Ultrastructure and molecular phylogenetic position of a new marine sand-dwelling dinoflagellate from British Columbia, Canada: Pseudadenoides polypyrenoides sp. nov. (Dinophyceae)

Mona Hoppenrath, Naoji Yubuki, Rowena Stern & Brian S. Leander

To cite this article: Mona Hoppenrath, Naoji Yubuki, Rowena Stern & Brian S. Leander (2017) Ultrastructure and molecular phylogenetic position of a new marine sand-dwelling dinoflagellate from British Columbia, Canada: Pseudadenoides polypyrenoides sp. nov. (Dinophyceae), European Journal of Phycology, 52:2, 208-224, DOI: 10.1080/09670262.2016.1274788

To link to this article: http://dx.doi.org/10.1080/09670262.2016.1274788
Ultrastructure and molecular phylogenetic position of a new marine sand-dwelling dinoflagellate from British Columbia, Canada: *Pseudadenoides polypyrenoides* sp. nov. (Dinophyceae)

Mona Hoppenrath*a,b, Naoji Yubuki*i,c, Rowena Stern*a,c and Brian S. Leander*a

*a Departments of Botany and Zoology, University of British Columbia, 6270 University Boulevard, Vancouver, BC, V6T 1Z4, Canada; b Current address: Senckenberg am Meer, Deutsches Zentrum für Marine Biodiversitätsforschung (DZMB), Südstrand 44, Wilhelmshaven, Germany; c Current address: Departmen of Parasitology and Zoology, Faculty of Science, Charles University, Vinicna 7, Prague, 128 44, Czech Republic; d Current address: Sir Alister Hardy Foundation for Ocean Science, The Laboratory, Citadel Hill, Plymouth PL1 2PB, UK

**ABSTRACT**
Two monospecific genera of marine benthic dinoflagellates, *Adenoides* and *Pseudadenoides*, have unusual thecal tabulation patterns (lack of cingular plates in the former; and no precingular plates and a complete posterior intercalary plate series in the latter) and are thus difficult to place within a phylogenetic framework. Although both genera share morphological similarities, they have not formed sister taxa in previous molecular phylogenetic analyses. We discovered and characterized a new species of *Pseudadenoides*, *P. polypyrenoides* sp. nov., at both the ultrastructural and molecular phylogenetic levels. Molecular phylogenetic analyses of SSU and LSU rDNA sequences demonstrated a close relationship between *P. polypyrenoides* sp. nov. and *Pseudadenoides kofoidii*, and *Adenoides* and *Pseudadenoides* formed sister taxa in phylogenetic trees inferred from LSU rDNA sequences. Comparisons of morphological traits, such as the apical pore complex (APC), demonstrated similarities between *Adenoides*, *Pseudadenoides* and several planktonic genera (e.g. *Heterocapsa*, *Azadinium* and *Amphidoma*). Molecular phylogenetic analyses of SSU and LSU rDNA sequences also demonstrated an undescribed species within *Adenoides*.

**ARTICLE HISTORY**
Received 23 September 2016; Revised 23 November 2016; Accepted 3 December 2016

**KEYWORDS** Benthic; morphology; phylogeny; *Pseudadenoides kofoidii*; taxonomy; ultrastructure

**Introduction**

Herdman (1922) described two *Amphidinium* species characterized by their depressed, small episme: *A. eludens* and *A. kofoidii*. *Amphidinium kofoidii* is round to square in shape with a striking starch-ring in the middle of the cell (Herdman, 1922, fig. 2). *Amphidinium eludens* Herdman is more oval with an inconspicuous episme and a bulge in the sulcal region (Herdman, 1922, fig. 1). Balech (1956) described the new thecate genus *Adenoides*, with *A. eludens* (Herdman) Balech as the type. Hoppenrath et al. (2003) re-investigated and revised the description of *Adenoides eludens* and discussed the taxonomical problem caused by the basionym selection from Balech in detail. *Amphidinium kofoidii* Herdman would have been the correct basionym as the described species *Adenoides eludens* was morphologically conspecific with it. Whether the second species (*Amphidinium eludens*) described by Herdman (1922) really exists was not clear until recently (Hoppenrath et al., 2014), aside from a brief textual account on the observation by Dodge & Lewis (1986), Gómez et al. (2015) discovered a new thecate taxon that under the light microscope looked like *Amphidinium eludens*. This new genus was morphologically different from *Adenoides* and also distinct at the molecular phylogenetic level (Gómez et al., 2015). The formal description of this genus was complicated because nomenclatural problems had to be solved. In accordance with the ICN (International Code of Nomenclature for Algae, Fungi, and Plants; McNeill et al., 2012), *Adenoides* has been redefined based on the emended description of the basionym *Amphidinium eludens* (Gómez et al., 2015) and the new combination *Pseudadenoides kofoidii* (Herdman) F.Gómez, R. Onuma, Artigas & T.Horiguchi has been proposed to accommodate *Amphidinium kofoidii* (*Adenoides eludens sensu Balech, 1956*).

Both *Adenoides* and *Pseudadenoides* have very unusual thecal tabulation patterns (Hoppenrath et al., 2003; Gómez et al., 2015), and the designation of plates, especially of the cingular and sulcal plates, depends largely on interpretation (summarized for *P. kofoidii* in Hoppenrath et al., 2003). *Pseudadenoides* lacks a precingular plate series, a feature only known from the also benthic genus...
Plagiodinium Faust & Balech (Faust & Balech, 1993; Hoppenrath et al., 2014). The classification of Pseudadenoides (as Adenoides) is still uncertain (Hoppenrath et al., 2003; not listed by Hoppenrath in Adl et al., 2012). Molecular phylogenetic analyses have shown Pseudadenoides (as Adenoides) to branch as the sister lineage to the Prorocentrum clade (e.g. Zhang et al., 2007; Hoppenrath & Leander, 2008; Orr et al., 2012; Hoppenrath et al., 2013), a relationship that is important for understanding character evolution in core dinoflagellates (Hoppenrath et al., 2013, 2014).

