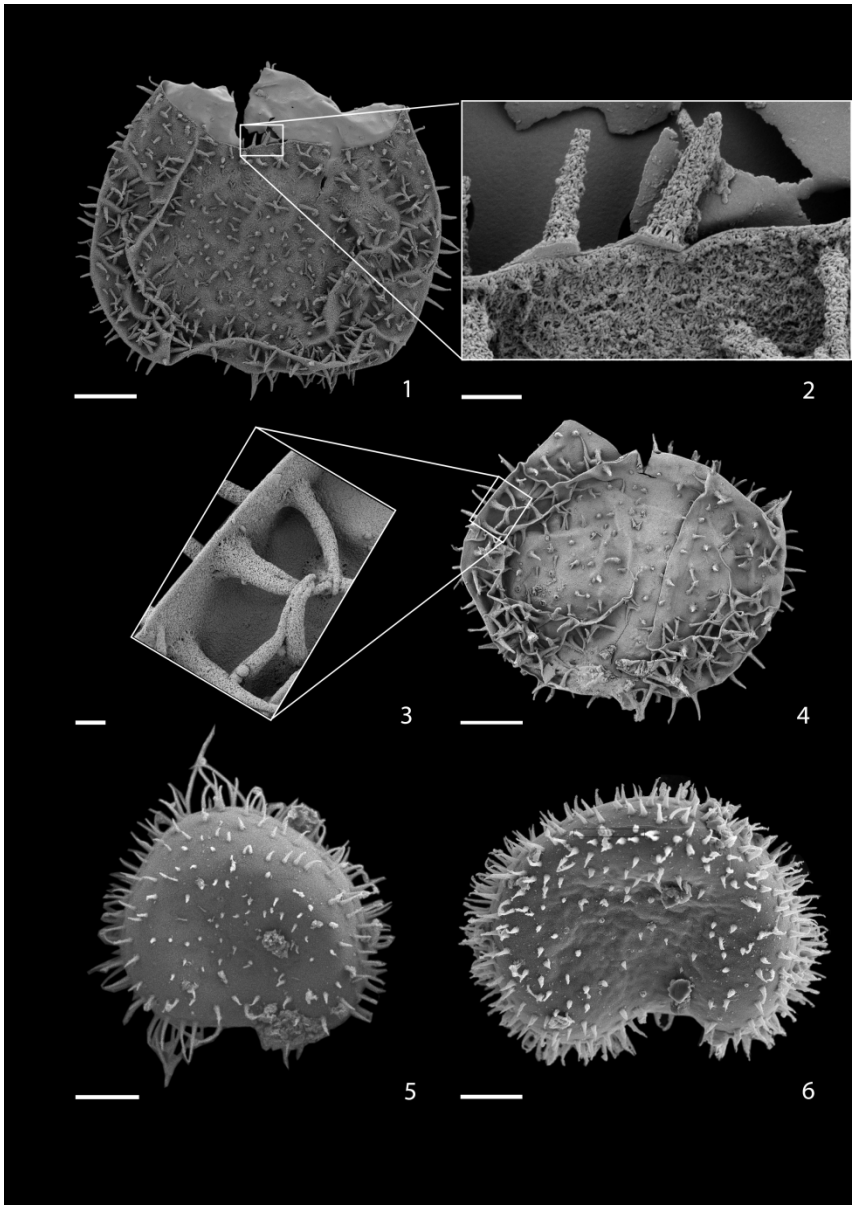

209x297mm (300 x 300 DPI)

Table 1. Biological (non-fossil) and geological (fossil) names of *Votadinium* species, including information about 2a shape and availability of ATR-FTIR data. Species studied in this paper are shown in bold.

