

Dedicated to the late Professor Jadwiga Siemińska

Auxospore wall structure and postsexual valve morphology in *Rhabdonema minutum* Kützing

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Article info

Received: 8 Mar. 2019
Revision received: 6 May 2019
Accepted: 9 May 2019
Published: 30 Jul. 2019

Associate Editor

Konrad Wołowski

Abstract. Several decades ago, three members of the araphid pennate genus *Rhabdonema* (*R. adriaticum*, *R. arcuatum*, *R. minutum*) were the first araphid diatoms studied using cultures and electron microscopical methods to determine auxospore structure and development. Of these, *R. minutum* was the least documented at that time. None have been reinvestigated until now. Here we present the structural elements of the mature auxospore and the initial and postsexual valve characteristics of *R. minutum*. Although in general the auxospore wall of this diatom is similar to that of the two other species examined (to the extent that they were documented), there are intriguing differences. Most unanticipated is the structure of the primary band of the longitudinal perizonium, which shows remarkable similarities to the raphid pennate diatom valve. The evolutionary implications of such a similarity are considered.

Key words: auxosporulation, araphid pennate diatom, incunabular scales, longitudinal perizonial bands, perizonium, evolution of raphe

Introduction

Three species of the araphid pennate genus *Rhabdonema* Kützing examined to date in terms of their sexuality demonstrate a unique mode of oogamy (Karsten 1899; von Stosch 1958, 1962) unknown in any other diatom. The species produce either two eggs (*R. arcuatum*, *R. minutum*) or one egg (*R. adriaticum*) per oogonium, and nonflagellate but motile male gametes known as spermatia. Furthermore, these spermatia are produced following depauperating mitoses, frequent among centric diatoms whose male gametes are flagellated but unknown among pennates. However, unlike flagellated centric diatom sperm, spermatia in *Rhabdonema* species are only capable of amoeboidal movement, which is slower and requires a substrate.

Light and transmission electron microscopy have been used to investigate the auxospore (the cell resulting from syngamy) of these diatoms, but the fine structure and development of this cell have been examined in detail in only two species, *R. adriaticum* and *R. arcuatum* (von Stosch 1962, 1982), which showed complex, thick walls even in mature auxospores, unlike in other pennate diatoms. The wall layers develop in sequence, depositing

structurally different, siliceous components as the auxospore grows. First, siliceous incunabular scales develop, embedded in mucilaginous layers. In the second stage of auxospore development, transverse perizonial bands are laid down, with the primordial loop being first in the series of many similar loops. The longitudinal perizonium is deposited next, but only in *R. arcuatum*, not *R. adriaticum* (von Stosch 1958, 1962). The auxospore wall structure of *Rhabdonema minutum* remains undocumented in all these stages.

The origin of perizonial bands in pennate diatoms is not clear. Two possible ways in which they might have evolved were postulated by von Stosch (1982). The first way would involve accretion of incunabular scales until they form a band. The second way would involve a modification of one incunabular scale with an annulus, with elongation of the annulus and the scale to eventually result in a structure that could be named a band. Perizonial bands are already known in polar centric diatoms. There, as in pennates, they play a role in shaping the initial valves of the sexual progeny by setting up limits on the size and shape of the postsexual frustules. There are several types of perizonial bands in diatoms, and they might have evolved independently within the polar centrics and then again within pennates. Most recently, a novel manner of development of transverse perizonial bands was documented in the unusually elongated, pennate-like polar centric diatom *Ardissonea crystallina* (Kaczmarska et al.

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2018). This and previously postulated hypotheses (von Stosch 1982) have raised interest in the structure of perizonial bands in polar diatoms, both centric and pennate.

The aim of this study was to investigate the mature auxospore wall's fine structure, its perizonial bands in particular, in a member of the "core" araphid pennates, and to compare them to other members of the genus, basal araphid pennates, and the polar diatoms in general.