A diversity survey using mitochondrial COI (cytochrome oxidase I) gene sequences revealed that Pseudadenoides (as Adenoides) also occurred in a plankton sample from Saanich Inlet (British Columbia, Canada) at 10 m depth (Stern et al., 2010), suggesting that the habitat distributions and species diversity within the genus is currently poorly understood.

During our survey of species diversity in marine sandy sediments in British Columbia, Canada, we discovered and characterized a second Pseudadenoides species at both the ultrastructural and molecular phylogenetic levels.

Materials and methods

Sampling

Sand samples were collected with a spoon during low tide at Centennial Beach, Boundary Bay, British Columbia, Canada during the years 2005 to 2007 (Supplementary Table 1). Pseudadenoides polypyrrenoides sp. nov. occurred together with P. kofoidii in most samples.

Sand samples were transported directly to the laboratory, and the flagellates were separated from the sand by extraction through a fine filter (mesh size 45 μm) using melting seawater-ice (Uhlig, 1964). The flagellates accumulated in a Petri dish beneath the filter and were then identified at ×40 to ×250 magnifications. Cells were isolated by micropipetting for the differential interference contrast (DIC) light microscopy and culture establishment as described below.

Culturing

Isolated cells (sample taken 9 May 2005) were washed in filtered seawater and transferred into a Petri dish containing f/2-medium (Guillard & Ryther, 1962). After establishment of the unialgal culture it was maintained in tissue flasks at 17°C under low light conditions in f/2-medium. Unfortunately, the culture died shortly after our first electron microscopical preparations at the end of 2007.

Light and electron microscopy

Cells were observed directly and micromanipulated with a Leica DMIL inverted microscope (Wetzlar, Germany). For DIC light microscopy, isolated cells were placed on a glass specimen slide and covered with a cover slip. Images were produced with a Zeiss Axiosplan 2 imaging microscope (Carl-Zeiss, Oberkochen, Germany) connected to a Leica DC500 colour digital camera.

For scanning electron microscopy (SEM), a part of the culture was fixed with several drops of acidic Lugol’s solution overnight at room temperature. Cells were transferred onto a polycarbonate membrane filter (Corning Separations Div., Acton, Massachusetts, USA) with 5 μm pore size, washed with distilled water, dehydrated with a graded series of ethanol (30, 50, 70, 80, 95, 100%) and 100% hexamethyldisilazane (HMDS) at the end, and air dried. Filters were mounted on stubs, sputter-coated with gold and viewed under a Hitachi S4700 Scanning Electron Microscope (Hitachi High-Technologies Corporation, Tokyo, Japan). SEM images were presented on a black background using Adobe Photoshop CS6.

For transmission electron microscopy of Pseudadenoides polypyrrenoides, cells were mixed with the same volume of fixative solution containing 4% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.2) at room temperature for 1 h. Cells were aggregated into a pellet by centrifugation at 1000 g for 5 min and rinsed with the buffer three times. These were then post-fixed in 1% OsO₄ in 0.2 M sodium cacodylate buffer at room temperature for 2 h followed by dehydration through an ethanol series (30, 50, 70, 80, 90, 95, 100%). Ethanol was replaced by 100% acetone before infiltrated with acetone-Epon 812 resin mixtures and 100% Epon 812 resin. Ultrathin sections were cut on a Leica EM UC6 ultramicrotome (Leica Microsystems, Wetzlar, Germany) and double-stained with 2% uranyl acetate and lead citrate (Reynolds, 1963). Ultrathin sections were observed using a Hitachi H7600 transmission electron microscope (Hitachi High-Technologies Corporation, Tokyo, Japan).

DNA extraction and polymerase chain reaction (PCR)

The cultures of Pseudadenoides species and additional cultured species (called Adenoides eludens) were obtained from the National Centre for Marine Algae and Microbiota (NCMA, formerly CCMP, Maine, USA) and the Microbial Culture Collection at National Institute for Environmental Studies (NIES, Tsukuba, Japan). Fifteen ml of culture were used for DNA extractions using a
DNeasy plant mini kit (Qiagen, Mississauga, Ontario, Canada) according to the manufacturer’s instructions. PCR amplification was carried out using Puretaq Ready-To-Go PCR beads (GE Lifesciences, New Jersey, USA) and Jumpstart Redtaq ReadyMix Reaction mix (Sigma-Aldrich, St. Louis, Missouri, USA) using 0.4 µmol (final concentration) of each primer (according to manufacturer’s instructions) in either 25 or 50 µl reactions. Amplification of large subunit (LSU) rDNA sequences was carried out using forward primers D1R or D3a (Scholin et al., 1994) and reverse primer LSU-R2 (Takano & Horiguchi, 2006). Sequencing reactions were performed with these primers and with the reverse primer LSU-25R1 (Takano & Horiguchi, 2006). Amplifications of small subunit (SSU) rDNA sequencing reactions were performed with the forward primer UPro18SF and the reverse primer U18R (Hong et al., 2008). Sequencing reactions were performed with these primers and the universal primers EK555F (forward) and EK1269R (reverse) (López-Garcia et al., 2001). Residual primers were removed from PCR reactions using the ExoSAPIT reagent (Affymetrix, USA). Sequencing was performed by Macrogen (Korea) and Source Bioscience (Nottingham, UK).