Motile name	**Cyst name**	Cell length (μm)	Cell width (μm)	**2a shape**	Cyst length (μm)	Cyst width (μm)	**Spine length**	**GenBank**	**FTIR**	**Reference**
Protoperidinium latidorsale	*Votadinium calvum*	112(116) 122	70 (72.4) 76	Hexa	45 (52.5) 60	46.5 (52.4) 58.5	0	MK499376 (LSU), MK522510.1 (SSU)	Yes	Sarai et al. 2013; Gurdebeke et al. 2020
Unknown	*Votadinium concavum*				65–85	65–85	0	MK499375, MK499374 (LSU)	No	Gurdebeke et al. 2020
Unknown	*Votadinium elongatum*				69.9–79.2	40.4–49.8	0	None	No	Gurdebeke et al. 2020
Unknown	*Votadinium nanhaiense*				59.7 (72.1) 79.4	44.4 (54.4) 67.3	0	None		Gurdebeke et al. 2020
Protoperidinium paraoblongum	*Votadinium pontifossatum*	108 (113.2) 118	60 (67.1) 78	Penta	71.6 (79.2) 86.6	61.1 (67.7) 75.7	0	AB255857, AB255858 (LSU)	Yes	Sarai et al. 2013; Gurdebeke et al. 2020
***Protoperidinium* sp.**	***Votadinium psilodora***	80.0 (82.5) 85.0	55.0 (57.5) 60.0	Quadra	69.0 (71.6) 76	60.0 (60.3) 61.0	0	OR879796–OR879797 (LSU)	No	This study
Protoperidinium steidingerae	*Votadinium reidii*	77.6 (103.6) 125.0	45.3 (63.2) 82.0	NA	68 (83) 95	48 (59) 68	0	EF152991, DQ444231 (LSU)	No	Gribble et al. 2009; Gurdebeke et al. 2020
Protoperidinium quadrioblongum	*Votadinium rhomboideum*	100 (103) 105	60 (65.6) 72	Quadra	61 (70) 84	59 (67) 80	0	DQ444230 (LSU)	No	Sarai et al. 2013
Protoperidinium carriae	***Votadinium multispinosum***	97.0 (109.6) 131.0	62.0 (53.6) 90.0	Quadra	58.5 (64.7) 71.1	56.1 (64.7) 75.1	2.8 (4.0) 7.7	OR879798–OR879800 (LSU)	Yes	This study
***Protoperidinium claudicans* ribotype 1**	***Votadinium spinosum***	49.0 (68.1) 82.0	34.3 (50.7) 60.8	Penta	46.7 (51.4) 56.2	47.3 (52.9) 55.1	3.9 (5.7) 8.1	OR879790–OR879792 (LSU)	Yes	This study
***Protoperidinium claudicans* ribotype 2**	***Votadinium spinosum***	71.4 (73.5) 75.6	42.9 (47.5) 52.1	Penta	43.2 (46.4) 50.2	40.0 (49.3) 59.4	4.4 (5.1) 6.3	OR879793–OR879795 (LSU)	No	This study

Table 2. Sampling stations with details on latitude, longitude and water depth and species studied.

Station	Location	Latitude	Longitude	Water depth (m)	Sampling data	Salinity (psu)	Temperature (°C)	Type of core	Sampled by	Species encountered	ON THE MAP
Patricia Bay St. 2	Canada	48° 38.975′N	123° 28.845′W	3	March 8, 2019	28	9	Van Veen Grab	VP	*P. carriae*	**A**
Maresclé	France	47° 27' 43" N	2° 30' 2.16" W	5	January 15, 2019	NA	NA	Petite Ponar Grab	KNM	*P. claudicans* ribotype 1	**B**
Fangchenggang, Guangxi	China	21°29.97′N	108°13.887′E	15	April 28, 2011	NA	NA	Petite Ponar Grab	HG	*P. claudicans* ribotype 2	**C**
Quanzhou, Taiwan Strait	China	24°42.54′N	118°55.00′E	31	November 18, 2018	NA	NA	Petite Ponar Grab	HG	*P. claudicans* ribotype 2	**D**
Ishikari, Hokkaido	Japan	43° 13′N	141° 18′E	surface	July 17, 2004	NA	20.5	Plankton net	AY	*P. claudicans* ribotype 2	**E**
Otaru, Hokkaido	Japan	43° 10′N	141° 01′E	surface	August 13, 2002	NA	21	Plankton net	AY	*P. claudicans* ribotype 2	**F**
Saroma Lake, Hokkaido	Japan	44° 07′N	143° 57′E	NA	September 9, 2004	NA	20.5	TFO gravity corer	AY	*P. claudicans* ribotype 2	**G**
Fangchenggang, Guangxi	China	21°29.97′N	108°13.887′E	15	April 28, 2011	NA	NA	Petite Ponar Grab	HG	*Votadinium psilodora*	**C**
Fangchenggang, Guangxi	China	21°29.97′N	108°13.887′E	15	April 28, 2011	NA	NA	Petite Ponar Grab	HG	*Votadinium* sp. 1	**C**
Fangchenggang, Guangxi	China	21°29.97′N	108°13.887′E	15	April 28, 2011	NA	NA	Petite Ponar Grab	HG	*Votadinium psilodora*	**C**
Lianyungang, Jiangshu	China	34°48.763′	119°31.627′	15	May 9, 2011	NA	NA	Petite Ponar Grab	HG	*P. claudicans* ribotype 2	**H**