Materials and methods

Culture establishment and auxosporulation

Seawater collected on 17 January 2006 at the shore of Cape Tormentine on the Gulf of St. Lawrence, New Brunswick, Canada (46.1325°N, 63.7810°W), contained short chains of *Rhabdonema* cells. One chain of cells was isolated by micropipetting for monoclonal culture method and grown in f/2 medium (Andersen 2005) based on the local seawater. The monoclonal culture (RhCTIII A4) was established and maintained in a growth cabinet at 12–22°C, irradiance of 30–60 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ and a 10 h photoperiod until enough biomass accumulated for harvesting for molecular analysis. DNA extraction, amplification and sequencing protocols are described in Moniz & Kaczmarska (2010). Internal transcribed spacer 2 (ITS-2) sequences and voucher images of the vegetative cells of the clone are deposited at Barcode of Life Data Systems (BOLD; www.boldsystems.org), labelled DITS319-08. Auxosporulation in the clone was non-induced and commenced unobserved. Consequently, only late and mature auxospores could be examined.

Microscopy analysis

Scanning electron microscopy (SEM) preparations of clonal isolates were made as soon as clone growth was established and cultured cell accumulation allowed sub-sampling (within 2 months after isolation) to capture natural frustule morphology. All data were collected from SEM images, with material prepared for examination following Kaczmarska et al. (2005). SEM was performed using a JEOL JSM-5600 SEM (JEOL USA, Peabody, MA, USA) operating at 10 kV and 8 mm working distance, at the Mount Allison University Digital Microscopy Facility. At least twenty representative valves of both the parental and progeny populations were measured for species diagnostic characters (apical and transapical length, density of striae and striae areolae, areolae on the copulae, number of rimoportulae) using dmfMeasure (www.mta.ca/dmf/dmf-measure.html). Terminology follows Ross et al. (1979) for frustule structures, and Kaczmarska et al. (2013) for the auxospore wall.

Results

Species identity

Rhabdonema minutum Kützing (Fig. 1A–H)

Synonym: *Rhabdonema* sp., clone RhCTIII A4; GenBank accession number GQ330431; Moniz & Kaczmarska (2010).

Chain-forming diatom. Parental frustules long, rectangular from girdle view, with up to 10 copulae per theca (Fig. 1A, B). Valves broadly lanceolate with broadly rounded apices, 9–17 μm in apical and 8–10 μm in transapical axes. Striae coarse, parallel, 8–13 in 10 μm throughout valve face. Striae areolae nearly as coarse as striae, 11–16 in 1 μm , occluded by cribra. Sternum narrow. Apices carrying pore fields built of parallel rows of small pores extending onto mantle, some with interruptions (Fig. 1A, B).

Progeny valves linear to having a slightly inflated mid-section with rounded apices; 41–72 μm in apical and 9.5–13 μm in transapical axes. Striae somewhat coarser than in smaller parental valves, 8–11.5 in 10 μm , but parallel throughout most of valve face. Striae areolae 10–15 in 10 μm , occluded by cribra. Sternum narrow. Apical pore fields built of parallel rows of small pores, extending onto mantle (Fig. 1D–F), in some valves interrupted by hyaline areas. External opening of rimoportulae indistinct. Internally these processes were very small, vestigial in appearance, and varying in number and location (Fig. 1D–F), particularly in parental valves. One to three processes might occur on the valve, but only a very few valves carried them on the sternum as shown for the genotype in Round et al. (1990). Still, there were many valves that had no rimoportula (Fig. 1B).

Parental and progeny thecae alike were associated with up to 10 copulae. Copulae were perforated by short striae (9–13 in 10 μm in parental, 9–10 in 10 μm in postsexual valves) or a row of areolae (Fig. 1A). Valvocopulae, and copulae closer to the valve were closed (complete), had a serrate edge on the advalvar side of postsexual bands (but not parental valves) and carried a large septum (Fig. 1C, D, H). In LM some of the copulae demonstrated the "hyaline" section (Hustedt 1959) on one of its sides, which is a section with no striae.

Overall, our specimens (parents and progeny together) were 9–72 μm long in apical and 8–13 μm in transapical axes, with 8–13 striae in 10 μm on the valve and copulae, 10–16 areolae in 10 μm of striae length, with up to 10 copulae, some with septa. Although some individual valves from among the parental population were smaller than the smallest published sizes for this species, the majority of parental and sexual progeny valve morphologies and metrics fit well within the range of diagnostic character variability, supporting the specific identity of our clone.