**Sequence alignments and phylogenetic analysis**

Partial DNA sequences were manually checked for errors, and constructed to their full length using BioEdit (Hall, 1999). Additionally the sequences were checked for their correct identity as either *Pseudadenoides* or *Adenoides* by the BLASTn algorithm (Altschul et al., 1990). SSU and LSU rDNA sequences were automatically aligned using MAFFT with L-INS-i option (Katoh et al., 2005; Katoh & Standley, 2013), as recommended for an analysis of a small alignment like this dataset. The two different datasets were then manually aligned and trimmed to exclude all ambiguous sites using Mesquite version 3.04 (Maddison & Maddison, 2015). The final datasets used in the analysis contained 64 taxa and 1564 unambiguously aligned sites for the SSU rDNA dataset, and 51 taxa and 1011 unambiguously aligned sites for the LSU rDNA dataset.

The phylogenetic trees were inferred using Maximum Likelihood (ML) with the program Garli 2.0 (Zwickl, 2006) under a GTR + I + G model for the SSU rDNA dataset and a TIM2 + I + G for the LSU rDNA dataset, both of which were selected by jModeltest 2.1.6 (Darriba et al., 2012). ML bootstrap analyses were carried out with 1000 pseudoreplicates. Bayesian analyses using MrBayes v3.2.5 (Ronquist et al., 2011) was performed on two independent groups of four Monte-Carlo-Markov Chains (MCMC), starting from random trees. A total of 1 000 000 MCMC generations were run, and the trees were sampled every 500th generation. The first 25% of the generations were discarded as burn-in. Posterior probabilities (PP) were calculated from the sampling points.

**Results**

*Pseudadenoides polypyrenoides Hoppenrath, Yubuki, R. Stern & B. S. Leander, sp. nov. (Figs 1–3, 7–24, 37–51)*

**Description:** Thecate species with laterally flattened, asymmetrical cells with the dorsal side of the posterior end longer. Button-like epitheca and large hypotheca with complete, not displaced, shallow anterior cingulum and very short sulcus. Plate formula: APC 4′ 6C 4S 5′′ 5p 1′′′. No precingular plate series. Complete posterior intercalary plate series. Three large pores containing small sieve-like pores on the dorsal side of the posterior end of the cell, one each on plates 3p, 4p and 1′′′. Specimens 28.8–38.3 µm long and 25.6–34.0 µm deep. Central nucleus. Typical dinoflagellate chloroplasts with several stalked pyrenoids.

**Holotype:** Specimen shown in Fig. 10, conserved on SEM stub designated CEDiT2016H55 deposited in the Centre of Excellence for Dinophyte Taxonomy, Senckenberg am Meer, Wilhelmshaven, Germany.

**Sequences:** Nearly complete SSU and partial LSU rDNA sequences (GenBank accession numbers: KU726886 and KU726887).

**Type Locality:** Boundary Bay, British Columbia, Canada (49°0.0’N, 123°8.0’W).

**Etymology:** *polypyrenoides* in Greek, meaning several pyrenoids in contrast to only two large pyrenoids in *Pseudadenoides kofoidii*.

**General morphology**

Cells were asymmetrically oval, longer dorsal than ventral, and flattened laterally (Figs 1–3). Specimens were 28.8–38.3 µm long and 25.6–34.0 µm deep (n = 11) and ~24 to 25 µm wide (n = 2). The button-like epitheca was inconspicuous (Figs 1–3, 7–16). The cingulum completely encircled the epitheca, was not displaced, was very slightly depressed, and was located at the anterior end of the cell (Figs 9, 10, 12–14, 17–19). The slightly depressed sulcus was located in the anterior third of the cell, neither extending onto the epitheca nor reaching the posterior end of the cell.
The large hypotheca covered most of the cell (Figs 1–3, 7–16). The large round to oval nucleus was situated in the centre of the cell (Figs 1, 2). The cells contained brown chloroplasts and several pyrenoids with starch sheaths (starch-rings) of different diameters (Figs 2, 3). These starch-rings were not easily recognizable.

The plate formula was APC 4’n 6C 4S 5”’ p 1’’’ (Figs 7–12, 25–28). The epitheca consisted of five plates (Figs 17–19, 27). The apical pore complex (APC) consisted of the round to angular apical pore plate (Po) with a central apical pore, covered by a small round cover plate (cp) (Fig. 18, Supplementary Fig. 3). A small, narrow plate (canal plate X?) connected the first apical plate (1’) with either the apical pore or the cover plate by traversing the Po plate (Fig. 18, Supplementary Fig. 3). In addition to the apical pore, the Po plate had normal thecal pores arranged around the apical pore (Supplementary Fig. 3, arrows). Four apical plates of very different shapes bordered the APC (Figs 17–19). Plates 1’ and 4’ were in contact with the anterior sulcal plate (Sa) (Figs 17, 18, 27). No precingular plates were present (Fig. 27). The shallow cingulum consisted of six plates (Figs 17–19, 27). Four sulcal plates surrounded the flagellar pore (Figs 20, 21, 27, 28). The hypotheca consisted of eleven plates (Figs 7–16, 25, 26, 28). The first (1p) and second (2p) postcingular plates were positioned on the left lateral side of the cell; the third postcingular plate (3’p) was positioned on the dorsal side of the cell; and the relatively large and posteriorly pointed fourth postcingular plate (4’p) and the small fifth (5’p) postcingular plate were positioned on the right lateral side of the cell (Figs 7–16, 25, 26). Five large posterior intercalary plates made up a series that completely surrounded and covered most of the hypotheca (Figs 7–16, 25, 26, 28). The first (1p)
and fifth (5p) posterior intercalary plates contacted each other in a long ventral suture and unusually bordered the posterior sulcus (Figs 12, 16). The third (3p) and fourth (4p) posterior intercalary plates met in a long dorsal suture (Figs 14, 15). One pentagonal antapical plate (1''′′′) was located at the posterior end of the cell (Figs 15, 16, 28). Three large pores with a sieve-like internal structure (Fig. 24) were present on the dorsal surface at the posterior end of the cell (Figs 10, 15, 26, 28). Plates 3p and 4p had the large pores at the posterior end, and plate 1''′′ had the large pore at the dorsal end (Figs 7–15, 28). The thecal plates were smooth with scattered pores (Figs 22–24, Supplementary Fig. 1). Wide sutures were sometimes transversally striated (Supplementary Figs 1, 2).
Morphological variability and plate pattern interpretations

