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MOLECULAR PHYLOGENY OF SELECTED MEMBERS OF THE ORDER THALASSIOSIRALES (BACILLARIOPHYTA) AND EVOLUTION OF THE FULTOPORTULA[1]

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Recent phylogenetic studies of the diatoms indicate that members of the order Thalassiosirales occupy an interesting position in the diatom evolutionary tree. Despite their radial morphology and scaly auxospores, they are consistently recovered in molecular analyses as a member of subdivision Bacillariophytina and a sister clade to non-fultoportulate and non-radial lithodesmioids. This study included 46 species from nine traditionally accepted extant genera, and analyzed 43 nuclear small subunit (SSU) rRNA sequences in parallel with a survey of the variation in fultoportula structure. Three possible scenarios leading to the evolution of the fultoportula are discussed in the context of molecular and morphological similarities between the examined Thalassiosirales and their SSU rRNA sister clade Lithodesmiales. We speculate that the fultoportula might be derived by a modification of either a cribrum in an areola (fultoportula within an areola), or structures similar to marginal ridges now seen in lithodesmioids around a cluster of poroids (fultoportula in a tube), or finally, that the central fultoportula may have an origin different from the marginal fultoportulae. Our data confirm that fultoportula-bearing diatoms constitute a natural phylogenetic group. The families Thalassiosiraceae, Skeletonemaceae, and Stephanodiscaceae and the genus *Thalassiosira* Cleve were unexpectedly found to be paraphyletic. Further, *Cyclotella* Kutz. and *Stephanodiscus* Ehr. may not be closely related and some species of these genera are more closely allied to other species of *Thalassiosira*. The generitype, *T. nordenskioeldii*, is embedded within a large poorly structured cluster of species that includes several members of *Thalassiosira*, *Planktoniella sol*, *Minidiscus trioculatus*, and two members of *Stephanodiscus*. An emendment of the order Lithodesmiales and the family Lauderiaceae are proposed.

Key index words: **development; fultoportula; Lithodesmiales; molecular phylogeny; morphology; Thalassiosirales**

Abbreviations: **AWI, Alfred–Wegener Institute; BI, Bayesian Inference; BT, bootstraps; ML, Maximum Likelihood; MP, Maximum Parsimony; MTA, Mount Allison University; NJ, Neighbor Joining; PP, posterior probabilities**

Despite their relatively recent origins (Rothpletz 1896, Kooistra and Medlin 1996), the Bacillariophyta are a successful, abundant, and diverse algal group found in most aquatic ecosystems. Diatoms are of great ecological importance, especially as a key contributor to global carbon and silica cycling (Treguer et al. 1995, Mann 1999). The classical view of diatom systematics, inferred mainly from morphological characteristics of their siliceous cell walls, places species into one of two or three classes: centric diatoms (radial and non-radial) and pennates (araphid or raphid) (Simonsen 1972, 1979, Round et al. 1990). Recent molecular analyses, however, have suggested extensive reorganization of the diatom classification system into different clades (Medlin and Kaczmarska 2004, Fig. 1). The first clade, based on nuclear small subunit (SSU) rRNA, includes only radial centric diatoms, whereas the second clade contains two distinct subclades: bipolar centrics plus the radial Thalassiosirales and all pennate forms (Medlin et al. 1993, 1996a, b). This segregation of diatom types is supported by analyses using several genes: nuclear SSU rRNA (Medlin et al. 1993, 1996a, b, 2000, Sinninghe Damsté et al. 2004), nuclear large subunit rRNA (Sörhannus et al. 1995), plastid *rbc*L and *tuf*A (D. G. Mann, unpublished data, Medlin et al. 1997, respectively), mitochondrial cytochrome c oxidase I, and *rpo*A (Ehara et al. 2000, Fox and Sörhannus 2003, respectively).

This segregation has been recently formalized, and two new subdivisions have been proposed for these clades: Coscinodiscophytina (Clade 1) and Bacillariophytina (Clade 2, Medlin and Kaczmarska 2004). Cos-

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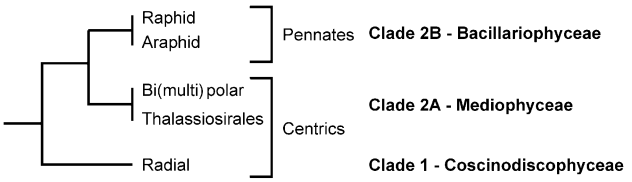

FIG. 1. Schematic representations of the molecular phylogeny from Medlin and Kaczmarska (2004) showing the relationship between the major lineages within the diatoms and their common names.

cinodiscophytina contains one class (Clade 1, radial centrics, except Thalassiosirales), whereas Bacillariophytina has been further separated into two subclades: Clade 2a containing the bi(multi)polar centrics plus the radial Thalassiosirales, and Clade 2b retaining the raphid and araphid pennate diatoms (Fig. 1, and Medlin et al. 1993, 1996a, b, 2000). Thus, the diatoms are now divided into two subdivisions (Coscinodiscophytina and Bacillariophytina) and three classes: Coscinodiscophyceae, Mediophyceae, and Bacillariophyceae (Medlin and Kaczmarska 2004).