Comparison to other reports on this species

The frustule and valve structure of our clone conforms to the descriptions and illustrations of Kützing (1884, p. 126; no published metric data) and Karsten (1899, p. 37, fig. 23; cited as a good illustration by Hustedt 1959), whose specimens were 20–60 μm long and 11 μm wide. Hustedt (1959 and translation 1985, p. 16–18) reported specimens 18–70 μm long and 12–20 μm wide, with 8–10 rows of striae and numerous striated copulae, with a hyaline area present on one side of some copulae. Cleve-Euler's specimens (1953, p. 6) were 20–80 μm long, 12–16 μm wide, with 7–10 striae in 10 μm , while Snoeijs & Vilbaste (1994, p. 185) reported valves 14.5–34 μm long, 8–13.5 μm

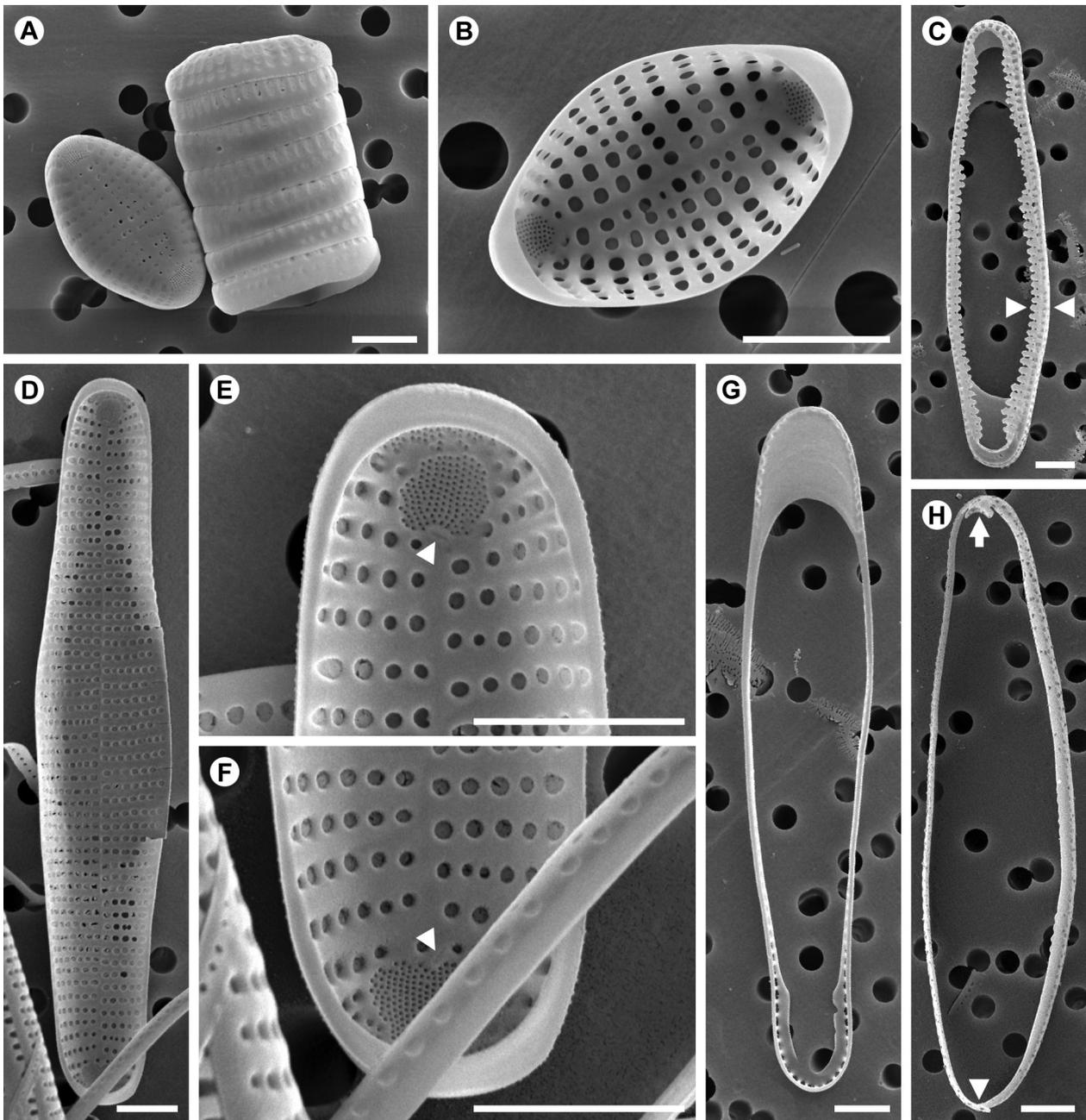


Figure 1. *Rhabdonema minutum*, SEM. A – representative parental valve and theca, showing external view of valve face and ornamentation of copulae; B – internal view of representative parental valve, note the absence of rimoportulae; C – valvocopulae and other copulae underneath, note inward-directed, areolated outgrowth of copular abvalvar rim with serrate edge (arrowheads), and septa; D – example of postsexual progeny valve in internal view; E, F – closeups of apices of valve shown in D, note very small (vestigial?) rimoportulae (arrowheads) and apical pore fields; G – closed copula with septum; H – open copula (arrowhead) with ligula (arrow) and no septum. Scales: A–H = 5 μm .