Table 3. Specimens that were measured using ATR FTIR. All are dinoflagellate cysts, except for *Halodinium verrucatum*, which is a ciliate cyst.

Taxon	Number of cysts	Order	Family	Sample location
Halodinium verrucatum	2	Prorodontida	/	Diana Lagoon, Corsica, France
Lejeunecysta sp.	6	Peridiniales	Protoperidiniaceae	Qinhuangdao, Bohai Sea, China
	1	Gymnodiniales	Polykrikaceae	Olhão Port, Portugal
	1	Gymnodiniales	Polykrikaceae	Aveiro, Portugal
*Polykrikos kofoidii**	2	Gymnodiniales	Polykrikaceae	Ōmura Bay, East China Sea, Japan
	3	Gymnodiniales	Polykrikaceae	Wadden Sea, Germany
	1	Gymnodiniales	Polykrikaceae	Isla San José, Mexico

*Polykrikos schwartzii**	1	Gymnodiniales	Polykrikaceae	Gulf of Naples, Italy
	1	Gymnodiniales	Polykrikaceae	Ōmura Bay, East China Sea, Japan
	1	Gymnodiniales	Polykrikaceae	Qinhuangdao, Bohai Sea, China
	2	Gymnodiniales	Polykrikaceae	Isla San José, Mexico
Quinquecuspis concreta	1	Peridiniales	Peridiniaceae	Qinhuangdao, Bohai Sea, China
	4	Peridiniales	Protoperidiniaceae	Aveiro, Portugal
	1	Peridiniales	Peridiniaceae	Wadden Sea, Germany
	1	Peridiniales	Protoperidiniaceae	Patricia Bay, Saanich inlet, Vancouver Island, Canada
Votadinium calvum	2	Peridiniales	Protoperidiniaceae	Ōmura Bay, East China Sea, Japan
Votadinium multispinosum	2	Peridiniales	Protoperidiniaceae	Saanich Inlet, Vancouver Island, Canada

19-Nov-2023

Dear Dr Mertens:

Hi Kenneth,
sorry for the delay in getting back to you. I have good news - only minor revisions needed - congratulations.
I already sent you a review by email right - let me know you got this please?
The other reviewer also loves your work. He/she commented that one "minus" was that your study is based on very few actual data.
I really hope that you agree with me that the feedback you have received is very constructive and extremely helpful. This feedback will significantly improve your paper. Using the comments you have been given, please revise your manuscript using the review, attending to every point that has been made. If you feel the need to rebut a reviewer comment, please eloquently justify this to me in your report.
When you resubmit, please include a comprehensive report to me on how the manuscript has been revised. I greatly look forward to receiving your resubmission, which I am sure will be a great credit to this journal.
Please consider thanking your reviewers in the acknowledgements.
As you see, there is an arbitrary timeframe for resubmission, but you can have as many extensions to this as you wish. Please take your time; I would rather have an excellent resubmission than a rushed one!
My best regards to you.
Jim Riding (Editor)

Dear Editor,
many thanks for the reviews, which were well received.
We have made a reply to the comments below.
Sincerely yours,
Kenneth Mertens

Your manuscript entitled "Revisiting the cyst-theca relationship for Votadinium spinosum: an examination of the Protoperidinium claudicans species complex", which you submitted to Palynology, has been reviewed. The reviewer comments are included at the bottom of this letter.