Members of the order Thalassiosirales, a morphologically radial centric group, do not belong to the SSU rRNA Clade 1 of radial centrics as might be anticipated from their valve morphology (Round et al. 1990, Medlin et al. 1993). Instead, members of this order have been consistently placed within Clade 2 together with bi(multi)-polar centrics and pennates (Fig. 1). Within this clade, the Thalassiosirales and its sister groups the Lithodesmiales and Hemiaulales (*sensu* Round et al. 1990, in part) appear to be among the earliest divergences. Whereas molecular evidence strongly supports the inclusion of the Thalassiosirales with bi(multi)polar diatoms, the cellular evidence is less consistent. Some aspects of protoplast ultrastructure (e.g. arrangement of the Golgi bodies) support the inclusion of Thalassiosirales with the non-radial centrics in Clade 2 (Schmid 1987, 1989, Medlin et al. 2000). In contrast, the circular shape, radially symmetric valve perforation pattern, scaly structure, and isometric architecture of the auxospores of this group are more similar to the radial than to the bi(multi)polar centric diatoms (von Stosch 1982, Kaczmarska et al. 2000, 2001).

With the advent of SEM, the distinctiveness of taxa that possess strutted processes, or fultoportulae, quickly became apparent (Ross and Sims 1972, Hasle 1973b). A conspicuous feature of the order Thalassiosirales (Gleser and Makarova 1986, Gleser et al. 1988), this character was used as the criterion for emending the family Thalassiosiraceae (Hasle 1973a, b). Presently, more than 30 genera are known to possess fultoportulae (Table 1), which are absent in all examined members of their sister groups, the Lithodesmiales and Hemiaulales.

Three genera belonging to Thalassiosirales (*Thalassiosira, Stephanodiscus*, and *Cyclotella*) in particular are unarguably the most systematically studied taxa in the history of diatom research and reflect the group's ecological importance and rich morphology. *Thalassiosira*, with about 180 described marine species and at least 12 reported freshwater species (Round et al. 1990, Silva and Hasle 1994), is one of the most speciose genera within the order. The genus is recognized by a combination of characters rather than specific diagnostic characteristics (Hasle 1972, 1973a, Makarova 1981, 1994), which typically include loculate areolae with external foramina and internal cribra, marginal and central strutted processes (fultoportulae, Hasle 1972, Hasle 1973a), and the peripheral labiate processes (rimoportulae). In addition, a number of girdle and valve face characteristics identified with light microscopy (LM) and SEM may be used to circumscribe species within the genus (Fryxell and Hasle 1972, 1979a, Hasle and Fryxell 1977a, Hasle 1980, 1983).

Despite the extensive volume of literature devoted to many aspects of *Thalassiosira* biology, fultoportulae have not been well investigated within the genus. To the best of our knowledge, there has been no systematic research on the types of fultoportulae since the work on *Lauderia annulata* by Syvertsen and Hasle (1982). Even the number of pores subtending this portula has not been consistently reported in recent accounts. Although there are serious logistical reasons for the lack of such knowledge, the absence of systematic attention to fultoportulae is quite surprising given the widespread consensus that it is a lineage-defining characteristic. As such, it should be considered a prime candidate for studies of the evolutionary history of these diatoms, as suggested a decade ago (Theriot and Serieyssol 1994).

The goals of our study were twofold. First, we reexamine the phylogenetic position of Thalassiosirales within the diatoms, specifically in terms of the internal architecture of the fultoportula and its relationship with the valve face structures in the non-fultoportulate species from its SSU rRNA sister clade, Lithodesmiales–Hemiaulales. We then hypothesize about the evolutionary roots of the fultoportula in the context of the molecular relatedness between the two sister lineages. Second, we investigate interspecific relationships within selected members of Thalassiosirales. Marine species of the genus *Thalassiosira*, emended by Hasle (1973a), constitute the majority of the diatoms investigated in this study. Additional strains, representing traditionally accepted genera from the order, were included to recover the major lineages among the fultoportulate species and to compare them with currently accepted taxonomies. Members of *Stephanodiscus* and *Cyclotella* (predominantly freshwater lineages) are presently under investigation in another laboratory (E. Theriot, personal communication), and so are included in the analyses but not investigated in-depth here. Comprehensive investigation of the phylogenetic relationships within the entire order will be considered in future work because our primary interest in this paper is the possible origin of the character defining the lineage.

TABLE 1. Summary of systematic affiliation and number of genera with fultoportula, in alphabetic order for ease of comparison.