An additional small triangular plate was observed between plates C1 and 1′′′ (Fig. 19). Alternative plate pattern interpretations were possible, especially in the sulcal area. The sixth cingular plate (C6) could be a right anterior sulcal plate (Sad); if so, then the anterior sulcal plate would become a left anterior sulcal plate (Sas), and the plate formula would change to: APC 4′ 5C 5S 5p 1′′′. Additionally, the fifth postcingular plate (5′′′) could be a sulcal plate, becoming the right sulcal plate (Sd). If so, then the right sulcal plate would be changed into a middle sulcal plate (Sm), and the plate formula would change to: APC 4′ 5C 6S 4′′ 5p 1′′′ (Figs 29–32). If the posterior intercalary plates must lie between the postcingular and antapical series (neither touching the cingulum nor the sulcus) and the antapical plates must border the sulcus and not touch the cingulum, then the hypothecal plates should be named as follows: 1p = 1′′′, 2p = 1p, 3p = 2p, 4p = 3p, 5p = 2′′′, 1′′′′ = 4p. If so, then the plate formula would change to: APC 4′ 6C 4S 5′′ 4p 1′′′ or APC 4′ 5C 5S 5′′ 4p 2′′′′ or APC 4′ 5C 6S 4′′ 4p 2′′′ (Figs 33–36).

Ultrastructure

Cells contained a typical dinokaryon with condensed chromosomes (Figs 37, 38), trichocysts below thecal pores (Fig. 39), developing stages of trichocysts close to dictyosomes (Fig. 40), and mitochondria with tubular cristae (Fig. 41). The Golgi apparatus was located near the nucleus (Fig. 40). Dinoflagellate chloroplasts associated with several pyrenoids were distributed at the cell periphery (Figs 37, 38). The chloroplasts contained parallel thylakoids (Fig. 42) in stacks of three (Fig. 46) and had three outer membranes. Single-stalked pyrenoids were covered with a starch sheath and were partly traversed by thylakoid pairs (Figs 43–45). An electron-dense plug-like structure was positioned beneath the apical pore and was surrounded by trichocysts (Fig. 47). A membranous network, possibly belonging to the pusule, was associated with the flagellar apparatus below the flagellar pore (Fig. 48). An accumulation of trichocysts and their primordia were positioned...
below the large pores with an internal sieve-like structure (Figs 49–51). Some trichocysts were observed extruding through these sieve pores (Fig. 50).

**Molecular phylogenetic analyses**

The phylogenetic tree inferred from SSU rDNA sequences using a maximum likelihood method demonstrated that the new species (KU726886) was a sister taxon to the *P. kofoidii* clade with high support (BP = 94 and PP = 0.99) (Fig. 52). The phylogenetic tree inferred from LSU rDNA sequences using a maximum likelihood method also showed that the new species (KU726887) formed a sister lineage to a clade comprising all *P. kofoidii* sequences from different localities (Fig. 53) with the highest statistical support (BP = 100 and PP = 1.00). The two species differed by eight bases in the SSU and 37 bases in the LSU rDNA sequences. The *P. kofoidii* clades contained *P. kofoidii* sequences (LC002843, LC002848) described by Gómez *et al.* (2015) from France plus sequences from CCMP2081 (KX000290, KX000294, Germany), CCMP1891 (KX000289, KX000293, Canada) and NIES-1367 (KX000291, KX000295, Japan) cultures from this study, which confirmed CCMP2081, 1891 and established NIES-1367 as *P. kofoidii* (Figs 52, 53). Additionally, the *P. kofoidii* clade inferred from SSU rDNA sequences (Fig. 52) contained a publicly available strain from CCCM 683 retrieved as *Adenoides eludens* (AF274249) but identified as *P. kofoidii* by Gómez *et al.* (2015). The phylogenetic positions of the SSU and LSU rDNA sequences (KX000292, KX000296) from the NIES-1402 culture (identified as *A. eludens* sensu Balech, now *P. kofoidii*) were unresolved, suggesting that this strain represents a new species of *Adenoides*, albeit with weak statistical support (Fig. 53).

Molecular phylogenetic analysis of the LSU rDNA sequences showed that the sister group to *Pseudadenoides* was a clade consisting of the new *Adenoides eludens* sequences (BP = 73 and PP = 1.00; Fig. 53). Although a clade of *Prorocentrum* taxa clustered close to the *Adenoides/Pseudadenoides* clade in the LSU rDNA tree (Fig. 53), this relationship did not receive statistical support in the tree inferred from SSU rDNA sequences (Fig. 52). The SSU phylogeny did not resolve the relationship between *Pseudadenoides* and *Adenoides*.