The reviews are in general favourable and suggest that, subject to minor revisions, your paper could be suitable for publication. Please consider these suggestions, and I look forwards to receiving your revision.

When you revise your manuscript please highlight the changes you make in the manuscript by using the track changes mode in MS Word or by using bold or coloured text.

To submit the revision, log into https://mc.manuscriptcentral.com/tpal and enter your Author Centre. Click on the purple 'Click here to submit a revision' link to start the revision process. If you have more than one manuscript awaiting revision, this will take you to a list of those papers and you can click on the Create a Revision' link for the paper you want to revise. Your manuscript number has been appended to denote a revision. Please enter your responses to the comments made by the reviewer(s) in the space provided. You can use this space to document any changes you made to the original manuscript. Please be as specific as possible in your response to the reviewer(s).

IMPORTANT: Your original files are available to you when you upload your revised manuscript. Please delete any redundant files before completing the submission.

Because we are trying to facilitate timely publication of manuscripts submitted to Palynology, your revised manuscript should be uploaded as soon as possible. If it is not possible for you to submit your revision in a reasonable amount of time, we may have to consider your paper as a new submission.

Once again, thank you for submitting your manuscript to Palynology and I look forward to receiving your revision.

Sincerely,
Dr Riding
Managing Editor, Palynology
jbri@bgs.ac.uk

Reviewer(s)' Comments to Author:

Reviewer: 1

Comments to the Author
Reviewer comments Palynology: Revisiting the cyst-theca relationship for Votadinium spinosum: an examination of the Protoperidinium claudicans species complex."
This is work of high standard, applying the full range of modern techniques to a palynological problem. The basic underlying problem is fundamental to dinoflagellate cyst palynology, and therefor meets the aims of the journal: do described fossil cysts represent biological species, and if different species can have morphologically indistinguishable cysts, do they represent different environments that could result in failed paleoenvironmental interpretations? The study is well-targeted on the cyst-theca relationship of living cysts with the cyst-based name Votadinium spinosum, since they previously were linked to motile cells of Protoperidinium claudicans, a species considered to include variations in thecal tabulation that might suggest polyphylogeny. The objective to examine cyst-theca relationships from widely spread global localities (France, Canada, China, and Japan) in search of morphologic variation is well conceived, and including molecular phylogenetics and macromolecular analysis of dinoflagellate cyst walls in addition to the usual incubation experiments sets the bar high for future work.
While this study produced interesting and significant results, these are based on very few actual documented observations. The limitations imposed by this, and the need to address this is included in detailed comments later here. If this was a well-funded project specifically designed to address this particular problem, the authors would need to include samples from more localities globally, and to carry out more incubation experiments. However, such a project would be difficult to fund, and the impression given is more of an opportunistic realization that the several authors working on samples from different regions saw the value of "pooling" their results and attempting "a first cut" at this problem arising out of other work? If this is the case, I support their efforts, not least due to the extra difficulties of working with heterotrophs. For me, this justifies a first attempt with otherwise unacceptably few results (e.g. a new species described from observing six specimens) – but it has to be explained in the text.

*Thanks for the encouraging comments. It is not so easy to get good sequences through hatching cysts, certainly for heterotrophic species, and it often takes many experiments before successful results are obtained, because of contaminations and so forth. Many similar studies have functioned like this, with only few sequences attached to the species description (see for instance Kawami et al. 2009, Phycological research for description of P. tricingulatum, only a few sequences; or Sarai et al. 2013, only a few sequences as well). We have to be very careful to only use the sequenced cysts/cells for the description of the species, otherwise we risk to have a species description that is too broad. We have added a short section at the start of the discussion: "*Although for the species descriptions only few specimens were hatched and few cells sequenced, we consider these sufficient to describe the species, since the morphological observations and sequences were reproducible. Sequencing heterotrophic species has a low success rate (e.g. Mertens et al. 2023, p. 16)."