	Hasle (1973a, b)	Simonsen (1979)	Gleser et al. (1988)	Round et al. (1990)	Nikolaev et al. (2001)	All genera compiled from literature
Order	Coscinodiscineae (suborder)	Centrales	Thalassiosirales	Thalassiosirales	1. Thalassiosirales 2. Stephanodiscales	Thalassiosirales
Family	Thalassiosiraceae	Thalassiosiraceae	A. Ectodictyonaceae B. Stephanodiscaceae C. Thalassiosiraceae	A. Lauderiaceae B. Skeletonemataceae C. Stephanodiscaceae D. Thalassiosiraceae	1A. Lauderiaceae 1B. Skeletonemataceae 1C. Thalassiosiraceae 2A. Ectodictyonaceae 2B. Stephanodiscaceae	
Genus	*Bacteriosira* *Cyclotella* *Detonula* *Lauderia* *Minidiscus* *Planktoniella* *Porosira* *Skeletonema* *Stephanodiscus* *Thalassiosira*	*Aulacosira*(?) *Bacteriosira* *Cyclotella* *Cymatodiscus* *Cymatotheca* *Detonula* *Lauderia* *Minidiscus* *Planktoniella* *Porosira* *Skeletonema* *Stephanodiscus* *Thalassiosira* *Trybliotychus*	A. *Ectodyction* B. *Concentrodiscus* *Cyclostephanos* *Cyclotella* *Stephanocostis* *Stephanodiscus* C. *Bacteriosira* *Cymatotheca* *Detonula* *Lauderia* *Minidiscus* *Planktoniella* *Porosira* *Skeletonema* *Thalassiosira*	A. *Lauderia* B. *Detonula* *Skeletonema* C. *Cyclostephanos* *Cyclotella* *Mesodictyon* *Pleurocyclus* *Stephanocostis* *Stephanodiscus* D. *Bacteriosira* *Minidiscus* *Planktoniella* *Porosira* *Thalassiosira*	1A. *Lauderia* 1B. *Skeletonema* 1C. *Bacteriosira* *Cymatotheca* *Detonula* *Lomonycus* *Minidiscus* *Nephrodiscus* *Planktoniella* *Porosira* *Thalassiosira* 2A. *Ectodyction* 2B. *Concentrodiscus* *Crateriportula* *Cyclostephanopsis* *Cyclostephanos* *Cyclotella* *Cyclotubicoalitus* *Mesodictyon* *Pleurocyclus* *Pliocеanicus* *Stephanocostis* *Stephanodiscus* *Tertiarius*	*Bacteriosira* *Coenobiodiscus* *Concentrodiscus* *Crateriportula* *Cyclostephanopsis* *Cyclostephanos* *Cyclotella* *Cyclotubicoalitus* *Cymatodiscus* *Cymatotheca* *Detonula* *Ectodyction* *Lauderia* *Lomonycus* *Mesodictyon* *Minidiscus* *Nephrodiscus* *Pelagodictyon* *Planktoniella* *Pleurocyclus* *Pliocеanicus* *Porosira* *Roundia* *Skeletonema* *Stephanocostis* *Stephanodiscus* *Takanoa* *Tertiarius* *Thalassiocyclus* *Thalassiosira* *Trybliotychus*

MATERIALS AND METHODS

Sequences from 46 strains from a variety of sources (Table 2) were initially considered but only 43 of them were included in the final analyses. Of these, 38 strains representing nine of the traditionally accepted extant genera in the order Thalassiosirales (Gleser and Makarova 1986, Gleser et al. 1988, Round et al. 1990) were obtained for analysis of nuclear SSU rRNA sequences at Alfred Wegener Institute (AWI) or Mount Allison University (MTA), Table 2. Whenever it was possible, generitypes were selected. Seventeen strains of *Thalassiosira* spp. representing a wide range of valve morphologies were studied. A few species were represented by more than one strain in order to confirm sequence stability. Species identity for some of the GenBank sequences was verified by SEM examination of the archival voucher samples, kindly made available by Dr. V. E. Armbrust. In some cases, samples were found to include more than one species or the species identity could not be confirmed. Those sequences were excluded from analysis but are listed in Table 2 for the reader's information. Four members of the lithodesmioid and hemiauloid species, each from a different genus (Round et al. 1990), and one diatom representing an outgroup were also included.

Cultures. Diatom cultures (Table 2) were obtained from several culture collections and grown at MTA or AWI in their recommended media. Warm water strains (*Thalassiosira tumida*, *T. pseudonana*, *T. oceanica*, *Minidiscus trioculatus*, *Cyclotella meneghiniana*, and *Stephanodiscus hantzschii/parvus*) were maintained at 20° C with an 18:6 LD in light intensity varying between 1.9 and 5 $\mu mol \cdot photons \cdot m^{-2} \cdot s^{-1}$. The cold water strains (*T. aestivalis*, *T. minima*, and *T. nordenskioeldii*) were grown at 5° C under a constant light exposure of approximately 13.2 $\mu mol \cdot photons \cdot m^{-2} \cdot s^{-1}$. Strains maintained at AWI were grown in f/2 media (Guillard 1975) under similar light and temperature conditions.

Microscopy. The identities of the diatom strains and purity of the cultures processed were confirmed using SEM or TEM. When possible, the original and amended descriptions were consulted (Hasle 1973a, 1980, 1983), in addition to widely used monographs and guides (Hustedt 1962, Ramirez 1981, Tomas 1997). Terminology for structures discussed here follows Ross and Sims (1972), Anonymous (1975), and Ross et al. (1975).

