

## PHYLOGENETIC POSITION OF *TOXARIUM*, A PENNATE-LIKE LINEAGE WITHIN CENTRIC DIATOMS (BACILLARIOPHYCEAE)<sup>1</sup>

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The diatom genus *Toxarium* Bailey has been treated as a pennate because of its elongate shape and benthic lifestyle (it grows attached to solid substrata in the marine sublittoral). Yet its valve face lacks all structures that would ally it with the pennates, such as apical labiate processes, a midrib (sternum) subtending secondary ribs and rows of pores extending perpendicularly out from the midrib, or a raphe system. Instead, pores are scattered irregularly over the valve face and only form two distinct rows along the perimeter of the valve face. In our nuclear small subunit rDNA phylogenies, *Toxarium* groups with bi- and multipolar centrics, as sister to *Lampriscus* A. Schmidt. Thus, the genus acquired a pennate-like shape and lifestyle independently from that of the true pennates. The two species known, *T. hennedyanum* Grunow and *T. undulatum* Bailey, differ only in a single feature: the valve perimeter of the former shows only a central expansion, whereas that of the latter possesses in addition a regular undulation. Yet both forms were observed in our monoclonal cultures, indicating that the two taxa represent extremes in a plasticity range. *Toxarium* resembles another elongate and supposedly araphid diatom, *Ardissonea* De Notaris, in being motile. Cells can move at speeds of up to  $4 \mu\text{m}\cdot\text{s}^{-1}$  through secretion of mucilage from the cell poles or they remain stationary for longer periods, when they form short polysaccharide stalks. Division during longer periods of quiescence leads to the formation of small colonies of linked or radiating cells.

**Key index words:** bipolar centrics; convergence; diatom; morphology; pennates; phylogeny; SSU rDNA; *Toxarium*

*Toxarium* J. W. Bailey (1854) is a diatom genus with highly elongate cells, which are generally considered typical of pennate diatoms. Its frustules have a flat valve face and the valve outline exhibits slight expan-

sions in the middle region and at the apices (Fig. 1; see also Round et al. 1990, p. 422–3). Small poroids are scattered irregularly over the valve face but form two well-defined rows along the valve face perimeter. The genus also has a growth form typical of many araphid pennates: cells are connected to various substrata, either individually or in small bunches, by means of apical mucilage pads. Only two species are currently recognized: *T. undulatum* Bailey and *T. hennedyanum* Grunow. They resemble each another in all but a single feature: the perimeter of the valve of *T. hennedyanum* shows only the central expansion mentioned above, whereas that of *T. undulatum* possesses in addition a regular undulation (Hustedt 1932, Round et al. 1990, Hasle and Syvertsen 1997).

Despite its pennate shape and lifestyle, the taxonomic position of *Toxarium* is uncertain. True pennates generally possess a sternum (midrib), which subtends a system of  $\pm$  parallel transapical ribs (costae) and striae (rows of pores). During valve formation, silica deposition commences from the sternum (Mann 1984, Pickett-Heaps et al. 1990). Within the pennates, a distinction is made between raphids and araphids. The former possess raphe slits, which are used for active movement (Harper 1977, Edgar and Pickett-Heaps 1984, Round et al. 1990), whereas the latter lack such an organelle but instead usually possess apical or median labiate processes (Round et al. 1990). Apical pore fields involved in mucus excretion are rare in raphids but abound in the araphid genera. *Toxarium* lacks all these pennate traits (Round et al. 1990).

Given the lack of a sternum, *Toxarium* might belong to the bi- or multipolar centric diatoms. Genera in this group (Medlin et al. 2000) generally possess either one central or two pericentral labiate or tubular processes and often have a pore field at each of the two or more poles. Ribs and striae radiate out, not from an axial sternum but from the circumference of a variously distorted ring-like pattern center, called an annulus. In their taxonomic reevaluation of diatom genera, Round et al. (1990) removed several elongate diatoms from the pennate group because they possessed multipolar centric traits, but *Toxarium* lacks these too. Hence, for lack of evidence, *Toxarium* was provisionally retained in

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the pennates. The row of poroids along the valve perimeter in *Toxarium* might indicate the presence of an expanded and deformed annulus (Mann 1984), in which case silica deposition should commence from this structure, but until now no information has been available on valve morphogenesis.

*Toxarium* could even be a radial centric diatom, albeit an extremely elongated one. Members of this group, which include *Coscinodiscus* C. G. Ehrenberg, *Actinocyclus* C. G. Ehrenberg, and *Aulacoseira* G. H. K. Twaites, are generally shaped like Petri dishes and often possess an array of labiate processes along the circumference of the valve face and ribs that radiate from a more or less circular annulus. They lack the pore fields found in multipolar centrics. Grouping *Toxarium* with the radial centrics would explain the lack of clearly defined pore fields in *Toxarium* but not the absence of labiate processes and a circular annulus.

Plastid shape and size are potentially helpful characters for identifying the taxonomic position of diatom genera. Centric diatoms usually possess many relatively small ovoid plastids, whereas pennates generally have only a few relatively large and variously lobed plastids (Round et al. 1990, Mann 1996); however, many araphids, such as *Licmophora* C. A. Agardh (Honeywill 1998), have small discoid plastids. The plastids of *Toxarium* have apparently never been described.

A molecular phylogenetic approach is needed to resolve the position of *Toxarium*. Medlin and coworkers (e.g. Medlin et al. 1991, 1993, 1996a,b, 2000) showed that phylogenies inferred from differences along the nuclear small subunit (SSU) rDNA sequences provide resolution at various levels across the diatom diversity. In these phylogenies raphid pennates form a clade within an araphid pennate grade. Pennates as a whole form a clade, which is sister to a multipolar centric clade or lies within a multipolar centric grade. Phylogenies inferred from nuclear large subunit rDNA (Sörhannus et al. 1995) corroborate these findings. Hence, the traditional classification of diatoms into "centric" and "pennate" groups or into "centric," "araphid pennate," and "raphid pennate" (Round et al. 1990) does not reflect phylogeny.

Moreover, it is becoming clear that some of the generalizations that used to be made about the biology of the major groups of diatoms are flawed. For example, it was previously thought that centric diatoms were entirely passive and that the only diatoms capable of active movement in the vegetative phase were those possessing a raphe system (e.g. Fritsch 1935). However, autonomous movements, albeit slow, have since been demonstrated in the centric genera *Actinocyclus* (Medlin et al. 1986), which has a circular valve, and *Odontella* C. A. Agardh (Pickett-Heaps et al. 1986), which has bipolar valves and in the genus *Ardissonea* G. De Notaris (Pickett-Heaps et al. 1991), which has highly elongate bipolar valves and is usually treated as a pennate diatom. There is also a report of movement in the araphid pennate *Tabularia* (Kützing) D. M. Williams & F. E. Round

(observations of Hopkins, referenced in Harper 1977), which needs confirmation.

Ongoing addition of taxa has resulted in a densely ramified SSU phylogeny in which the position of newly added diatoms can be located fairly precisely. Here we incorporate an SSU sequence of *Toxarium* and other new pennate diatom sequences into an existing data set to unravel the phylogenetic position of *Toxarium*. We have also studied motility, frustule morphology and ontogeny, and plastid shape.

#### MATERIALS AND METHODS

*Cell isolation and culture.* Single cells of *Toxarium* were isolated from two marine samples: an algal turf on the island of Ischia, Italy (Mediterranean Sea) and sparse epilithic growths in the sublittoral (approximately 6 m below chart datum) in Loch Carron, Wester Ross, Scotland (UK grid reference NG861353). For the Ischia material, the cell was washed through a series of sterile seawater droplets using sterile Pasteur pipettes. A culture from this cell was then grown in plastic Petri dishes in half-strength *f/2*-enriched seawater (36 psu) with silica addition (Guillard 1983) at 16° C at an irradiance of 25–50  $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  provided by fluorescent tubes (Osram 36W/19 daylight, spectral range;  $\pm 300\text{--}700\text{ nm}$  (Osram GmbH, Munich, Germany)), with a 12:12-h light:dark cycle. The Loch Carron isolate was isolated by pipette, washed, and grown in Roshchin medium (see Chepurnov and Mann 1997) at 15° C with approximately 5  $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ , with a 12:12-h or 14:10-h light:dark cycle.