The main result reveals a species complex including at least four suggested cyst types and their equivalent motiles. This is similar to other early-described species that proved to represent a species complex, and it improves taxonomy and understanding of phylogeny of the group. I would expect some colleagues to question if V. multispinosum and V. spinosum can be differentiated visually in the microscope (I am not fully convinced from the limited information here). Future attempts to apply the results to "working palynology" will show how far this goes to answering the underlying questions, but it certainly moves the subject forward.

Thanks for the interesting comments.

Specific comments
1. Title: We need a standardized term for these sort of experiments that include more than thecal examination of species that sometimes are not even thecate. Suggested alternative "New investigation of the cyst-motile relationship for Votadinium spinosum reveals a Protoperidinium claudicans species complex"

Thanks for the suggestion, we have followed it.

2. "dinoflagellate" is missing from Key words, and also could be included in title – for non-cyst worker readers?

"dinoflagellate" has been added to the keywords and we added Dinophyceae, Peridiniales to the title.

3. Introduction. Having shown the problems caused by different authors providing better descriptions for species from different localities than type locality – would be a good place to include a point about the particular value of targeting incubation experiments like these to the type locality (or nearby region), as with France in this case?

We have added this sentence: "We targeted cysts from France, which is not too far from the type localities of both the cyst- and motile-defined species (Ireland and Denmark respectively)."

4. 97 – "room temperature"?

Corrected to 16°C.

5. 116 – "hatchling" – need to standardize terms within the ms – E.g. 146. – "germinated motile cell ". Do you think this should replace terms previously used in other publications?

We have now used "hatchling" throughout. No I don't think this needs to be used everywhere.

6. 135-6 – is it clear further in the ms which are from hatched?

Yes, there is no confusion about this.

7. 177 – in this work are cyst measurements from palynology or living material significantly different (possible effect of processing increasing size?).

As the reviewer can see, the cyst measurements from palynology (see V. multispinosum) and from incubation (see P. carriae) are very similar.

8. 225 – delete "as well" here and elsewhere in ms.

Done.

9. 277 and elsewhere – "beset" = more concentrated processes or optical effect from sulcal depression "reducing surface area" seen?

No we do not consider to be an optical effect.

10 Results. For each sampled region: should show how many incubation experiments attempted, hatched, and produced motiles.

This is written in the text: in section 3.1: " Undescribed motile cells, here assigned to *Protoperidinium carriae* n. sp., emerged from cordate, spinose cysts isolated from surface sediments from Patricia Bay, Saanich Inlet, BC, Canada (six specimens identified)" and *in section 3.2: "*Undescribed motile cells, here assigned to *Protoperidinium claudicans*, emerged from cordate, spinose cysts isolated from surface sediments from Maresclé, Morbihan, France (six specimens identified), one cyst from Fangchenggang, Guangxi, China and one cyst from Quanzhou, Taiwan Strait, China and one cyst, one motile cell hatched from a cyst and one motile isolated from plankton from Hokkaido, Japan (Figure 1 and Table 2)."

Each species description: should include relevant comparisons with other species (how differ). Ideally should also include morphologic variation (but not well documented from so few here).

The comparison with other species is handled in discussion: 4.1 and 4.2.

11. 258-9 – delete "reached", and replace "to" with "than" (and elsewhere -).

Corrected.

12. 267 – description from incubations only, or from sediments after palynology treatment?

This is mentioned at the end of the paragraph: "The description is based on cysts used in the incubation experiments.", the description of V. multispinosum is based on palynological treatments.