Valves in various stages of development were selected for examination of the portula internal structure. The fultoportula

TABLE 2. Strains and species of diatoms used in this study and their GenBank accession numbers, when available.

Species	Strain or DNA prep (Pxxx)	Accession	Reference
Bellerochea malleus (Bright.) V. Heurck	CCAP 1008/1	AF525670	4
Cyclotella meneghiniana Kützing	Black Sea, V. Chepurnov	AJ535172	4
C. meneghiniana	Cme13	B. Beszteri (unpublished)	7
C. meneghiniana	CCMP 337	DQ093371	8
Detonula confervacea (Cleve) Gran	R Crawford	AF525672	4
Ditylum brightwellii (West) Grun.	CCAP 1022/1	X85386[a]	4
Lampriscus kittonii Schmidt	A.M. Schmid	AF525667	4
Lauderia annulata Cleve	CCAP 1044/1	X85399	3, 5
Lithodesmium undulatum Ehr.	CCMP 474	Y10569	6
Minidiscus trioculatus (Taylor) Hasle	CCMP 495	DQ093363	8
Planktoniella sol (Wallich) Schütt	A.M. Schmid	AJ535173	4
Porosira glacialis (Grun.) Jørg.	n/a P186	X85398	3
Skeletonema grethae Zingone et Sarno	Unknown	X52086	7, 10
S. grethae	CCAP 1077/4	X52006	3, 10
S. grethae	CCAP 1077/3	X85395	3, 10
S. marinoi Sarno et Zingone	CCMP 1009	AJ535165	4, 10
S. marinoi (incorrect in GenBank as *S. pseudocostatum*)	Unknown	AF462059	2, 10
S. menzelii Guillard, Carpenter & Reimer	CCMP 790	AJ535168	4
S. menzelii	CCMP 787	AJ536450f	7
S. pseudocostatum Medlin	CCAP 1077/7	X85394	7
S. pseudocostatum	CS-76	X85393	3
S. pseudocostatum	SZN-B77	AJ632207	3
S. subsalsum (A. Cleve) Bethge	CCAP 1077/8	AJ535166	4
Stephanodiscus niagarae Ehr.	Okamanpeedan L.	E. Theriot (unpublished)	8
S. parvus/hantzschii (as *S. hantzschii* Grun.)	CCAP 1079/4	DQ093370	8
Streptotheca thamesis Shrubsole (as *Helicotheca thamesis*)	CCAP 1076/1	X85385	3
Thalassiosira aestivalis Gran	CCMP 975	DQ093369	8
T. anguste-lineata (A. Schmidt) Fryxell & Hasle	M. Hoppenrath	AJ810854	9
T. antarctica Comber (incorrect in GenBank–raphid diatom(s) sequence)[b]	n/a	AF374482	1
T. eccentrica (Ehr.) Cleve	n/a P108	X85396	3
T. fluviatilis Hustedt	n/a P928	AJ535170	7
T. guillardii Hasle[b]	CCMP 988	AF374478	1
T. hendeyi Hasle & Fryxell	M. Hoppenrath	AM050629	9
T. minima Gaarder	CCMP 991	DQ093366	8
T. nordenskioeldii Cleve	CCMP 997	DQ093365	8
T. oceanica (Hust.) Hasle & Heimdal[b]	CCMP 1005	AF374479	1
T. oceanica	CCMP 1001	DQ093364	8
T. profunda (Hend.) Hasle	n/a	S. Douglas (unpublished)	4
T. pseudonana Hasle & Heimdal	n/a P11	AJ535169	4
T. pseudonana	CCMP 1007	DQ093367	8
T. pseudonana	CCMP 1335	AF374481	1
T. rotula Meunier	CCMP 1647	AF374480	1
T. rotula	CCAP 108414	X85397	3, 5
Thalassiosira sp.	CCMP 1281	AJ535171	10
T. tumida (Jan.) Hasle	CCMP 1465	DQ093368	8
T. weissflogii (Grun.) G. Fryxell & Hasle	CCMP 1336	AF374477	1

n/a, culture no longer available but voucher specimens available upon request; 1, Armburst and Galindo (2001); 2, GenBank; 3, Kooistra and Medlin (1996); 4, Medlin and Kaczmarska (2004); 5, Medlin et al. (1996b); 6, Chesnick et al. (1997); 7, present study (AWI); 8, present study (MTA); 9, from M. Hoppenrath; 10, Sarno et al. (2005).

[a]Sequence corrected in GenBank since its original deposition.

[b]Strains not included in analysis.

internal architecture of 20 species included in our molecular analysis was determined using culture, wild material, or explicitly published evidence. They represent all the molecular groupings outlined below.