wide, both with no information regarding striae. Canadian specimens reported by Poulin et al. (1984, p. 287) had striae (6–10 in 10 μm) coarser than ours.

In metrics but not morphology our valves were also somewhat similar to *R. crassum* (Hendey 1964, p. 172; 12–23 μm long, 8–13 μm wide, with 6–9 striae in 10 μm and small septa). Hendey considered *R. crassum* close to *R. minutum* but emphasised the different valve outline (broadly lanceolate), hyaline spaces at the apices (absent in *R. minutum*), and asymmetrical septa. Alvarez-Blanco & Blanco (2014) give the following measurements for Mediterranean specimens of *R. crassum*: 14.5–16.3 μm long, 7.5–7.9 μm wide, with 9–10 striae and 12–16 areolae in 10 μm , which makes it more similar to specimens of

Poulin et al. (1984), although the nature of the septa of the Canadian specimens is not available.

Auxosporulation and auxospore wall structure

Mature auxospore walls contained three types of siliceous components: incunabular scales (IS) of various size and shape (in addition to the organic matrix they were embedded in), and transverse (TP) and longitudinal (LP) perizonial bands. Due to the high silicification of these structures, their micro-architecture could be well documented. The majority of the incunabular scales had a well-developed annulus (Fig. 2A); some scales were elongated as a result of linear accretion of several nearly circular scales (Fig. 2A, upper left corner). Transverse