*Microscopy and taxonomic identification.* Culture material was examined using LM and SEM. *Toxarium* cells are extremely long (in the Loch Carron clone, cells were initially approximately 470  $\mu\text{m}$  long). Hence, to avoid undue restriction of movement by the slide and coverslip during studies of locomotion, cells were examined directly in their culture dishes by use of a water immersion lens (Leitz  $\times 50$ , N.A. 1.0, Leica Camera AG, Solms, Germany); the metal parts of the objective were coated with "Ozokerit" type 377 R wax as recommended by von Stosch and Fecher (1979; the wax used was sent to D. G. M. by the late H. A. von Stosch). For SEM examination, cells were cleaned with 10%  $\text{HNO}_3$  and 40%  $\text{H}_2\text{SO}_4$  and washed with distilled water. A drop of the material was placed on aluminum stubs, coated with gold, and observed with a scanning electron microscope (model 505, Philips NV, Eindhoven, the Netherlands). For identification, micrographs were compared with pictures and descriptions in Hustedt (1932), Round et al. (1990), and Hasle and Syvertsen (1997).

*DNA extraction and purification.* Samples of 20 mL of medium containing growing cells were filtered through 0.45- $\mu\text{m}$  pore diameter membrane filters (Millipore SA, Molsheim, France). Filters were immersed in 500  $\mu\text{L}$  DNA extraction buffer containing 2% (w/v) CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, pH 8, 0.2% (w/v) PVP, 0.01% (w/v) SDS, and 0.2%  $\beta$ -mercaptoethanol. Immersed filters were incubated at 65° C for 5 min, vortexed for a few seconds, and then discarded. Subsequently, the buffer was cooled briefly on ice. DNA was extracted with an equal volume of chloroform-isoamyl alcohol (24:1 [v/v]) and centrifuged in a table-top Eppendorf microfuge (Eppendorf AG, Hamburg, Germany) at maximum speed (14,000 rpm) for 10 min. The aqueous phase was collected, reextracted with chloroform-isoamyl alcohol and centrifuged as above. Next, the aqueous phase was mixed thoroughly with 0.8 volumes ice-cold 100% isopropanol, then left on ice for 5 min, and subsequently centrifuged in a precooled Eppendorf microfuge under maximum speed for 15 min. DNA pellets were washed in 500  $\mu\text{L}$  70% (v/v) ethanol, centrifuged 6 min, and, after decanting of the ethanol, allowed to dry on air. DNA pellets were dissolved overnight in 100  $\mu\text{L}$  water. Quantity and quality of DNA were examined by agarose gel electrophoresis against known standards.

**PCR and sequencing.** The targeted marker sequence comprises the SSU rDNA within the nuclear rDNA cistron. The marker was PCR amplified in 25- $\mu$ L volumes containing 10 ng DNA, 1 mM dNTPs, 0.5  $\mu$ M of forward primer, 0.5  $\mu$ M of reverse primer, 1 $\times$  Roche diagnostics PCR reaction buffer (Roche Diagnostics, GmbH, Mannheim, Germany), and 1 unit *Taq* DNA Polymerase (Roche). The PCR cycling comprised an initial 2-min heating step at 94 $^{\circ}$  C, followed by 35 cycles of 94 $^{\circ}$  C for 35 s, 50 $^{\circ}$  C for 35 s, and 72 $^{\circ}$  C for 2 min, and a final extension at 72 $^{\circ}$  C for 5 min. Because generating sufficient quantity of full-length SSU rDNA products proved to be challenging, two overlapping PCR products were generated: one with forward primer A and 1055R and one with 528F and reverse primer B (Medlin et al. 1988). Quantity and length of products were examined by agarose gel electrophoresis against known standards. PCR products were purified using low melting agarose TAE buffer gel electrophoresis, excision of the target band under low UV light, and subsequent purification using the QIAEX II Gel Extraction kit 500 (Qiagen GmbH, Hilden, Germany), following manufacturer's instructions. Purified PCR products were sequenced on a Beckman Ceq 2000 automated sequencer (Beckman Instruments, Fullerton, CA, USA) according to manufacturer's instructions and using all sequence primers in Medlin et al. (1988).

**Data analysis.** Forward and reverse sequences were combined in DNASTAR (DNASTAR, Madison, WI, USA) and aligned with diatom sequences available in our database (Medlin et al. 2000, and this study) and in GenBank using Se-Al v1.d1 (Rambaut 1995). *Bolidomonas mediterranea* Guillou & Chre'tiennot-Dinet and *B. pacifica* Guillou & Chre'tiennot-Dinet (Guillou et al. 1999) were included as outgroups. Phylogenetic signal among parsimony-informative sites was assessed by comparing the measure of skewedness ( $g_1$ -value, PAUP\* version 4.0b10, Swofford 2002) with the empirical threshold values provided by Hillis and Huelsenbeck (1992). To determine which model of sequence evolution fits the data best, hierarchical likelihood ratio tests (hLRTs) were performed using Modeltest Version 3.0 (Posada and Crandall 1998). Phylogenies were reconstructed with PAUP\* using maximum likelihood (ML) constrained with obtained Modeltest parameters or neighbor joining (NJ) of likewise constrained pair-wise ML distances. Nodal support was estimated using NJ bootstrap analyses using the same settings (1000 replicates). Alternative topological arrangements for *Toxarium* were evaluated against that in the best tree using Kishino and Hasegawa (1989) in PAUP\*. Maximum parsimony trees were generated using weighted maximum parsimony (full heuristics, TBR, Goloboff fit criterion  $K = 2$ ); bootstrap values (1000 replicates) were determined without weighting.

## RESULTS

**SEM and LM.** Micrographs of *Toxarium* (Fig. 1) originated from Ischia culture material grown from a single cell. Only Figure 1, C and N, was taken from field material and are representative of cells observed in culture material. Cells were observed alone or adhering to their neighbors' valve faces (Fig. 1, A and D) and either detached or standing straight up on a mucilage pad (cf. Fig. 5). A valvar view (Fig. 1B) of the whole cell illustrates the central and peripheral convex regions characteristic for the genus. The central bulge in the lateral exposure of Figure 1A is an artifact caused by the convex central region being squashed between the object and cover slide. Such a bulge is not visible in a lateral SEM exposure (Fig. 1C). Cells contain numerous small elongate plastids (Fig. 1D; see also Fig. 5, of Loch Carron material) when healthy (upper cell in Fig. 1D).

Many aspects of valve morphology have already been described and illustrated by Round et al. (1990)

and are not repeated here. A close-up of the apical region illustrates the organization of valves and girdle bands *in situ* (Fig. 1E). The valve apex lacks pore fields or labiate processes (Fig. 1, F–H); the valve face bears simple irregularly placed poroids, as elsewhere on the valve, and there are two rows of regularly spaced and positioned poroids, one of either side of the valve face–mantle junction.

A close-up of the girdle in girdle view (Fig. 1I) reveals how the girdle band 1, the valvocopula (depicted from above in Fig. 1J and from below in Fig. 1K), fits precisely under the valve mantle, with a single line of poroids at the junction between pars interior and pars exterior (Fig. 1I). The pars exterior of this band is apparently plain. Girdle band 2 also has a well-defined transverse (*sensu* von Stosch 1975) row of poroids at the junction between pars interior and pars exterior, but in addition it bears striae containing poroids (Fig. 1, I and L). Bands 1 and 2 are of comparable width, but band 3 is much narrower. It bears a single row of poroids at the junction of pars interior and pars exterior (Fig. 1E). The pars interior of band 2 is fimbriate (Fig. 1L). All bands are closed (figure not shown).

The girdle is modified at the poles. The valvocopula is bent inward ("notched") at the pars interior–pars exterior junction, and the poroids are slightly larger in this region (Fig. 1J, also visible in Fig. 1E) and open inwardly via elongate slits (Fig. 1K). Band 2 is also modified at the pole, with larger more slit-like pores (Fig. 1E).