Samples were passed through a Millipore vacuum filtration apparatus containing a 3 µm polytetrafluoroethylene membrane (Poretics, Osmonics Inc. Minnesota, MN, USA). The collected material was first washed with distilled water, re-suspended in distilled water, and cleaned by adding 10 mL each of concentrated sulfuric and nitric acid in a boiling water bath for 1 h. Samples were again washed with distilled water, re-suspended, and finally dispersed on 3 µm filters, dried, and mounted on aluminum stubs using double-sided tape and colloidal carbon. Specimens were coated with approximately 10 nm of gold in a Hummer 6.2 sputtering unit (Anatech Ltd., Springfield, VA, USA) and examined using a JEOL JSM-5600 SEM (JEOL USA, Peabody, MA, USA) operating at 10 kV and 8 mm working distance at the Digital Microscopy Facility at MTA. Cultures grown at AWI were cleaned according to Simonsen (1974) and similarly mounted for SEM or mounted in hyrax for LM observation. The TEM preparations followed that of Hasle (1972), and were examined using a Zeiss 109 TEM (Zeiss, Oberkochen, Germany). SEM preparations for *Mediopyxis* are as in Kühn et al. (2006). SEM preparations for the fossilized diatom remains are given in Kaczmarska (1982).

DNA methods. Genomic DNA was isolated from cells of flourishing cultures using a *N*-Cetyl-*N*,*N*,*N*-trimethyl-ammonium bromide (CTAB) DNA organic extraction procedure

(Saghai-Maroof et al. 1984). Typically, DNA extractions were performed using 50 mL of dense culture. Diatom frustules of pelleted cells were mechanically disrupted by vortexing with glass beads prior to a 30 min incubation of the cellular material in 2× CTAB buffer (warmed to approximately 60° C). The DNA from cultures grown at AWI was extracted according to Medlin et al. (1993). Genomic DNA from a strain of *Stephanodiscus niagarae* was isolated in a similar manner (E. Theriot, personal communication) and made available for our analysis.

Nuclear SSU rRNA was amplified in 25 μL reactions by using Ready-To-Go PCR beads (Amersham Biosciences, Picataway, NJ, USA). Amplifications were performed using standard primers of Medlin et al. (1988) for the amplification and Elwood et al. (1985) for the internal sequencing primers, each at a final concentration of 0.8 μM under the following cycling conditions: 95° C for 3 min, 35 cycles of 95° C for 30 s, 53° C for 30 s, 72° C for 100 s, and 72° C for 3 min. The PCR amplifications at AWI were performed with the primers listed in Medlin et al. (1988) following the protocol outlined there.

Excess primers were removed from amplification products using GFX spin columns (Amersham Biosciences) prior to sequencing fragments in both directions directly using a Perkin–Elmer Big Dye termination kit (PerkinElmer, Foster City, CA, USA) and an ABI PRISM-377 (Applied Biosystems, Foster City, CA, USA) automated sequencer at the University of Guelph Molecular Supercentre. Sequences determined at AWI were performed with a LI-COR sequencer (LI-COR Biosciences, Bad Homburg, Germany) following the preparation of the PCR products and the sequencing reactions as outlined in Medlin et al. (2000).

Sequence analysis. Nuclear SSU rRNA gene sequences were manually aligned against 46 diatom species (representing all diatom classes), one bolidophyte, and one oomycete rRNAs from the rRNA WWW server (Wuyts et al. 2002) using the Se-Al alignment editor (Rambaut 1996) on a Macintosh G3 computer. The bipolar diatom *Lampriscus kittonii* was selected as the outgroup because it belongs in the sister group to the clade containing Thalassiosirales and Lithodesmiales (Medlin and Kaczmarska 2004). Phylogenetic inferences were made using two software packages: PAUP 4.0* (phylogenetic analyses using parsimony) (Swofford 2002) and MrBayes (V. 3.0b4) (Huelsenbeck et al. 2001). All trees were then calculated based on variation at 1814 aligned nucleotide sites because the entire length of the sequence could be unambiguously aligned.

The data set was subjected to the model test program (V. 3.06) to ascertain the appropriate model of evolution for our data set (Posada and Crandall 1998). Using this program, two models of evolution were selected: the model TrN + I + G was selected by the heirarchical likliwood ratio test (HLRT) test and the TIM + I + G were selected by Akaike information criterion. The values for the parameters of each of these models were implemented for the maximum likelihood (ML) and neighbor joining (NJ) analyses using PAUP 4.0*. Model 1 parameter values were Lset Base = (0.2682 0.1963 0.2547), Rmat = (1.0000 2.7018 1.0000 1.0000 4.0307), Rates = gamma, Shape = 0.6675, and Pinvar = 0.4698. Model 2 values were Lset Base = (0.2672 0.1961 0.2557), Rmat = (1.0000 3.0759 1.2768 1.2768 4.5884), Rates = gamma, Shape = 0.6685, and Pinvar = 0.4705. For the NJ and ML analyses, the Tamura–Nei model was used for the TrN model and the general time-reversible (GTR) model was used for the TIM model because it was not significantly different from the TIM in the log-likelihood table.