Nonetheless, the phylogenetic trees inferred from both the SSU and LSU rDNA sequence datasets demonstrated that *P. kofoidii*, *A. eludens* and *P. polypyrenoides* sp. nov. are distinct from each other.

**Discussion**

The most similar species to *P. polypyrenoides* sp. nov. is *P. kofoidii* (basionym: *Amphidinium kofoidii*, synonym: *Adenoides eludens* sensu Balech); both species have identical thecal tabulation patterns, a button-like epitheca, a shallow anterior cingulum, a very short sulcus, one flagellar pore, and a large hypotheca that is longer dorsally than ventrally (Balech, 1956; Hoppenrath *et al.*, 2003; Gómez *et al.*, 2015) (Table 1). In both species, the thecal plates are smooth with scattered pores. They have overlapping cell sizes but *P. polypyrenoides* is generally larger, more rectangular and more elongated in mixed environmental samples (Figs 1–6; Supplementary Table 1). The most reliable feature
to distinguish the two species under the light microscope is the number and size of the pyrenoids; *P. kofoidii* possesses two large lateral pyrenoids (easily visible as the starch sheath appears as a ring-like structure), whereas *P. polypyrenoides* has several inconspicuous pyrenoids of different diameters distributed through the cell (Figs 1–6). The nucleus in *P. polypyrenoides* is located in the cell centre, in *P. kofoidii* in the lower dorsal cell half (Figs 1–6). Furthermore, *P. polypyrenoides* differs from *P. kofoidii* in having three large pores with an internal sieve on the dorsal side of the posterior end of the cell on plates 3p, 4p and 1′′′ (Figs 10, 15; Table 1); *P. kofoidii* has only two of these pores on plates 3p and 4p (Hoppenrath et al., 2003). The two species are easily distinguishable by molecular phylogenetic analyses within a highly supported monophyletic group (Figs 52, 53).

The general ultrastructure of *P. polypyrenoides* sp. nov. is typical for dinoflagellates. One of the main differences between *P. polypyrenoides* sp. nov. and *P. kofoidii* is the size and number of
the pyrenoids. It has been suggested previously that pyrenoid ultrastructure is at most a species-level character, and different types of pyrenoids have been described for several dinoflagellate species (e.g. Dodge & Crawford, 1971; Hansen & Moestrup, 1998; Schnepf & Elbrächter, 1999; Hoppenrath & Leander, 2008). However, some traits associated with the pyrenoid structure can reflect phylogenetic relationships above the species level (Hansen & Moestrup, 1998). For instance, despite differences in the size and number, the pyrenoids in both species of *Pseudadenoides* share the same basic structure (i.e. single-stalked pyrenoids with internal pairs of thylakoids and external starch rings). Although single-stalked pyrenoids (type C) have been described for a few other species (e.g. *Heterocapsa rotundata* (Lohmann) Hansen, *Peridiniella catenata* (Levander) Balech), they are not common in dinoflagellates (Dodge & Crawford, 1971; Hansen, 1989; Hansen & Moestrup, 1998). Moreover, pyrenoids with internal pairs of thylakoids have also been observed in *Prorocentrum cordatum* (Ostenfeld) Dodge (as *Exuviaella mariae-lebouriae* Parke et Ballantine) and *Amphidinium carterae* Hulburt (Dodge & Crawford, 1971). Whether or not these shared traits reflect homology remains to be determined with more robust molecular phylogenetic analyses.

We also characterized traits associated with trichocysts, pusules and the apical pore region in *P. polypyrenoides* sp. nov. which are similar to those described previously in other species of dinoflagellates. The earliest stage of trichocyst ontogeny involves primordia in vesicles containing homogeneous material and a crystalline lattice (Bouck & Sweeney, 1966). Developmental stages of trichocysts were previously described in detail in *P. kofoidii* (Hoppenrath et al., 2003); similar stages of trichocyst development were also evident in *P. polypyrenoides* sp. nov. (Supplementary Fig. 11). Different types of pusules have been distinguished

**Figs 37–41.** Transmission electron micrographs (TEM) showing general ultrastructural characteristics of *Pseudadenoides polypyrenoides* sp. nov. **Fig. 37.** Longitudinal image. **Fig. 38.** Transverse image through the nucleus and the pyrenoides. **Fig. 39.** Longitudinal TEM of trichocysts below a thecal pore. **Fig. 40.** High magnification TEM showing immature trichocysts adjacent to Golgi apparatus near the nucleus. **Fig. 41.** High magnification view of the mitochondrion with tubular cristae. G = Golgi body; M = mitochondrion, n = nucleus, T = trichocyst. Arrows show pyrenoids. Scale bars =10 µm (37, 38), 1 µm (39, 40), and 500 nm (41).
at the ultrastructural level (Cachon et al., 1970; Dodge, 1972). The network of membranes reported here for *P. polypyrenoides* sp. nov. is interpreted to be a collapsed pusule most similar to so-called ‘sack pusules’ (Dodge, 1972) (Supplementary Fig. 12). Sack pusules have also been found in *Prorocentrum* species. The plug-like structure in the apical pore region described here for *P. polypyrenoides* sp. nov. is most similar to the dark-staining material in the same region in *Peridiniella catenata* (Hansen & Moestrup, 1998). Whether or not these shared traits reflect homology remains to be determined with more robust molecular phylogenetic analyses.