Despite the fact that the Ischia culture was monoclonal, variation in valve shape was discernible. Some cells possessed straight margins, generally considered typical of *T. hennedyanum* (Fig. 1M), whereas others had more or less undulate margins, as in *T. undulatum* (Fig. 1N). Valves consisted of a single layer of silica (Fig. 1O), though a network of rib-like thickenings was visible on the inside of the valve (Fig. 1P). Stages in valve formation were observed (Fig. 1, Q and R) but were not conclusive concerning morphogenesis because the earliest stages were not seen. Two things are certain: silicification does not commence from a mid-rib as in most pennates nor does it begin from a small central annulus, as in many radial and multipolar centrics (e.g. Pickett-Heaps et al. 1990, Round et al. 1990). The valve face appears to form as a single irregularly perforated plate (Fig. 1Q), which then "spills over" to form the more regularly structured valve mantle (Fig. 1R). We did not find evidence for centripetal silicification from an annulus lying at the valve face–mantle junction, as might have been expected from the pattern in completed valves (Fig. 1, Q and R), but this possibility cannot be excluded without thin section data or discovery of earlier stages in valve formation.

**Motility.** Observations of the Loch Carron culture revealed that cells were generally scattered across the whole of the Petri dish, even when the initial inoculum had been highly localized. By contrast, other

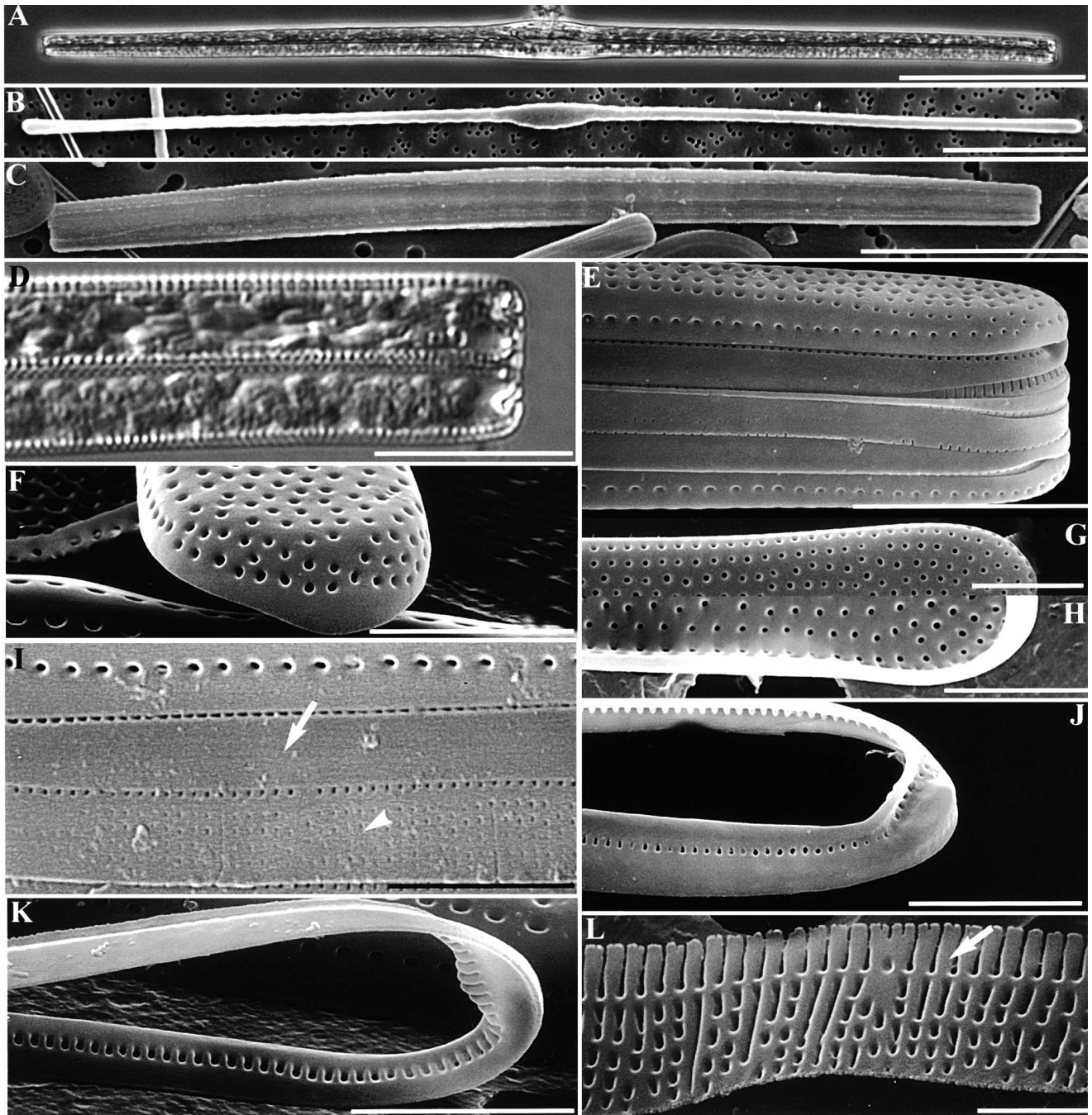


FIG. 1. LM and SEM images of *Toxarium undulatum*. Entire valve in LM (A), SEM valvar view (B), and lateral view (C, field material). LM image of valve apex in lateral view showing the slightly elongated plastids (D). SEM images of similar region (E). Seventy-five degree tilted images of valve at apex (F). External (G) and internal (H) details of valve face near apex. Detail of girdle (cingulum) with first girdle band (valvocopula, marked with arrow) and second girdle band (pleura, marked with arrowhead). Details of first girdle band in external (J) and internal view (K). Structure of the second girdle band (L) showing fimbriae (arrow). Seventy-five degree tilted images of the central part of a valve with both smooth (M) and undulating pattern (N, field material of well-developed case). Detail of a valve section (O). Internal view of valve surface showing network of ribs, called costae (P). Growth pattern of the valve in central (Q) and apical region (R). Scale bars, 100  $\mu\text{m}$  (A–C), 10  $\mu\text{m}$  (D, E, H, I, M, and N), 5  $\mu\text{m}$  (F, G, J, and K), 2  $\mu\text{m}$  (L and R), and 1  $\mu\text{m}$  (O–Q).

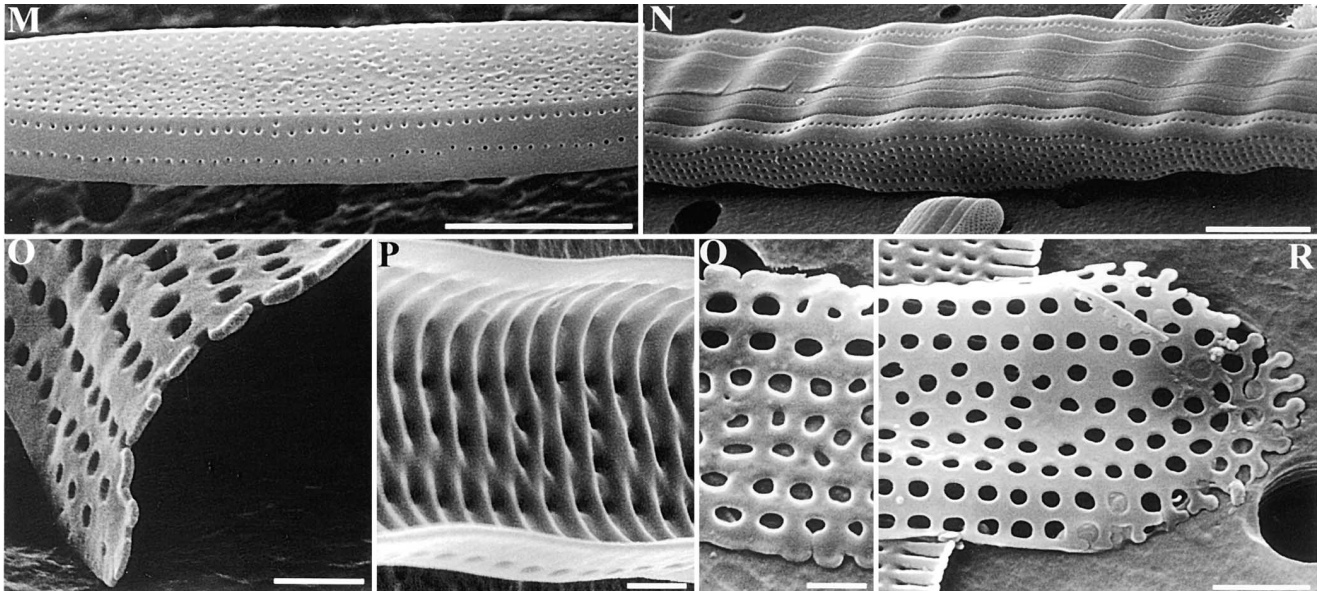


FIG. 1. Continued.

raphid pennate diatoms in culture at Edinburgh and Naples (e.g. *Synedra* C. G. Ehrenberg, *Diatoma* J. B. M. Bory de St.-Vincent, *Tabellaria* C. G. Ehrenberg) and also some attached raphid diatoms (e.g. *Achnanthes*) tended to form clusters where they had fallen after inoculation. Hence, given the previous report of autonomous movement in *Ardissonea* (Pickett-Heaps et al. 1991), *Toxarium* cells were examined for motility.