Bayesian analysis was performed using MrBayes (http://morphbank.ebc.uu.se/mrbayes/). Bayesian, like ML, is a probabilistic method that uses a given model of evolution and analysis for the best set of trees that are consistent with the model and the data set. The advantages of Bayesian inference (BI) are that it is relatively fast even when large data sets are used, and it generates probabilistic measures of tree strength, which gives posterior probabilities (PPs) for phylogenetic stability. These values are more straightforward to interpret than bootstrap (BT) values because they can be taken as the probability that the topology of a tree is most likely and represents the best-estimated phylogeny. We ran the Bayesian search using the GTR model with an undefined gamma distribution, during 1,000,000 generations, and saved every 1000th tree. We did not define gamma, the number of invariant positions, or the rate substitutions to allow the program to optimize these values itself. We discarded the first 100 trees, and the remaining 900 trees, all with higher PPs, were used to construct a 50% majority rule consensus tree. On this tree, PPs for each clade are shown, which represent the percentage, out of those 900 trees, having the corresponding clades.

Maximum parsimony (MP) analyses were implemented with the PAUP 4.0* program, using 343 informative sites. Introduced gaps were treated as missing data, and informative characters were treated as multistate and unordered. Unweighted MP trees were obtained using the tree-bisection-reconnection branch swapping option and a heuristic search with random additions of the taxa. The characters were re-weighted using a re-scaled consistency index, and a weighted analysis was performed on the data. Thirty-nine MP trees were recovered, all with essentially the same topology except for one polytomy in clade D, as illustrated below. One thousand BT replications were performed with models 1 and 2 for the NJ analysis and for the weighted MP data set.

RESULTS

Fultoportula architecture. In the species examined (Table 2), the fultoportulae show the same basic design. The fultoportula (*sensu stricto* or *s.s.*) or strutted process consists of the perforations of the base silica layer and associated thickenings (tubular or otherwise) around the pores (Figs. 2A–C). The base silica layer is the first layer produced during valve formation (Fig. 2A, as the internal layer of the valve in Schmid 1984). Therefore, when the external surface of the valve is observed, fultoportulae *s.s.* are obscured by siliceous layers deposited later, during the formation of the loculate areolae (Figs. 2A–D). In much of the current literature, however, the term fultoportula encompasses either a second, usually larger tube (Figs. 2D and 3A, B) or less commonly, a polygonal chamber of an areola (Figs. 3C, D) that surrounds external openings of all satellite pores and fultoportulae *s.s.* The fultoportula *s.s.* (a strutted process) and the external tube (or areola) are ontogenetically different structures and can exist (but probably not function) independent of each other. This second tube or the areolar chamber develops later after much of the fultoportula *s.s.* is already in place (Figs. 3A, C), and does not penetrate the base silica layer but rests upon its external surface. Because of the commonality of the use of the term encompassing both the fultoportula *s.s.* and the external tube or areola, we conserve this name here with the now fuller understanding of its development and structure and refer to it as the fultoportula *sensu lato* or *s.l.*

The fultoportula *s.l.* consists of a strutted tube, associated satellite pores and a superimposed external