13. P. carriae n. sp. – Holotype from Saanich Inlet, B.C. (215-18). Its cyst is described from there (267-280), and is equivalent to V. multispinosum sp. Nov. (holotype described from different locality, Vancover Island (292-347). Two separate names for cyst and motile of one new species described from different type localities in the same publication - will really stoke up the fires of those advocating one species name. The two separate names are generally accepted by many, but it would be worth including an explanation for why a separate type locality was chosen for the cyst.

*We follow an approach explained in Head et al. (Palynology in submission) that outlines how dual nomenclature can be used. We have added: "*The type localities of *P. carriae* and its equivalent *V. multispinosum* are not identical but are both from around Vancouver Island, where both are considered widely distributed."

The cyst is described twice. Lacking adequate data for morphological variation in size and process density, these cyst descriptions are so alike that including both is superfluous – it is sufficient to say the cyst of P. carriae conforms to V. multispinosum.

We beg to differ. The description in P. carriae is for the cysts of the incubation experiments, and in V. multispinosum it is related to the fossil specimens. We consider that both are necessary to document that they are similar.

14. 320-21 – pronounced apex not shown in plate.

Yes, but since it is considered equivalent to P. carriae, the cyst of the latter shown in Plate 1, fig. 1, which do shows a pronounced apex, does show this feature.

15. 370 onwards- Here too only very few results from incubation experiments (nine specimens from 4 different localities in France, China, Taiwan Strait and Japan)

Although few incubation experiments, the results were reproducible and therefore considered reliable.

446 – delete “processes”.

Removed.

478 – on - two further new species were suggested, but not formally proposed since too few specimens - this is worth including here as presented, to be followed up in future.

Thanks for agreeing.

16. Discussion. Section 4.2 Morphologic comparisons between the cysts of four suggested species is limited by the lack of sufficient data on morphologic variation, and this should be clearly stated, since it is arguably the most important point for palynology. These cysts were included in the one V. spinosum previously, and it remains to be seen if the differences suggested here allow differentiation when applied to routine palynology or if, in practice, they will stand as examples of different biological species with one cyst-based species. Similarly, biogeographic work should eventually show any paleoenvironmental significance to conclusions here.

We have added this to section 4.2: "V. spinosum and V. multispinosum were both previously identified as V. spinosum, but this study demonstrates that they are two different species and can be distinguished using morphological criteria." The biogeography is discussed in 4.6.

Section 3.4 adds another useful few data to the relatively new "tool" (cyst-wall analysis) for cyst work that offers potential for improving our phylogenetic understanding, as do the molecular analysis results here. Indicating that the processes on these cysts may have evolved at different times (640) is particularly interesting since fossil cyst genera have been erected sometimes according to presence/absence of processes.

*We have added a note: "*This implies that the presence or absence of processes can be used to separate species, but not on a higher taxonomic level."

The observation that species with pentagonal or hexagonal 2a plates cluster together in a monophyletic group questions previous significance sometimes placed on plate shape in thecal-based classification.

This has been discussed in section 4.5: "On a group level, the phylogeny demonstrates also that species that have been assigned to the Oceanica group cluster together in a monophyletic group, despite some of them having a pentagonal or hexagonal 2a. There does not seem to be any clear logic in the positions of species that have a quadra, penta or hexa configuration."

17. Figures: Not clear in all cases which theca or cysts are from which locality in figs. 2 and 3 (data points from separate countries and from separate localities in a country should be identified by different symbols). Captions should indicate that only cysts and theca from incubations are included in these Figs. It is notable that in some cases there are not equal numbers of cysts and motiles from experiments?

Thanks for spotting this. We have clarified this in the caption.

18. Plates: Captions – Patricia Bay should not be shortened to Pat Bay.

Corrected.

1.6-15 caption missing.

Added.

2.7-12 caption should include locality and that it is from a sediment sample (only number given with no indication if palynological prepared or not).

Added.

Pl. 3 caption should include that experiments of P. claudicans (naming species same as for Pl.2), and similarly for Pl. 4. Caption should indicate that only some cysts here are from experiments (4 and 10-11?).Pl. 6. Are 2-6 all different cysts? 7-9 are from which cyst? Pl. 7. Are these all from the same cyst?
Many of the captions fall disappointingly short of the required standard, and must be "cleaned up".