*Toxarium* cells moved in straight (Figs. 2, A and C, and 3), bent (Fig. 2B), or curved (Fig. 2C) paths. During movement, only one tip of the cell was in contact with the substratum, so that the long axis of the cell was not necessarily parallel to the direction of movement (Fig. 3, I-P), in contrast to what is usual in raphid diatoms (e.g. Edgar and Pickett-Heaps 1984, Round et al. 1990). However, the deviation between

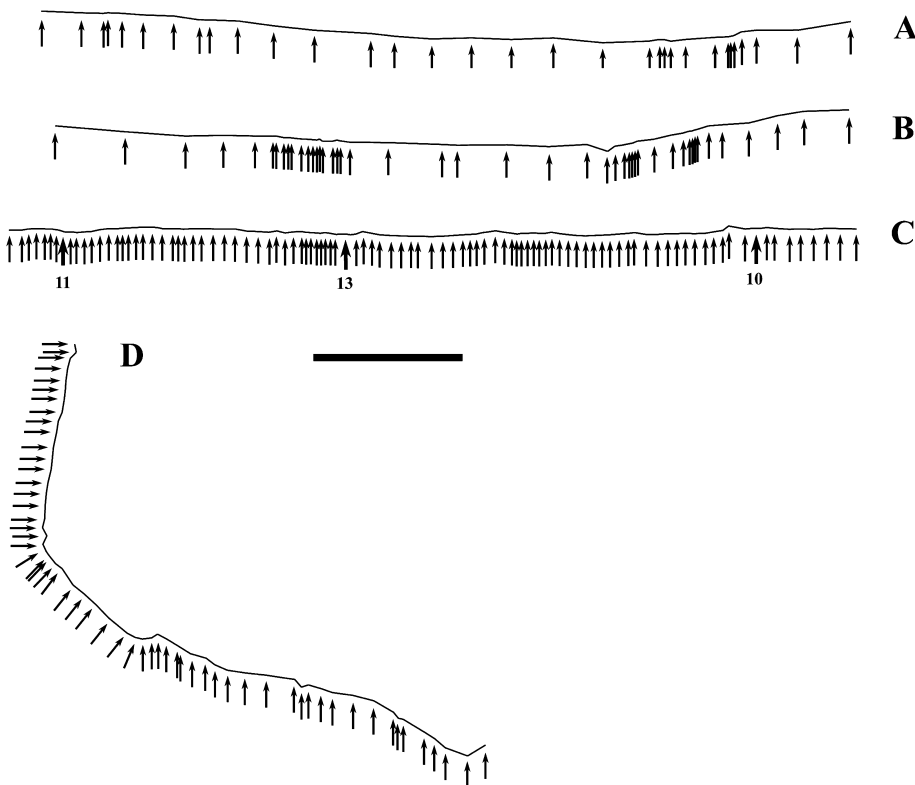


FIG. 2. Tracks of moving *Toxarium undulatum* cells, with arrows marking the positions of the posterior basal pole of each cell (i.e. the pole in contact with the substratum) at 10-s intervals. (A and B) Rapid straight-line movement interrupted by two (A) or three (B) brief periods of much slower progression. (C) Steady slow movement interrupted by three stops (the numbers indicate stasis for 110, 130, and 100 s, respectively). (D) Slow movement along a curved track, following reorientation of the cell. Total times for A, B, C, and D are, respectively, 310, 440, 1330, and 570 s. Scale bar, 100  $\mu$ m.

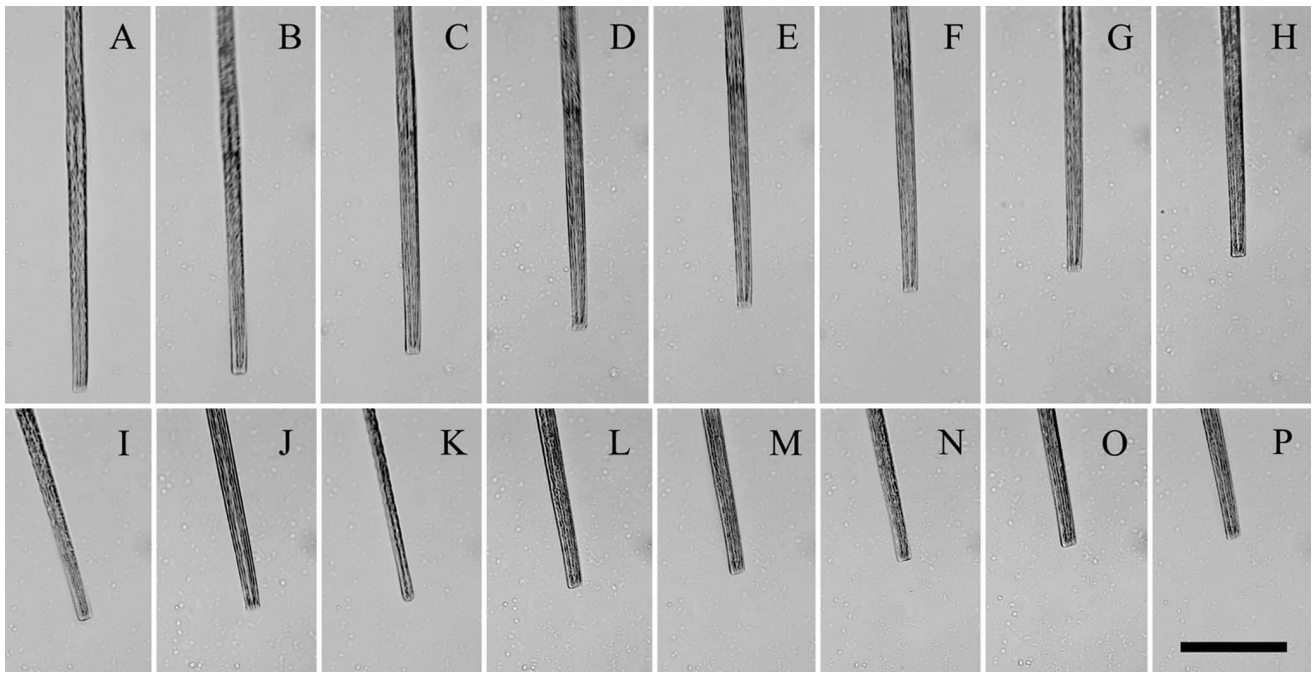


FIG. 3. Time-lapse sequence for a moving, recently divided *Toxarium undulatum* cell during a total of 259 s. Times between frames varied between 11 and 39 s. The top, left, and right margins of all photographs are coincident. Note the marked reorientation of the cell in the 11-s interval between H and I, as the cell rotated about its attached pole. Despite this, the cell continued to move in a straight line, that is, the axis of the cell was not parallel to the direction of movement in I–P. In most frames, the cell is orientated in girdle view, and at the pole shown the whole of the girdle and the valve mantles was in contact with the Petri dish; the lack of contact elsewhere is shown by the fact that the cell could not be focused throughout its length (e.g. in I). In K the cell has rotated into an oblique view and here only the edge of one valve can be touching the substratum. Scale bar, 100  $\mu\text{m}$ .

the axis of movement and the long axis of the cell was usually small (Fig. 4A). Cell usually contacted the substratum via the apex of both valves and the intervening girdle, so that cells moving on the Petri dish bottom or a slide presented themselves in girdle view; however, movement was also possible when the area of contact was much less, involving only one valve and the adjacent girdle (Fig. 3K). Recently divided cells could move (Fig. 3).