Both species of *Pseudadenoides* have two unusual thecal features: (1) they lack a precingular plate series, a feature only known from the benthic genus *Plagiodinium* M.A. Faust & Balech (Faust & Balech, 1993; Hoppenrath et al., 2014); and (2) they have a complete posterior intercalary plate series (completely encircling the cell, the first and last posterior intercalary plates touching each other ventrally), which is novel. As described in the results, different hypothecal plate interpretations are possible when following strict definitions of posterior intercalary and antapical plates. However, the alternative pattern (4p 2‴‴) would result in antapical plates located ventrally (not reaching the antapex) and the intercalary plates not being arranged in a series, but instead arranged in a cluster. To the best of our knowledge, only one species, *Pyrophacus steinii* Schiller, is known to have a posterior intercalary plate ‘cluster’ (Balech, 1978).

The recently emended *Adenoides* Balech emend. F.Gómez, R.Onuma, Artigas & T.Horiguchi shows
similarities with *Pseudadenoides* (Gómez et al., 2015) (Table 1). The APC morphology is nearly identical to *Pseudadenoides*. Moreover, *Adenoides eludens* F.Gómez, R.Onuma, Artigas & T. Horiguchi has three distinct areas with densely arranged small pores (called ‘pore fields’) (Gómez et al., 2015) that correspond to the three large pores with an internal sieve-plate on the dorsal side of the posterior end of *Pseudadenoides*. *Adenoides* is distinguished from *Pseudadenoides* by the possession of a precingular plate series, the lack of cingular plates, and by only three posterior intercalary plates (Hoppenrath et al., 2003; Gómez et al., 2015) (Table 1).

The APC construction of the two genera (Figs 57, 58) resembles *Azadinium* Elbrächter & Tillmann (e.g. Tillmann et al., 2009, 2012a, 2014) and *Amphidoma languida* Tillmann, Salas & Elbrächter (Tillmann et al., 2012b) (Figs 59, 60). The size and location of the canal plate (X) connecting the first apical plate with the cover plate by traversing the Po plate is special. In contrast to *Pseudadenoides* and *Adenoides*, the Po plate in *Azadinium* and *Amphidoma* is smooth without normal thecal pores. *Heterocapsa* Stein has an APC intermediate to the *Pseudadenoides/Adenoides* version and peridinoid APCs (Tillmann & Hoppenrath, unpubl. obs.) (Fig. 56). The *Heterocapsa* APC has a canal plate resembling peridinoid taxa (Fig. 54) but the location differs (the first apical plate has contact to Po) and an additional structure (plate?) similar to the canal plate as observed for *Pseudadenoides/Adenoides* (Figs 57, 58). The structure has been described for

Figs 47–51. Transmission electron micrographs (TEM) of *Pseudadenoides polypyrrenoides* sp. nov. Fig. 47. Longitudinal TEM through the apical pore. Fig. 48. A longitudinal section of flagellar pore. Fig. 49. The longitudinal sections of the posterior dorsal depression. Fig. 50. High magnification TEM of the posterior depressions. Fig. 51. Tangential TEM section of the inner sieve-like structure. F = flagellum, Pl = plug-like structure. Arrows and arrowheads indicate an inner sieve-like structure and a membrane network, respectively. Scale bars = 1 µm (47, 50, 51) and 2 µm (48, 49).
**Heterocapsa minima** Pomroy as an extra structure acting as a hinge or connection and marked as ‘?’ (Salas et al., 2014). Tiny connecting structures between the X-plane and the cp-plane were also visible in *Azadinium* species (e.g. Tillmann et al., 2014) and *Amphidoma languida* (Tillmann et al., 2012b).

A large antapical (dorsal) pore with depressed field of small pores (= sieve plate) has been described for *Amphidoma languida* (Tillmann et al., 2012b). Interestingly, small pore fields close to the antapical spines in some *Azadinium* species are located in a comparable region of the cell (e.g. Tillmann et al., 2009, 2012a) and can be present also in species without an antapical spine, like *Azadinium poporum* (Tillmann et al., 2016). *Azadinium* species can also have stalked pyrenoids but ultrastructural data are not available yet (e.g. Tillmann et al., 2014).

Another species with an antapical pore field is *Peridiniella danica* (Paulsen) Okolodkov & Dodge (Okolodkov & Dodge, 1995). Similar depressions with sieve plates and pore fields were described in a few benthic *Prorocentrum* species as well, which may be homologous with that of *Pseudadenoides* (Hoppenrath et al., 2013).

The combination of morphological characters in *Pseudadenoides* overlap the traits found in both peridinioid and gonyaulacoid dinoflagellates (e.g.

---

**Fig. 52.** Phylogenetic tree inferred from SSU rDNA sequences using a maximum likelihood model. Numbers by branches represent bootstrap support (over 50) from 1000 replicates. Bayesian posterior probabilities over 0.95 are represented by thick lines. Taxa included in this study are highlighted in bold.
the apical pore complex ultrastructure (Figs 54–60) and no clearly assignable tabulation pattern; Table 2). The genus has been placed by different authors in different orders and families (Hoppenrath et al., 2003). Other genera with this combination of traits include Azadinium, Amphidoma, Heterocapsa and Peridiniella (Mesomorpha = Incerta sedis in Hoppenrath, 2016). Therefore, these taxa have an affiliation with both peridinioids and gonyaulacoids (Table 2) so are critical for understanding broad patterns of character evolution within dinoflagellates.