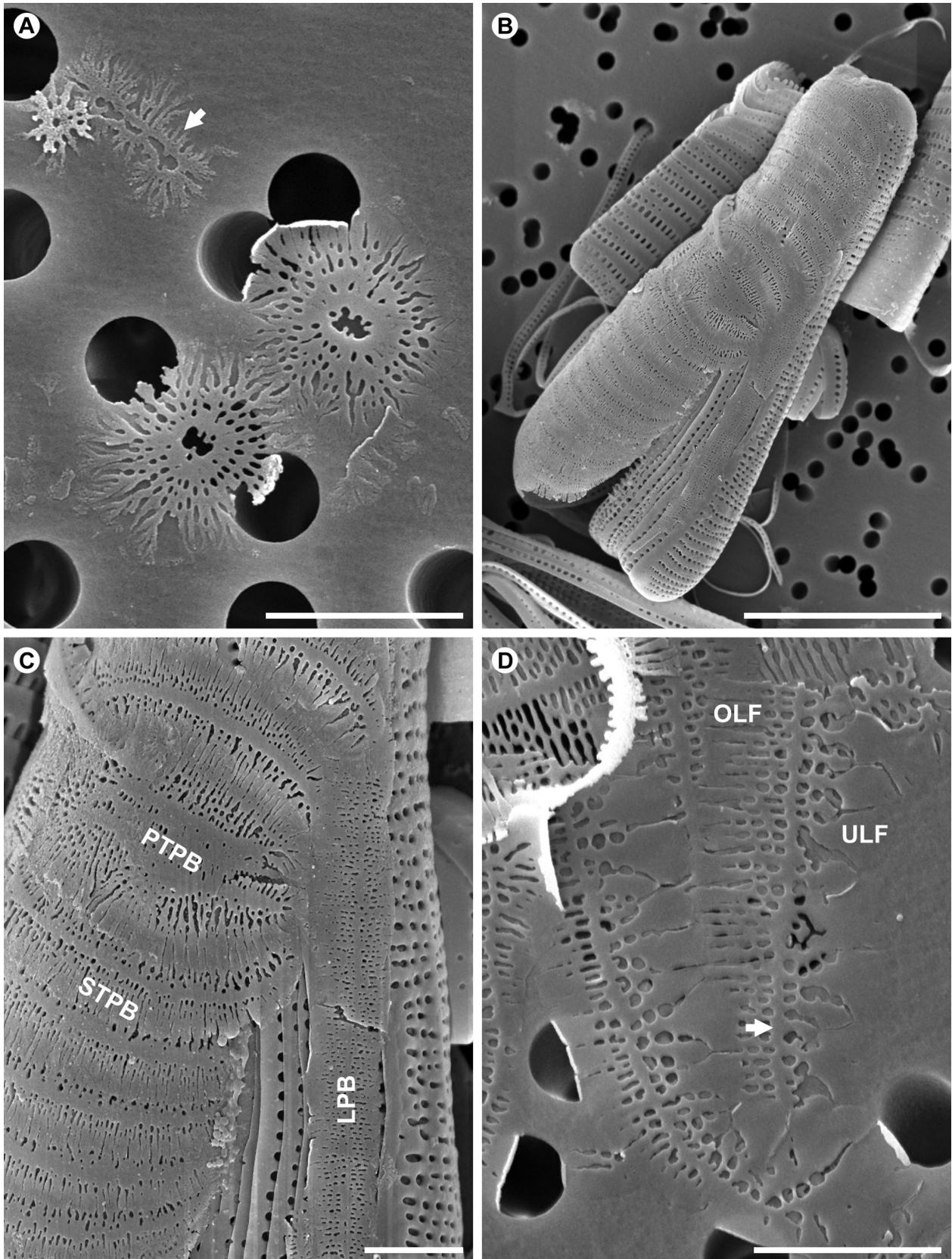


Figure 2. *Rhabdonema minutum*, SEM. A – examples of incunabular scales from auxospore wall, note chain of scales and multiple annuli (arrow); B – mature auxospore split open by divided initial cell; C – closeup of midsection of auxospore from B, note primary transverse perizonial band (PTPB), secondary transverse perizonial bands (one labelled STPB) and longitudinal perizonial band (LPB); D – disarticulated transverse perizonial bands with axial midrib (arrow), and overlapping lateral fimbriae (OLF) differing from underlapping lateral fimbria (ULF) which are spatulate in shape. Scales: A = 5 μ m, B = 25 μ m, C–D = 5 μ m.