A maximum speed of approximately  $6 \mu\text{m}\cdot\text{s}^{-1}$  was recorded, but this was maintained for less than 10 s. Cells tracked for 1 min showed a maximum average speed of  $3.5 \mu\text{m}\cdot\text{s}^{-1}$  ( $N = 96$ ), but speeds of  $0.5$ – $2 \mu\text{m}\cdot\text{s}^{-1}$  were more common. Cells tracked for longer periods exhibited periods of  $\pm$  steady forward movement alternating with periods of stasis or very slow progression (Fig. 2, A–C). During forward movement (Fig. 3, H and I) or when virtually still (Fig. 4B), cells sometimes swiveled around the pole in contact with the substratum.

No trail was seen behind moving *Toxarium* cells, but this probably reflects the LM optics used. Sessile cells produced obvious masses of mucilage, apparently from the valve mantle or girdle at the poles, because the masses did not extend around onto the valve face (Fig. 5); this contrasts, for example, with *Rhoicosphenia* A. Grunow (Mann 1982, Fig. 6), where there is an obvious contribution of mucilage from pores on the valve face.

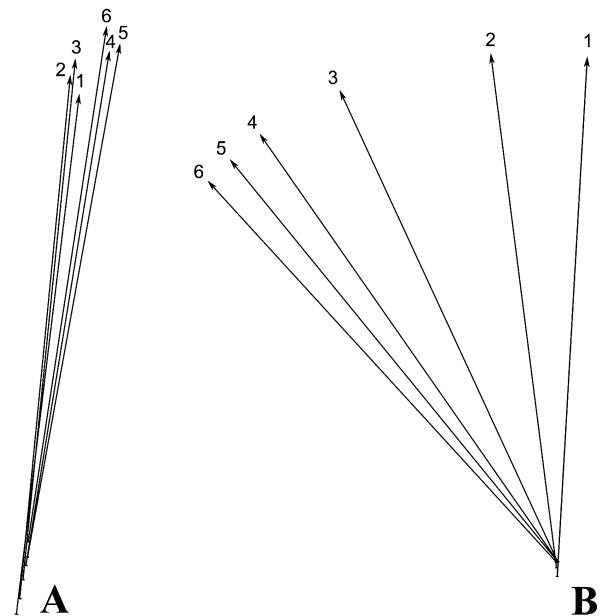


FIG. 4. Orientations and positions of the apical axes of two *Toxarium undulatum* cells at 10-s intervals (total duration 1 min each). (A) Apical axis remaining essentially parallel to the direction of movement. (B) Cell swiveling about the posterior attached pole.



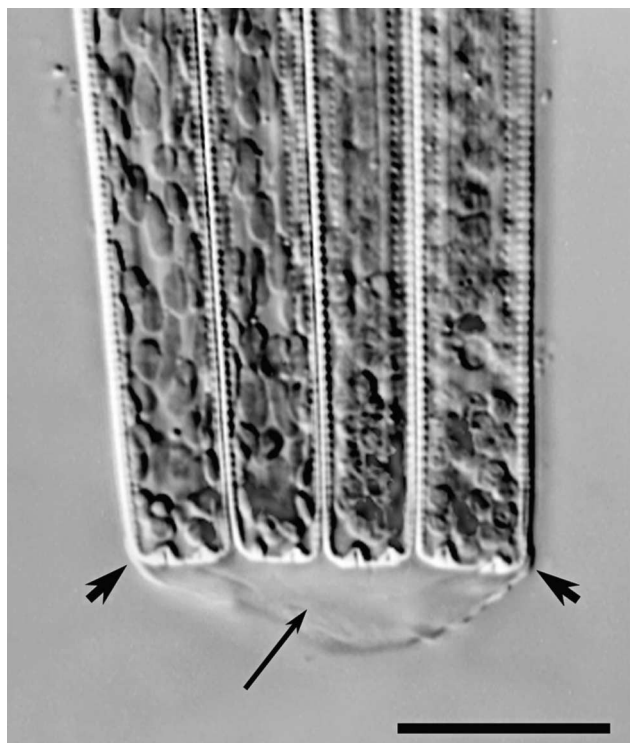


FIG. 5. Basal attached pole of a four-cell colony. A large amorphous mass of mucilage (long arrow) has been secreted from the cell apices; note that this does not extend past the valve face-mantle junction (arrows) of the outermost cells. Numerous small slightly elongate plastids are visible within the cells. Scale bar, 20  $\mu\text{m}$ .

SEM observations (not shown) did not reveal the exact route of mucilage secretion.

**Molecular analyses.** An alignment among the nuclear SSU rDNA sequences of 98 diatoms (Medlin et al. 2000) and two outgroup taxa showed significant phylogenetic structure at 677 parsimony-informative sites ( $g_1 = -0.40$ , whereas the 99% threshold would only be  $-0.08$  for 500 sites and 25 taxa). The hLRTs carried out on this data set chose a general time-reversible model of base substitutions, unequal distribution of base changes, and unequal base frequencies. The C $\leftrightarrow$ T substitutions occurred more than 1.5 times as frequently as the A $\leftrightarrow$ G substitutions. Modeltest constrained pair-wise ML distances showed several values exceeding 0.2, especially among radial centrics but also between some radial centrics and the other lineages.

An initial NJ bootstrap phylogram (not shown) inferred from the pair-wise distances among all these taxa revealed a principal dichotomy in a clade of radial centrics (referred to as Clade 1 in Medlin et al. 2000 and earlier papers) and a weakly supported clade with multipolar centrics and pennates (referred to as Clade 2 in Medlin et al. 2000 and earlier papers). The latter clade consisted of a polytomy containing several clades and individual branches with multipo-

lar centric taxa, including Thalassiosirales and one firmly supported clade with pennate diatoms. *Toxarium* was recovered on a single branch dropping straight on this polytomy. Results of Kishino-Hasegawa tests did not reject *Toxarium* as sister to multipolar centrics + pennates, or to multipolar centrics, or to some clades within multipolar centrics. Notably, recovery with *Lampriscus* A. Schmidt was definitely not significantly worse.

The above NJ analysis was rather crude, of course, and the resulting resolution therefore remained imprecise. Analysis could be done more accurately using full heuristics in ML, but assessing robustness of relationships among 100 taxa would take years. To obtain results in a more practical time interval, we had to delete taxa. Many radial centric taxa but also some multipolar centric ones (*Skeletonema* R. K. Greville, *Porosira* E. Jørgensen, *Lauderia* P. T. Cleve, and some among *Chaetoceros* C. G. Ehrenberg) were recovered on long branches, and ML inferred distances among them often exceeded 0.2. All these taxa were removed. Kishino-Hasegawa test results definitely rejected *Toxarium* as sister to radial centrics or to the diatoms as a whole. Furthermore, *Toxarium* was not sister to the raphid diatoms or to the remainder of the araphids. So, most taxa among the radial centrics and pennates could be deleted as well, without impairing recovery of the phylogenetic position of *Toxarium*. Of the radial centrics, only *Stephanopyxis nipponica* Gran & Yendo and *Rhizosolenia setigera* Brightwell were retained, because they were recovered on short branches. *Bolidomonas* taxa were left in as outgroup for the same reason. A restricted number of pennates were retained as representatives of the various clades and branches recovered in the first tree.

The data set with the remaining 38 diatoms and two outgroup taxa (Table 1) showed significant phylogenetic structure in 412 parsimony informative sites ( $g_1 = -0.76$ , whereas the 99% threshold would only be  $-0.09$  for 250 sites and 25 taxa). The hLRTs carried out on this data set chose a general time-reversible model of base substitutions, unequal distribution of base changes and unequal base frequencies, with parameter settings (Fig. 6) comparable with those recovered using the full data set. All Modeltest constrained pair-wise ML distances remained below 0.2.

The resulting ML tree (Fig. 6) showed raphid pennates in a well-supported clade within a paraphyletic araphid group. The pennates as a whole, excluding *Toxarium*, were in their turn monophyletic within an apparently paraphyletic group of multipolar centric diatoms. The latter group was essentially composed of a series of clades that collapsed in a polytomy because their basal dichotomies remained unsupported. Pennates and multipolar centrics in their turn formed a weakly supported clade, which was sister to radial centrics.