Gómez et al. (2015) treated Pseudadenoides and Adenoides as distantly related genera because of apparent evolutionary distance in their molecular phylogenetic positions. In light of the completely missing statistical support of the branches deeper in the tree, this interpretation is not supported by the data. As shown in the present study using partial LSU rDNA sequences, both genera cluster together as sister lineages with modest but compelling statistical support in the tree (Fig. 53), which is concordant with comparative morphology. The plate pattern of Pseudadenoides and Adenoides is very similar and the distinction of both genera may
be debatable. Interpreting the plate series of Adenoides differently (Supplementary Figs 13–16), considering possible plate homologies, a new ‘one genus hypothesis’ could be formed (Supplementary Figs 13–24). It is a matter of interpretation and for them to remain kept as separate genera (as discussed above) until further data about Adenoides eludens and additional Adenoides species become available.

The molecular phylogenetic analyses demonstrated that a culture (NIES-1402), originally identified as Adenoides eludens (Herdman) Balech, now Pseudadenoides kofoidii, most likely represents a new Adenoides species. The species was isolated from a beach in Wakayama, Japan, in 2003. Unfortunately, at the time it was not possible to investigate the culture in detail; the only available morphological information is light microscopic observations that show an outer shape similar to the new A. eludens with two large pyrenoids (Gómez et al., 2015), the cells contain several smaller pyrenoids (Supplementary Fig. 6). These observations are consistent with the molecular data.

Table 1. Morphological features of the Pseudadenoides and Adenoides species.

<table>
<thead>
<tr>
<th></th>
<th>P. kofoidii¹⁄²</th>
<th>P. polypyrenoides²</th>
<th>A. eludens³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroplasts</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Pyrenoids</td>
<td>2 large starch sheath</td>
<td>several small starch sheath</td>
<td>2 large starch sheath</td>
</tr>
<tr>
<td>Nucleus</td>
<td>lower dorsal cell half</td>
<td>cell centre</td>
<td>posterior</td>
</tr>
<tr>
<td>Epitheca</td>
<td>tiny, button-like</td>
<td>tiny, button-like</td>
<td>dorsi ally longer</td>
</tr>
<tr>
<td>Hypotheca</td>
<td>dorsi ally longer</td>
<td>dorsi ally longer</td>
<td>~1/3 cell length</td>
</tr>
<tr>
<td>Ventral hump</td>
<td>no</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>Flagellar pore(s)</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Cell length [µm]</td>
<td>30–37</td>
<td>29–38</td>
<td>27–37</td>
</tr>
<tr>
<td>Cell depth [µm]</td>
<td>21–29</td>
<td>26–34</td>
<td>5</td>
</tr>
<tr>
<td>APC</td>
<td>Po, cp, X</td>
<td>Po, cp, X</td>
<td>Po, cp, X</td>
</tr>
<tr>
<td>Apical plates</td>
<td>4</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Precingular plates</td>
<td>0</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Cingular plates</td>
<td>6</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Sulcal plates</td>
<td>4</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Postcingular plates</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Posterior intercalary plates</td>
<td>5</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Antapical plates</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Thecal ornamentation</td>
<td>no, smooth</td>
<td>no, smooth</td>
<td>no, smooth</td>
</tr>
<tr>
<td>Pore fields posterior dorsal</td>
<td>2 in depression</td>
<td>3 in depression</td>
<td>3</td>
</tr>
<tr>
<td>on plates</td>
<td>3p, 4p</td>
<td>3p, 4p, 1''''</td>
<td></td>
</tr>
</tbody>
</table>

¹ Hoppenrath et al. (2003), ² present study, ³ Gómez et al. (2015).

Figs 54–60. Apical pore complex (APC) construction of Pseudadenoides, further genera of the ‘Mesomorpha’ group, Peridiniales and Gonyaulacales. Fig. 54. Protoperidinium (after Dodge & Hermes 1981), Peridiniales. Dotted line = rim around the apical pore. Fig. 55. Ceratium (after Dodge & Hermes 1981), Gonyaulacales. Figs 56–60. Mesomorpha. Fig. 56. Heterocapsa triquetra. Fig. 57. Pseudadenoides. Fig. 58. Adenoides eludens. Fig. 59. Azadinium. Fig. 60. Amphidoma languida. Po = apical pore plate; cp (white area) = cover plate; X = canal plate; ? = a not yet described structure connecting X and cp; 1’–6’ = apical plates; black area = apical pore.
Table 2. Morphological features of the genera Pseudadenoides, Adenoides, Azadinium, Heterocapsa and characters of the dinoflagellate orders Peridiniiales and Gonyaulacales (after Fensome et al., 1993).