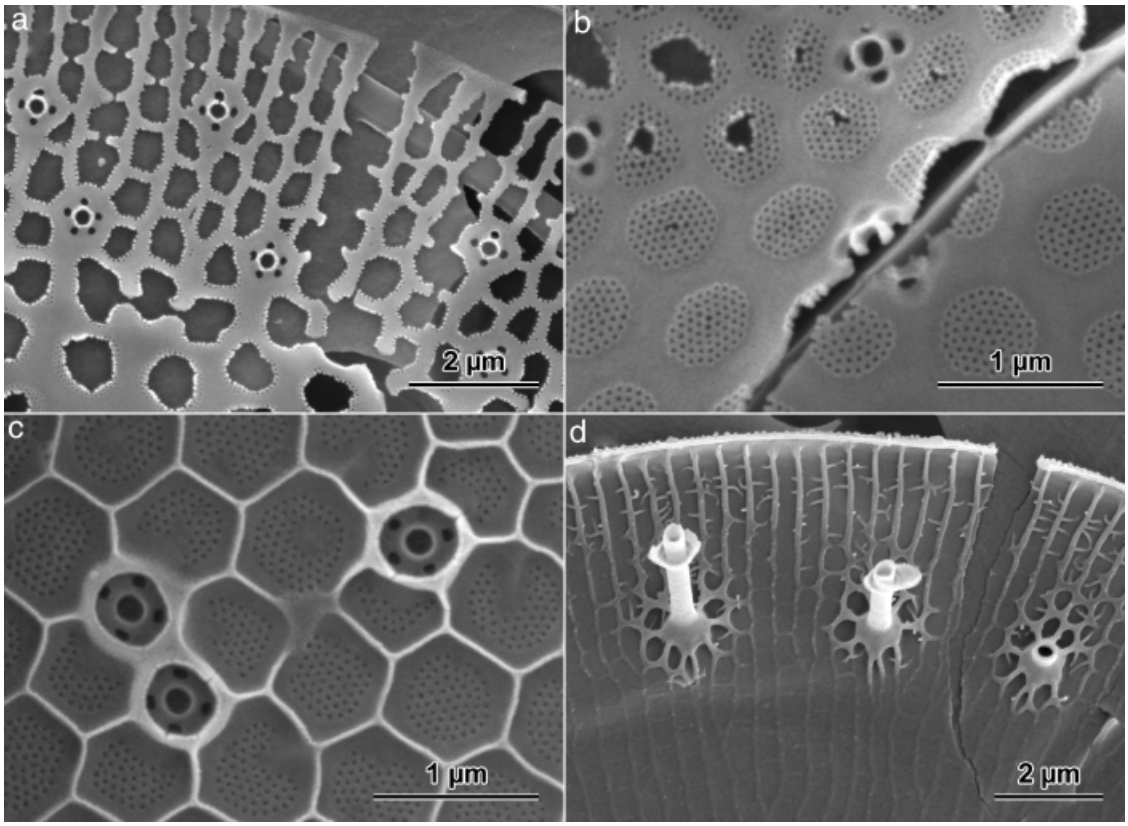

FIG. 2. Scanning electron microscopy showing development of a fultoportula *s.l.* (A) The early stage of fultoportula *s.s.* development in *T. hendeyi*; note separate perforations for the strutted tube and satellite pores. (B) Proximal ends of a completed fultoportula *s.s.* in the same species; note one portula split in half denominating the independent nature of the pores and the strutted tube. (C) Distal end of an older, completed fultoportula *s.s.* in *T. hendeyi* is seen underneath the developing base of the structure superimposed on it during the formation of areolae network. At this stage, the fultoportula that will develop into a fultoportula within a tube resembles a fultoportula within an areola. (D) A complete external tube of a fultoportula *s.l.* with a skirt in *Thalassiosira nordenskioeldii*.

tube (Fig. 3A) or areola (Fig. 3C). The narrower, strutted tube delimits the central pore of the fultoportula *s.s.* and extends above and below the base silica layer. The length and shape of this tube, on either side of the base silica layer vary depending on the species. In all diatoms examined here, the internal extension of the strutted tube (proximal opening) was longer than the external opening (distal opening, Figs. 3B, D). A variable number of satellite pores surrounds the strutted tube and their internal openings may be associated with a variety of minute structures (see some illustrations by Round et al. 1990 and Theriot and Serieyssol 1994 and Figs. 4A–C). There are also additional microstructures on the external tube of the fultoportula *s.l.*, which, along with the number of satellite pores, may either define species, groups of species, or even genera in the Thalassiosirales (Theriot and Serieyssol 1994). These additional variations do not alter the basic structure of the fultoportulae as being either one of two types, a fultoportula within an external tube (Figs. 3A, B and all the species investigated in this report), or fultoportula within an areola (Figs. 3C, D in *T. mala*). The external tube or the areola may be seen as a ''chitin-dispenser'' posed over the fultoportula *s.s.*

In all species examined in this study, satellite pores perforate the base silica separately from, outside of, and parallel to the strutted tube (as in Anonymous 1975, Fig. 34, Herth 1979, Fig. 3D, Theriot and Stoermer 1981, Fig. 17, Syvertsen and Hasle 1982, Schmid 1984). Pores open independently from the strutted tube on both sides of the base silica layer (Figs. 2A, B and 3A, C). The proximal openings of the strutted tubes and their satellite pores are relatively accessible to examination and are the familiar ''strutted processes'' of the internal surface of valve faces (Ramirez 1981, Round et al. 1990, Gleser et al. 1992). The opposite (distal) openings, the satellite pores, and the fultoportulae *s.s.* are rarely shown because they are obscured by the base of a wider external tube or are enclosed in a chamber of an areola produced later in valve development, and can be seen only when those external superstructures are, for whatever reason, absent (Figs. 2C and 3).

Considerable variation exists in the specifics of the internal endings of the fultoportula *s.s.* and the external tube of fultoportula *s.l.* These structures are frequently illustrated (Ramirez 1981, Round et al. 1990 and Figs. 2 and 4A). Their relative size, shape, number