In Figure 6, *Toxarium* is sister to *Lampriscus* with moderate bootstrap support. However, results of Kishino-Hasegawa tests (Table 2) showed that topologies with *Toxarium* as sister to various other multipolar centric

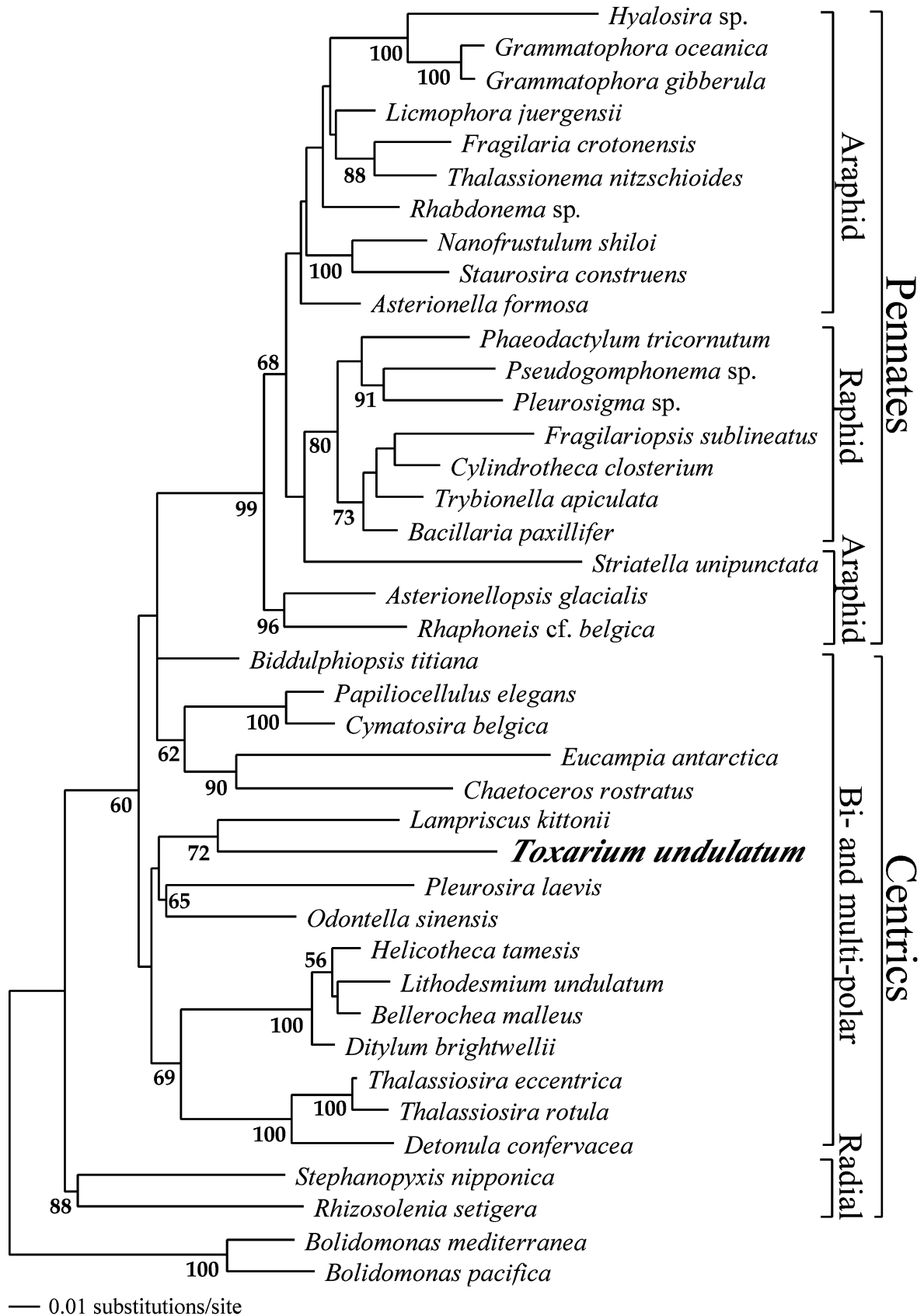


FIG. 6. ML tree inferred from base differences across SSU rDNA sequences listed in Table 1. Calculations were constrained using recovered Modeltest parameter settings. Base frequencies: A = 0.2576, C = 0.1784, G = 0.2413, T = 0.3227; base substitution frequencies (G↔set to 1.000): A↔C = 0.9394, A↔G = 2.4904, A↔T = 1.1368, C↔G = 1.1279, C↔T = 3.7238; proportion of invariable sites I = 0.3360; gamma shape parameter = 0.5716. Bootstrap values (1000 replicates) generated with NJ analysis of pair-wise Modeltest constrained ML distances. Bolidophyceae are designed as outgroup.



TABLE 1. Taxa used and their GenBank numbers. Taxa are listed in the same order as in which they appear in the tree to facilitate comparison.

Taxon	GenBank No.
<i>Hyalosira</i> sp. F. T. Kützing <sup>a</sup>	AF525654
<i>Grammatophora oceanica</i> Ehrenberg	AF525655
<i>Grammatophora gibberula</i> Kützing	AF525656
<i>Licmophora juergensii</i> Agardh	AF525661
<i>Fragilaria crotonensis</i> Kitton	AF525662
<i>Thalassionema nitzschioides</i> (Grunow) Hustedt	X77702
<i>Rhabdonema</i> sp. F. T. Kützing	AF525660
<i>Nanofrustulum shiloi</i> (Lee, Reimer & McEmery) Round, Hallsteinsen et Paasche	AF525658
<i>Staurosira construens</i> Ehrenberg	AF525659
<i>Asterionella formosa</i> Hassall	AF525657
<i>Phaeodactylum tricornutum</i> Bohlin	AJ269501
<i>Pseudogomphonema</i> sp. L. Medlin	AF525663
<i>Pleurosigma</i> sp. W. Smith	AF525664
<i>Fragilariopsis sublinearis</i> Hasle	AF525665
<i>Cylindrotheca closterium</i> (Ehrenberg) Reimann et Lewin	M87326
<i>Tryblionella apiculata</i> Gregory	M87334
<i>Bacillaria paxillifer</i> (O.F. Müller) Hendey	M87325
<i>Striatella unipunctata</i> (Lyngbye) Agardh	AF525666
<i>Asterionellopsis glacialis</i> (Castracane) Round	X77701
<i>Rhaphoneis belgica</i> (Grunow in van Heurck) Grunow in van Heurck	X77703
<i>Biddulphiopsis titiana</i> (Grunow) von Stosch et Simonsen	AF525669
<i>Papiliocellulus elegans</i> Hasle, von Stosch et Syvertsen	X85388
<i>Cymatosira belgica</i> Grunow	X85387
<i>Eucampia antarctica</i> (Castracane) Mangin	X85389
<i>Chaetoceros rostratus</i> Lauder	X85391
<i>Lampriscus kittonii</i> Schmidt	AF525667
<i>Toxarium undulatum</i> Bailey	AF525668
<i>Pleurosira laevis</i> (Ehrenberg) Compère	AF525670
<i>Odontella sinensis</i> (Greville) Grunow	Y10570
<i>Helicotheca tamesis</i> (Schrubsole) Ricard	X85385
<i>Lithodesmium undulatum</i> Ehrenberg	Y10569
<i>Bellerochea malleus</i> (Brightwell) van Heurck	AF525671
<i>Ditylum brightwellii</i> (West) Grunow in Van Heurck	X85386
<i>Thalassiosira eccentrica</i> (Ehrenberg) Cleve	X85396
<i>Thalassiosira rotula</i> Muenier	X85397
<i>Detonula confervacea</i> (Cleve) Gran	AF525672
<i>Stephanopyxis nipponica</i> Gran et Yendo	M87330
<i>Rhizosolenia setigera</i> Brightwell	M87329
<i>Bolidomonas mediterranea</i> Guillou et Chre'tiennot-Dinet	AF123596
<i>Bolidomonas pacifica</i> Guillou et Chre'tiennot-Dinet	AF123595

<sup>a</sup>See Navarro and Williams (1991).

clades were borderline or not significantly worse. Nonetheless, both Figure 6 and Table 2 reject this genus as sister to pennates, radial centrics, or a clade comprising Lithodesmiales and Thalassiosirales.