<table>
<thead>
<tr>
<th></th>
<th>Pseudadenoides</th>
<th>Adenoides</th>
<th>Peridiniiales</th>
<th>Gonyaulacales</th>
<th>Azadinium</th>
<th>Heterocapsa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apical pore complex (APC)</td>
<td>Po, cp, X symmetric</td>
<td>Po, cp, X symmetric</td>
<td>Po, cp, X symmetric</td>
<td>Po, cp, symmetric</td>
<td>Po, cp, X, ?-str. symmetric</td>
<td>Po, cp, X, ?-str. (a) symmetric</td>
</tr>
<tr>
<td>Po in contact with 1’ plate</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>First apical plate 1’</td>
<td>pentagonal</td>
<td>pentagonal</td>
<td>hexagonal</td>
<td>hexagonal</td>
<td>hexagonal</td>
<td>penta-(hexa-?)</td>
</tr>
<tr>
<td>First apical plate 1’ symmetry</td>
<td>asymmetric</td>
<td>asymmetric</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ventral pore</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>Apical plates</td>
<td>4</td>
<td>5</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>5/4*</td>
</tr>
<tr>
<td># anterior intercalary plates</td>
<td>0</td>
<td>0</td>
<td>yes (2-4)</td>
<td>no (0)</td>
<td>3</td>
<td>3/2*</td>
</tr>
<tr>
<td>Precingular plates</td>
<td>0</td>
<td>6</td>
<td>7</td>
<td>6</td>
<td>6</td>
<td>7/6*</td>
</tr>
<tr>
<td>Cingular plates</td>
<td>6</td>
<td>0</td>
<td>3-6</td>
<td>descending</td>
<td>weakly descending</td>
<td>weakly descending</td>
</tr>
<tr>
<td>Cingular displacement</td>
<td>no</td>
<td>n.a.</td>
<td>no, or weakly ascending/descending</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Postcingular plates</td>
<td>5</td>
<td>5</td>
<td>6</td>
<td>6</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td># posterior intercalary plates</td>
<td>5</td>
<td>3</td>
<td>yes (1)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Antapical plates</td>
<td>1’’’’</td>
<td>1’’’’</td>
<td>2’’’’ (1’’’’’’’’’’’’)</td>
<td>1p &amp; 1’’’’</td>
<td>2’’’’’</td>
<td>2’’’’’</td>
</tr>
<tr>
<td>Antapical plates symmetry</td>
<td>n.a.</td>
<td>n.a.</td>
<td>symmetric</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flagellar pore Sulcal plates or area</td>
<td>in contact with Sp</td>
<td>in contact with Sp</td>
<td>in contact with Sp</td>
<td>not in contact with Sp</td>
<td>not in contact with Sp</td>
<td>no t &amp; acc plates</td>
</tr>
<tr>
<td>Growth bands</td>
<td>only on overlapping plate margins desmoschisis</td>
<td>?</td>
<td>on any plate margin</td>
<td>only on overlapping plate margins predominantly desmoschisis</td>
<td>only on overlapping plate margins desmoschisis</td>
<td>desmoschisis</td>
</tr>
<tr>
<td>Cell division</td>
<td>?</td>
<td>predominantly eleutheroschisis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Type species (Tillmann & Hoppenrath unpubl. data); † stated in the description (Gómez et al., 2015); ? = not known; n.a. = not applicable.

Acknowledgements

We would like to thank J. McNeill, Royal Botanic Garden, Edinburgh, UK, for his advice on the nomenclatural actions that were taken to rename the genus Adenoides (now Pseudadenoides) and N. Chomérat, IFREMER Concarneau, for help with the Latin species name. We are grateful to M. Schweikert, University of Stuttgart, Germany, for discussions about the morphology of taxa with uncertain classification.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by a scholarship to MH from the Deutsche Forschungsgemeinschaft (grant Ho3267/1-1) and by grants to BSL from the National Science and Engineering Research Council of Canada (NSERC 2014-05258) and the Canadian Institute for Advanced Research, Program in Integrated Microbial Biodiversity. RS was also supported by the Canadian Barcode of Life Project.

Author contributions

M. Hoppenrath: sampling, culturing, light and scanning electron microscopy, drafting and editing the manuscript; N. Yubuki: transmission electron microscopy, phylogenetic analyses, drafting and editing the manuscript; R. Stern: DNA extraction, PCR, phylogenetic analyses, editing the manuscript; B.S. Leander: infrastructure and salary support, editing the manuscript.

Supplementary Information

The following supplementary material is accessible via the Supplementary Content tab on the article’s online page at http://dx.doi.org/10.1080/09670262.2016.1274788

Supplementary Table 1. Records of Pseudadenoides species at Boundary Bay, British Columbia, Canada.

Supplementary Figs 1–3. Scanning electron micrographs of Pseudadenoides polyprenoides sp. nov. Figs 1,2. Transversely striated intercalary bands. Fig. 3. Apical pore complex consisting of the apical pore plate (Po), a cover plate (cp) and a canal plate (X). Note the normal thecal pores in the pore plate (arrows). Scale bars = 10 µm (Fig. 1) and 1 µm (Figs 2, 3).

Supplementary Figs 4–10. Light micrographs of cells from the NIES cultures represented as DNA sequences in the molecular phylogenies. Figs 4–6. NIES-1402, originally identified as Adenoides eludens (now Pseudadenoides kofoedii) and isolated from a sandy beach sample in Wakayama,
Japan. Figs 7–10. NIES-1367, originally identified as Adenoides eludens (now Pseudadenoides kofoidii) and isolated from the coast in Suzu Ishikawa, Japan. n = nucleus, p = pusule, arrows pointing at pyrenoids with starch sheath, small arrow pointing at the flagellar insertion, arrowhead pointing at a ventral hump in the sulcal area, double arrowheads pointing at the button-like epitheca. Scale bars = 10 μm.

Supplementary Figs 11–12. Transmission electron micrographs of Pseudadenoides polypleurooides sp. nov. Fig. 11. Different developmental stages and sizes of trichocysts. Fig. 12. Membrane net-work of the tentative sack pusule in the collapsed condition. Scale bars = 500 nm.

Supplementary Figs 13–24. Line drawings of the theca of Adenoides eludens (Figs 13–16), Pseudadenoides polypleurooides sp. nov. (Figs 17–20) and Pseudadenoides kofoidii (Figs 21–24). Figs 13, 17, 21. Left lateral. Figs 14, 18, 22. Right lateral. Figs 15, 19, 23. Apical. Figs 16, 20, 24. Antapical. 1′–5′ = apical plate series; C1–6 = cingular plate series; 1′′–5′′ = postcingular plate series; 1p–5p = posterior intercalary plate series; 1′′′ = antapical plate; Sa = anterior sulcal plate; Ss = left sulcal plate; Sd = right sulcal plate; Sp = posterior sulcal plate;

References