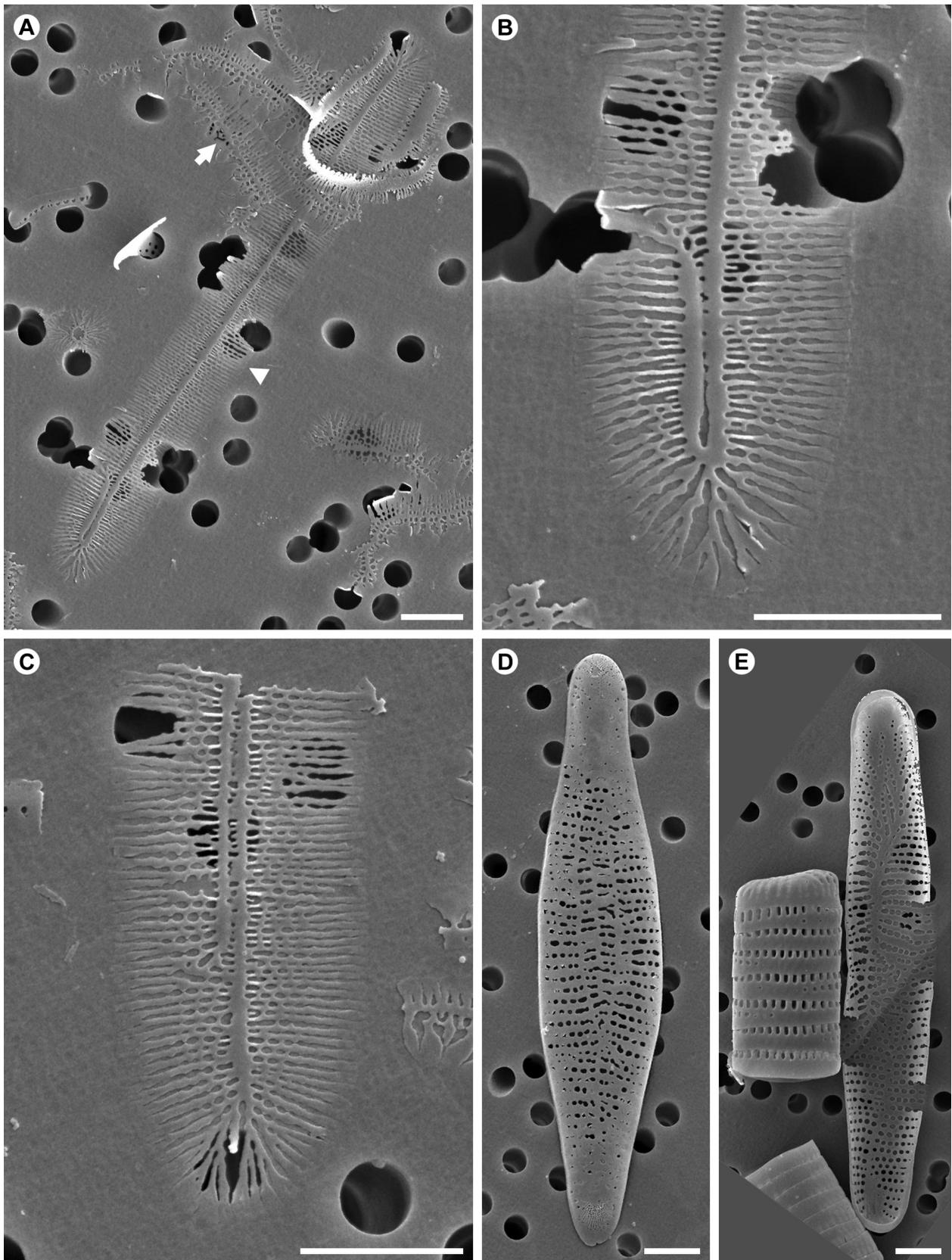


Figure 3. *Rhabdonema minutum*, SEM. A – primary longitudinal perizonial band (PLPB; arrowhead) still associated with remnants of transverse perizonium (arrow), note how its structure differs from that of transverse perizonial bands; B – closeup of lower end of PLPB from A, showing axial rib folded back, note branching fimbriae; C – fragment of another PLPB, showing two parallel axial ribs; D – one of the initial valves with abnormal valve ornamentation and branching sternum; E – another initial valve with disrupted striation and misplaced sternum. Scales: A–E = 5 μ m.

perizonial bands were open, with the openings lining up on one side of the auxospore, producing a ventral seam or suture (Fig. 2B–D). The first band was located in the midsection of the elongating auxospore and was much wider than all others (Fig. 2B, C). The striation of this band was symmetrical, with fimbriae on both sides of the centrally located wide axial rib, in contrast to all other bands. The successive bands were slightly slanted towards the suture and wrapped around the overlying ventral ends of the preceding bands (Fig. 2C, D). Transverse perizonial bands were perforated by pores that varied in size and shape, both on the area of their wide axial ribs and between lateral fimbriae. The fimbriae differed in size and shape. Distal (overlying) fimbriae were numerous, branching, perforated and thin (similar to those on the primary TP band; 29–31 in 10 μm). The underlying (proximal) fimbriae were fewer, less perforated, and spatulate (widening) toward their free ends (Fig. 2D).