DISCUSSION

*Taxonomy.* Our cultures indicate that *T. undulatum* and *T. hennedyanum* are probably nothing more than two extreme forms in the morphological plasticity range of a single species. Undulation appears to have little to do with genetics and environmental conditions because the variation was recovered from a clonal culture. Because *T. undulatum* was described first by Bailey (1854; see Hustedt 1932), we propose to

TABLE 2. Kishino-Hasegawa test results for alternative topologies in comparison with the -Ln likelihood of the best tree (=13985.63853) in Figure 6.

<i>Toxarium</i> as sister to	Diff -ln L	SD (diff)	P
Pennates	95.47780	21.34565	<0.0001 <sup>a</sup>
<i>Biddulphiopsis</i>	10.43513	8.37905	0.2131
Cymatosiraceae, <i>Eucampia</i> et <i>Chaetoceros</i>	10.79075	8.48373	0.2036
Cymatosiraceae	14.72472	9.30186	0.1136
<i>Eucampia</i> et <i>Chaetoceros</i>	14.85788	9.30485	0.1105
<i>Pleurosira</i> et <i>Odontella</i>	12.43309	6.08697	0.0412 <sup>a</sup>
Lithodesmiales et Thalassiosirales	14.00679	6.28097	0.0259 <sup>a</sup>
Radial centrics	32.73110	11.63253	0.0049 <sup>a</sup>

<sup>a</sup><0.05.

merge *T. hennedyanum* into *T. undulatum*. Undulation seems to be generated *de novo* and can then be amplified or moderated with ongoing mitotic divisions. Similar undulation in other species might be generated the same way and have little taxonomic meaning, for example, in *Synedra montana* Krasske (Hustedt 1932), *Neosynedra tortosa* (Grunow) Williams and Round (Williams and Round 1986), Tabellariaceae, *Meridion* C. A. Agardh, *Staurosira* (C. G. Ehrenberg) D. M. Williams et F. E. Round, *Pseudostaurosira* (Grunow) D. M. Williams et F. E. Round, *Staurosirella* D. M. Williams et F. E. Round, and fossil *Pseudorutilaria* (E. Grove et G. Sturt ex De Toni et Levi) E. Grove et G. Sturt ex De Toni (Round et al. 1990).

*Sequence information and phylogenetic analysis.* ML analysis of large data sets like the one we have assembled becomes prohibitively time consuming. Therefore, we opted for an initial NJ bootstrap analysis of ML distances constrained with Modeltest parameters. Despite being imprecise, the procedure and associated results of Kishino-Hasegawa tests among alternative topologies helped us to identify taxonomic groups that could be deleted without hampering accurate recovery of the phylogenetic position of *Toxarium* in a more sophisticated ML analysis among a more focused group of taxa.

Kooistra and Medlin (1996) identified significantly elevated substitution rates among nuclear SSU rDNA sequences of many radial centrics and some multipolar ones. Such anomalously fast-evolving sequences not only tend to attract one another in phylogenies but also can affect topology in shorter clades (Swofford et al. 1996, Page and Holmes 1998, Wiens and Hollingsworth 2000). Such effects become more profound with increasing distances among sequences. Because most pair-wise distances among the sequences do not exceed 0.2, one would not expect much distortion if SSU sequences evolved in a stochastic manner. Unfortunately, however, variations in SSU sequences are not stochastic (Neefs et al. 1991): changes concentrate in variable regions and C↔T transitions occur more frequently than others. Although overall distances among our diatom SSU sequences do not exceed 0.2, distances would do so if one only included

fast evolving regions in the comparisons. The amount of homoplasy, and hence its potentially distorting effects upon the phylogeny, would thus be underestimated if one assumed positional rate homogeneity. Of course, application of the best fitting model for the data set at hand is expected to alleviate such problems, yet Modeltest provides only a best fit given the data, not a perfect solution. To alleviate problems related to branch attraction, we eliminated all the significantly fast evolving sequences from the data set before ML analysis.

Goldman et al. (2000) reported statistical problems associated with the Kishino and Hasegawa (1989) test in the case of one of the competing topologies being the optimal solution, as in the present instance. Nevertheless, we used this test because results still show which alternative hypotheses are extremely unlikely and which are definitely not significantly worse. The results of these tests corroborate the ill-resolved basal topology of the multipolar centrics, because *Toxarium* can be grouped almost anywhere in this group.

*Character evolution in Toxarium.* Recovery of *Toxarium* among the multipolar centrics implies that this species has adopted a pennate-like shape and life style during evolution from a bi- or multipolar centric ancestor and that this occurred independently from the similar trend accompanying the evolution of the monophyletic true pennates. The general valve shape of *Toxarium* resembles that of the unrelated true araphid pennates *Synedra montana* (see Hustedt 1932, p. 205), *Thalassionema bacillare* (Heiden in Heiden and Kolbe) Kolbe (Hasle and Syvertsen 1997, p. 258) and *Reimerothrix floridensis* Prasad (in Prasad et al. 2001) and it is therefore a homoplasy. Because all these species are relatively large and occur in bunches attached to various surfaces by mucilage pads, similar environmental constraints may have forced convergence.

The recovery of *Toxarium* within the multipolar centrics is consistent with the plastid data. The many small oval plastids observed in this study (Fig. 1D) resemble those found in multipolar and radial centrics. However, these small plastids do not provide any additional support for placement of *Toxarium* in the centrics because many araphid pennates also possess small round plastids.

Although shared lack of a trait is a bad criterion for grouping taxa together in phylogenetic exercises, both *Toxarium* and its putative sister taxon *Lampriscus* share a lack of labiate processes, suggesting that these structures were lost in their common ancestor. Apical pore fields are also absent in *Toxarium*, but secondary loss of this feature has also happened several times elsewhere in the multipolar centrics (e.g. in *Biddulphiopsis* H. A. von Stosch et R. Simonsen and Thalassiosirales). Labiate processes and apical pore fields have also been lost secondarily in various groups of pennates, especially in raphids (Round et al. 1990), where the raphe attaches the cells to the substratum during movement; however, reacquisition of apical pore fields

has occurred in several raphid lineages (Round et al. 1990).

Loss of mucilage-exuding organelles from the valve face of *Toxarium* is curious because *Toxarium* nevertheless attaches to surfaces by means of mucilage pads. It appears to have developed an entirely new exudation site, which is most probably the row of enlarged pores in the apical invagination of the first girdle band, judging by the topographic distribution of the mucilage secreted and the very close resemblance between the girdle morphologies of *Toxarium* and *Ardissonea* (Round et al. 1990, Pickett-Heaps et al. 1991). *Ardissonea* produces mucilage for attachment and for motility (Pickett-Heaps et al. 1991), just as in *Toxarium*. The characteristics of *Ardissonea* locomotion are very similar to those in *Toxarium*. Movement takes place through interaction between the cell apex and the substratum; as a result, the long axes of the cells are not always parallel to the direction of movement and cells can swivel about the attached pole. Contact usually takes place via the whole of the girdle and valve mantles, but cells can rotate slightly into a more oblique orientation. Gliding speed is variable—perhaps a little slower than in *Toxarium* (Pickett-Heaps et al. 1991 recorded a maximum of  $2.6 \mu\text{m}\cdot\text{s}^{-1}$ )—with frequent stops. Motion can take place in a straight line or along a curve. Pickett-Heaps et al. recorded reversal of the direction of movement (as a result of the site of attachment and motility generation swapping from one pole to the other), which was not evident in the cells we tracked. This may be because we observed cells directly in the culture vessel, using a water immersion objective, rather than cells sandwiched between slide and coverslip. *Ardissonea* exhibits exactly the same apical groove and pores in the valvocopula as is seen in *Toxarium*, and this exact correspondence suggests a close functional similarity and phylogenetic relationship. *Ardissonea* also lacks apical pore fields and labiate processes. Why the lineage containing these two genera should have lost its pore fields and processes and developed an alternative pathway of mucilage excretion remains to be clarified, but movement may be an explanation.