We have significantly expanded the captions and tried to be more clear.

19. References have not been checked by me.

We have checked all references and made corrections where necessary.

Recommendation: acceptance after minor revision (after consideration of these comments)

Thanks for the constructive review.

Reviewer 2
Review of:
Mertens et al. Revisiting the cyst-theca relationship for *Votadinium spinosum*: an examination of the *Protoperidinium claudicans* species complex.

Revisiting cyst-theca relationships within the cyst defined genus Votadinium with a re-examination of the Protoperidinium claudicans species complex

The paper is not just limited to V. spinosum, but also discusses cordate species of Votadinium. I suggest modify the title - a suggestion above.

We have modified the title according to the suggestions of the other reviewer. Thanks for the suggestion though.

Initiate the paper with a comment that the study is an extension to the revision of the genus Votadinium by Gurdebeke et al. 2020. I found the paper hard to follow on my first read.

We have added this sentence to the final paragraph on the introduction: "Here we pursue research on *Votadinium* reinitiated most recently by Gurdebeke et al. (2020), and"

It would benefit from a revised Table 1. that summarises the current situation of known species attributed to Protoperidinium and Votadinium to include those defined, refered to in this paper in bold and the others in lower case. Divide the table into spiny and non spiny species. Use existing Table 1 headings to columns or edit/abbreviate to fit the categories included.

		SPINY				
Motile	Cyst	2a shape	RIB	DNA	AFT	FTIR Ref
1. **P. claudicans**	?**V.spinosum**	**Penta**	**X**			
2. **P. carriae sp nov.**	**V.multispinosum sp nov**	**Quadra**	**X**	**X**		
		WITHOUT SPINES				
3. P?		**V psilidora**		**X**		
4. P?		**V. sp.**		**X**		
5. P.latidorsale		**V. calvum**	**X**		**X**	
6. P. ?		V. concavum	X			

5. List the remaining cordate species and relevant information.

We have modified the table accordingly.

Lines 67 to 69 could then be omitted or shortened.

Done.

Include a brief background to the table and structure of the paper. Perhaps bringing part or the whole of the last paragraph of the introduction to the front (lines 75 to 83). I recognise of course that it is normally the last paragraph of the Introduction to a paper that outlines the subsequent content. In this case it was not enough to distinguish the separate bits of work which is an amalgam of different studies by the various authors in their home labs with not all methods applied in every case.

Thanks for the suggestion. We did not decide to move the final paragraph to the front, but we have tried to make the flow better and moved around some of the paragraph.

Confusion would also be removed if all the taxonomic descriptions (systematics) in the paper were within one section at the end and independent of the rest of the text. While important, as at present they break up the flow of the paper.

We have looked at recent publications in Palynology (e.g. Lindstrom 2023; Baranyi et al. 2021) and all seems to keep the systematic section as in our paper, so we have kept it as is.

The plates are superb.

Thank you.

Plate 1. The edge of Fig. 9 needs tidying up, and the alignment of the mag. bars on Figs 7 to 9. Ditto plate 2 Figs 7-9 and plate 6 Figs 7-9. although this may be fine in the originals.

Plate 1: The edge of Fig. 9 has been cleaned, and the scale bars have been aligned. The scale bars have been aligned on plate 2 and plate 6.

See additional comments on the enclosed manuscript. Funding for a SEM is acknowledged at the end of the paper even though there is no mention of work with a SEM. It would have been useful to include some SEM imagery of the surface ornamentation of both the spiny and smooth cysts. I understand the reason for including the acknowledgement, nevertheless.

We have added an extra plate with SEM micrographs, and included their description in the text.

I enjoyed reading the paper, which maintains progress by the authors in resolving the mysteries of motile/cyst relationships. The paper should be published after the author's have taken note of the comments and addressed as they and the editor see fit.

Thanks for your encouragement.

Replies to the manuscript annotated by Chris Reid.

We have made the corrections suggested.

** GenBank accession numbers have been added, to the text and the figures.*