The longitudinal perizonium also consisted of a wide primary band and successive half-bands positioned on each side of the primary band (Fig. 2D). The primary band in the longitudinal perizonium was simpler but showed similarities to the transverse bands in quantitative characters (number of fimbriae, pores). Lateral fimbriae in the primary longitudinal band were structurally similar to those in the transverse series; all have rows of pores between the fimbriae, and they may branch. There were 32–38 such fimbriae in 10 μm in both the TP and LP bands. However, the primary LP-band axial rib was narrow and better defined (Fig. 3A) than that in TP. In some specimens the axial rib of LP folded back on itself (Fig. 3B). In some other cases there was a pair of axial ribs running more or less parallel to each other (Fig. 3C) over some portion of the length of the band. There was either a fissure or a row of pores between axial ribs of LP when they occurred in pairs (Fig. 3A–C).

Initial valve morphology deviated from that of normal vegetative valves in shape, the number and position of the sternum, the orientation of striae, and the irregularly arranged pores in the apical fields (Fig. 3D, E).

Discussion

Mature auxospores of *R. minutum* and their wall structures are similar, in a general way, to those of two congeners examined by von Stosch (1958, 1962, 1982). All three major siliceous structural elements of the auxospore wall present in *R. arcuatum* and *R. adriaticum* are also found in *R. minutum*. These are incunabular scales (IS), transverse (TP) and longitudinal (LP) perizonia. However, the fine structure of the wall elements seen in *R. minutum* is difficult to compare to the two other species in sufficient detail because the documentation in earlier studies is fragmentary. From the published image data available, we can only conclude that their incunabular scales and transverse perizonial band structure is generally similar to those we observed in our *R. minutum* clone. The TP bands in *R. arcuatum* have an axial rib and well-developed lateral fimbriae on both sides, as in our species. There are also rows of pores between the fimbriae close to the

axial rib (von Stosch 1982, fig. 4b) in the TP bands of the two congeners previously examined and in our species.

Transverse perizonial bands in polar diatom auxospores vary in structure and ornamentation pattern. In polar centrics examined under SEM resolution sufficient to determine these features, at least three different forms of TP bands can be found. First, in some polar centrics, such as in *Chaetoceros acadianus* and the cymatosiroid *Brockmanniella brockmannii*, the TP bands are similar to the araphid pennate *Dimeregramma acutumontgo* and two species of *Plagiogramma* examined (Kaczmarek et al. 2017). In those cases the band centers bore slits or striae bordered by solid margins on the adapical and abapical sides. A second pattern is known from a centric diatom with exceptionally elongated valves, *Ardissonea crystallina*. In this diatom, TP bands resulting from accretion of several scales with no obvious annulus expand in width by growing in a nearly fractal manner. Consequently, the bands show no discernible slits, fimbriae, or perforation patterns upon completion of their development. A third type can be found in some other polar centrics, where the TP bands possess loosely defined axial ribs and lateral fimbriae on each side (e.g. *Biddulphia pulchella* and *Odontella aurita*; von Stosch 1982).

Both the axial ribs and the fimbriae are better defined in pennates (polar by definition) than in polar centrics thus far examined. A few pennate diatoms have likely lost their polarity secondarily (e.g. *Astrosyne* and *Psammomiscus*) but their auxospore structure is not yet documented. Pennate TP bands in general have an axial rib and similarly structured lateral fimbriae on both sides. Examples include the araphid *Rhabdonema arcuatum* (von Stosch 1982), *Grammatophora marina* (Sato et al. 2008a), *Gephyria media* (Sato et al. 2004), *Tabularia parva* (Sato et al. 2008b) and *Pseudostriatella oceanica* (Sato et al. 2008c). Raphid pennates examined under sufficient resolution that fit this category as well include *Pseudo-nitzschia multiseries* (Kaczmarek et al. 2000), *Nitzschia longissima* (Kaczmarek et al. 2007), the *Neidium ampliatus* species complex (Mann & Pouličková 2009) and *Rhoicosphenia curvata* (Mann 1982), although the fimbriate sides are not symmetrical in the last-mentioned species. This form of TP band therefore occurs in a wide range of orders, families and genera of araphid and raphid pennates, and in a less refined form in some polar centrics.