The locomotory abilities of *Toxarium* and *Ardissonea* are intermediate between those of the centrics *Actinocyclus* and *Odontella* and those of the raphid diatoms (we do not imply any sense of evolutionary “progression”). *Actinocyclus*, for example, moves at only  $1 \mu\text{m}\cdot\text{s}^{-1}$  and rotates at the same time (Medlin et al. 1986), so that control over the direction of movement cannot be great. *Odontella* merely performs continuous, small, highly irregular shuffling, rocking, and twitching movements (Pickett-Heaps et al. 1986). By contrast, some raphid diatoms can achieve top speeds of up to  $29 \mu\text{m}\cdot\text{s}^{-1}$  (Edgar and Pickett-Heaps 1984), and their movements are clearly directional, allowing rapid vertical migration through sediments, for example, in response to the tidal cycle in intertidal species (see Harper 1977). The paths individual cells take sometimes correspond closely to the course of the raphe

system (e.g. straight in *Navicula*, sigmoid in *Pleurosigma*) (Edgar and Pickett-Heaps 1984, Round et al. 1990), which is unsurprising if the mechanism of raphe motility postulated by Edgar and Pickett-Heaps (1984), involving active displacement of trail polysaccharide along the raphe slits, is correct. Motion is also readily and rapidly reversible. In contrast, movement through polar secretion, as in *Ardissonea* and *Toxarium*, will only propel the cell one way (with the attached pole at the posterior: reversal requires the opposing pole to become attached and functional in secretion), does not allow fine control over the direction of movement, and is likely to be much less efficient than the raphe system in terms of the distance moved per unit mass of trail carbohydrate. In these respects, the locomotion of *Ardissonea* and *Toxarium* resembles that of placoderm desmids (Häder and Hoiczky 1992).

The nature of the pattern center of *Toxarium* is partly revealed by our study. If the orderly rows of poroids encircling the valve face reflected the presence of an elongate distorted elliptical annulus (Mann 1984) reminiscent of those encountered in some bipolar centrics (e.g. *Odontella*: Pickett-Heaps et al. 1990, Fig. 40e), then silica precipitation would be expected to commence there. We have not observed a stage in which only an annulus is present. Instead, in the earliest stages available to us, silica precipitation has already taken place over most of the valve face (up to and including the first regular row of pores at the edge of the valve face), producing a flat sheet of silica perforated by irregularly scattered pores, except at its periphery, where the arrangement is more orderly. Silicification then proceeds out toward the valve rim, producing the mantle and apical regions. In any way, these figures show that the peripheral rim with its orderly spaced pores is not silicified first because in the central region of the valve silicification has already proceeded into the mantle, whereas along the apical parts the valve perimeter still remains to be silicified. Yet precipitation itself should not be confused with the construction of organic templates preceding the precipitation process. The order in which the organic matrix is laid down may differ from that in which precipitation takes place. It is certain, however, that no sternum is present.

*Phylogenetic position of Toxarium.* Removal of *Toxarium* from the pennates increases the morphological homogeneity of the latter. If *Toxarium* and its putative close allies (see below) are excluded, the monophyletic "true pennate" clade can be characterized by possession of a single sternum subtending parallel ribs and intervening rows of pores; in addition, there is either a raphe (in raphid pennates) or apical (rarely subcentral or axial, e.g. in *Rhabdonema* F. T. Kützing, *Thalassioneis* F. E. Round, *Tetracyclus* J. Ralfs and *Tabellaria*) labiate processes, or both (Round et al. 1990). The raphe may have evolved from the labiate processes, because raphids are monophyletic within paraphyletic araphids and because of similarities in struc-

ture and morphogenesis (Hasle 1974, Mann 1984, Pickett-Heaps et al. 1990). Inclusion of *Toxarium* in multipolar centrics does not really affect the taxonomic circumscription of the multipolar centrics, because this group is already a morphologically diverse (Round et al. 1990) and possibly paraphyletic assemblage (Medlin et al. 2000).

Relatives of *Toxarium* must now be sought. As noted above, *Ardissonea* may be particularly close to *Toxarium*, despite *Ardissonea*'s two-layered chambered valve. *Climacosphenia* C. G. Ehrenberg and *Synedrosphenia* (Peragallo) F. Azpeitia Moros are also likely candidates because they too lack labiate processes, obvious apical pore fields, and median sterna. *Ardissonea*, *Climacosphenia*, and *Synedrosphenia* resemble each other and differ from *Toxarium* in that the pores in their valve face are organized into regular rows perpendicular to the apical plane. The ribs between them are subtended by two parallel longitudinal ribs, each of which lies approximately halfway from axis to margin and which curve around and apparently fuse at the apices. This system of longitudinal ribs is called a bifacial annulus (Mann 1984), because it subtends transapical ribs on both sides, instead of only outwardly, as in a true annulus. If these three genera and *Toxarium* are all related, then the regular rows of pores along the valve perimeter of *Toxarium* might be a vestigial bifacial annulus and the scattered distribution of pores over the central parts of the valve face could result from secondary loss of organization.

The independent acquisition of a pennate lifestyle in *Toxarium* and its putative relatives renders these genera prime targets for life history studies. To our knowledge, their meiosis and auxospore formation have not been studied. Centrics all show oogamy with large macrogametes and the production of many small flagellated microgametes by each microgametangium, whereas in pennate diatoms the gametes are never flagellate, only one or two gametes are formed per gametangium, and the gametes are usually alike in size and morphology (there may be differences in activity) (Round et al. 1990); the exception is *Rhabdonema*, where a large egg cell is fertilized by a small amoeboid male cell (von Stosch 1958). In view of the phylogenetic position of *Toxarium*, oogamy would be the expected mode of reproduction. Auxospore development also needs to be examined in *Toxarium*. Given the highly elongate shape, one might predict that auxospore expansion will be analogous to that in true pennates, where transverse perizonial bands are laid down one after the other during auxospore development to constrain expansion and produce an elongated form (Round et al. 1990). However, if *Toxarium* and true pennates have evolved independently, the methods by which they generate their elongate shapes may show significant differences.

The fossil stratigraphy and molecular phylogenies reveal that pennates emerged well after the centrics (Gersonde and Harwood 1990, Kooistra and Medlin 1996). The earliest pennates known are apparently mem-

bers of the Rhabdioneidae and date from the Upper Cretaceous and Paleocene (Hajós 1975, Strel'nikova 1993). These have the axial sternum typical of true pennates and they also possess apical pore fields and labiate processes, as do their later relatives, such as the Eocene *Rhabdioneis atlantica* G. W. Andrews (Andrews 1989) or Oligocene *Dickensoniiforma* R. Scherer (Scherer 1997). No Cretaceous or Paleocene fossils are known with a *Toxarium*- or *Ardissonea*-like morphology or pattern centre. The *Toxarium*-*Ardissonea* group first appears in the Upper Eocene Oamaru flora, judging by the micrographs of "*Synedra baculus* Gregory" and "*S. hennedyana* Gregory" published by Desikachary and Sreelatha (1989, plate 110, Figs. 1–3); these seem to show two submarginal longitudinal pattern centres as in *Ardissonea*, *Climacosphenia*, and *Synedrosphenia*. We suspect that *Toxarium*, together with *Ardissonea*, *Climacosphenia*, and *Synedrosphenia*, are the last surviving members of a once far more diverse pennate-like diversity that is largely unrepresented in the fossil record, perhaps because they occupied habitats unsuitable for diatom preservation. However, it is also possible that they evolved from multipolar centric forebears relatively late, in the Paleocene or Eocene; the fossil record of multipolar centrals extends back to the Mesozoic (Strel'nikova 1993).

Finally, our documentation of movement in *Toxarium* reemphasizes that motility in diatoms is not restricted to the raphid lineages. Motility may be present in most major lineages, centric and pennate, and it should now be sought in any that have benthic representatives. Surface-associated movement is not obviously useful for species that are restricted to planktonic habitats and, not surprisingly, it is greatly reduced in those raphid taxa that have adapted secondarily to a planktonic mode of life, such as *Fragilariaopsis* F. Hustedt. Although most centric genera and many araphid pennates are planktonic, there are several species in the benthos, where orientated autonomous motility mechanisms might evolve (or an ancestral capacity for movement retained) to allow active microhabitat selection, for light acquisition, attachment, mating, or avoidance of burial.

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