Von Stosch (1982) considered possible ways for the TP to have evolved from incunabular scales to become perizonial bands. He suggested two ways: accretion of incunabular scales or elongation of one scale such that its annulus becomes an axial rib of the future perizonial band. We observed that the accretion of scales may proceed bidirectionally to form a chain (three scales that could illustrate such an accretion are shown in Fig. 2A, upper left corner), or multidirectionally (as in *A. crystallina*) to form a wide ribbon made by the accretion of more than one row of incunabular scales. Should the scales fuse linearly (in a chain, so to speak), flattened and fused annuli may be modified to form an axial rib and lateral fimbriae similar to those observed in *R. minutum* and other

Rhabdonema species. Other modes of transverse band origination may have occurred in other diatom groups.

The most significant differences in auxospore wall structure between our species and its congeners are in the primary longitudinal perizonial bands. Available representations of the primary LP bands in other species of the genus indicate that they are very narrow (von Stosch 1962, fig. 5, 1982, fig. 1), with ornamentation similar to that of the secondary TP bands (von Stosch 1982, figs 1, 4c). In *R. minutum* the secondary LP bands are quite similar to those in the TP series, but the primary band is very different. The primary band is several times wider than other LP bands, and has a narrow, distinct axial rib. In addition, primary band lateral fimbriae have pores between them, organised in a manner similar to the pores of the striae. However, the number of fimbriae per 10 µm and the number of pores between the individual fimbriae is closer to those of TP bands, and they are both several times finer than the striae of vegetative valves of our species. Moreover, there may be more than one axial rib in the primary LP band of *R. minutum* over some portion of its length, or the rib may fold back on itself at the apices. In the folded regions, fimbriae are present on one side of the rib only. It is because of all these characteristics that we consider these structures to be primary LP bands rather than the initial epivalve.

An LP band with such structure has not been reported before in diatoms, but it bears an intriguing similarity to certain stages of valve morphogenesis in some raphid pennate diatoms (compare Fig. 3B to Idei et al. 2018, fig. 3j). Indeed, the axial ribs of the primary LP band in *R. minutum* are very well defined and reminiscent of a sternum. Pennate valves show structural similarity to perizonial bands, those of araphid pennates in particular. It has been suggested (Mann 1984) that the raphe may have evolved through the “ontogenetic and phylogenetic fusion” of the araphid sternum and two secondary, originally lateral, pattern centers associated with the raphe slits, which may have developed from rimoportulae (Hasle 1974, Round et al. 1990, p. 42). This hypothesis clearly links them to the valves (not perizonial bands) of the diatom group that has rimoportulae. However, the ontogeny of the raphid valve in *Diploneis smithii* (Idei et al. 2018) demonstrates first a single elongated structure which is then folded back on itself to expand towards the valve midsection, similar to what is observed in the primary longitudinal perizonial band in our diatom, not rimoportulae. More consideration of the possible reason(s) behind such a similarity is clearly warranted, following Round et al. (1990, p. 42), who, further down on the same page cited above, noted this: “Clearly, however, there are other possible interpretations of the raphe-sternum and how it might have arisen.”

In this work we documented novel structural elements in the mature auxospore wall of the araphid diatom *R. minutum*. Although in general the auxospore wall of this species is similar to that of two congeners previously examined, the novel and unanticipated character found in *R. minutum* is the intriguing similarity of the primary longitudinal perizonial band to the vegetative valves of

some raphid pennate diatoms in their earliest stages of ontogenetic development. If the noted similarities represent evolutionary relationships, some of the raphes in raphid pennates may have evolved with no involvement from the labiate processes (or rimoportulae).

Acknowledgements

This work was supported by a Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grant and a Mount Allison University Marjorie Young Bell Faculty Fund (Sabbatical) Grant to IK.

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