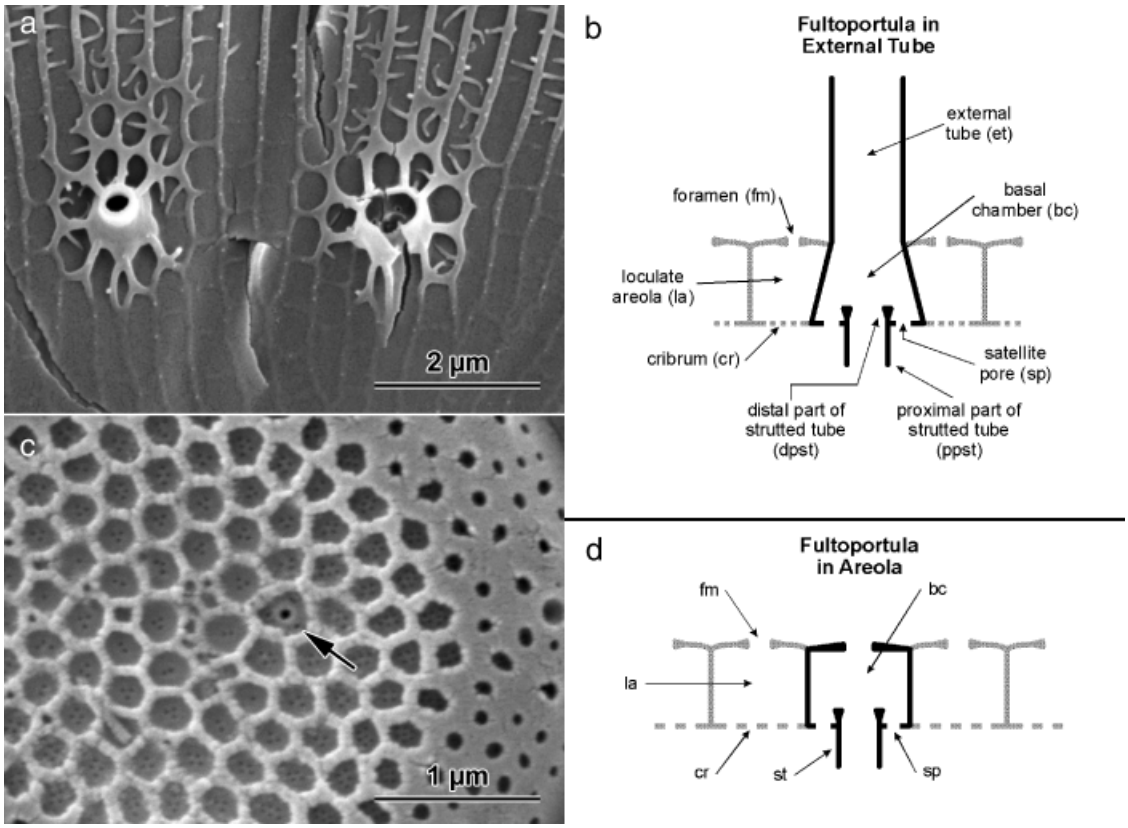

FIG. 3. Fultoportulae *s.s.* (A) Distal ends of two complete fultoportulae *s.s.* located underneath the base of an external tube; tubes are various stages of development in *T. nordenskioeldii* (SEM). (B) A diagrammatic representation of a long section of a fultoportula within an external tube, based on a simpler diagram from Syvertsen and Hasle (1982). (C) Distal ends of a completed fultoportula *s.s.* within a locular areola in *Thalassiosira mala*; note that when the foramens of the areolae are completed, those areolae that carry fultoportulae (arrow) are barely distinguishable from all others (SEM). (D) A diagrammatic representation of a long section of a fultoportula within an areola.

of satellite pores, and internal openings of satellite pores vary and are likely species specific or define groups of species, both extant and extinct (Figs. 4B, C). For example, all of the *Thalassiosira* spp. with long internal extensions of their fultoportulae also have rimoportulae away from the valve margin (Fryxell and Hasle 1979b). Less variation is associated with the fultoportulae *s.l.* within areola where the external foramen of the areola is often slightly larger than those of neighboring, regular areolae (Fig. 3C). A critical examination of the fultoportula structure of many additional thalassiosiroid species is obviously needed (Round et al. 1990, Theriot and Serieyssol 1994), but it is beyond the scope of this study. We ascertained the fultoportula structure for the species included in our molecular analysis.

Valve microarchitecture of selected taxa. Valve microstructures of selected related genera (*Ditylum* and *Mediopyxis*) are illustrated and summarized in Figs. 4D and 5 and Table 3. Phylogenetic significance of the siliceous valve face protrusions (spines, tubular, and hair-like projections and siliceous flaps) in these taxa and in thalassiosiroids (Fig. 4A) will be further discussed below in the context of their relationship with fultoportulae.

Kühn et al. (2006) have recently found a new diatom genus, *Mediopyxis*, in clade A (Figs. 5A–H). *Mediopyxis* contains marginal ridges and an assortment of tubes in its valve center: occluded processes, bilabiate processes with and without an external central tube, or microlabiate processes and stages intermediate between the bilabiates and microlabiates (Figs. 5C–H). *Mediopyxis* also carries a number of separate siliceous flaps and protrusions throughout the valve face (Fig. 5A). A variety of the valve face surface protuberances are also present in several other members of clade A, e.g. *Ditylum* (Fig. 4D), *Lithodesmium*, and *Lithodesmioides* (von Stosch 1986). The presence of all of these structures in a single diatom outside the Thalassiosirales has a bearing on the evolution of the group as discussed below, and illustrates the type of microstructural plasticity that the lineage still harbors.

Molecular phylogeny. Among the 43 strains included in the molecular analysis (Figs. 6 and 7), the amplified SSU rRNA fragment ranged in length from 1627 to 1810 nucleotides (nt), with much of the length variation because of the loss of small amounts of sequence at the 5′ or 3′ ends of the SSU rRNA gene. Most insertions and deletions were only 1–3 nt in length, except for one 9–12 nt insertion found in

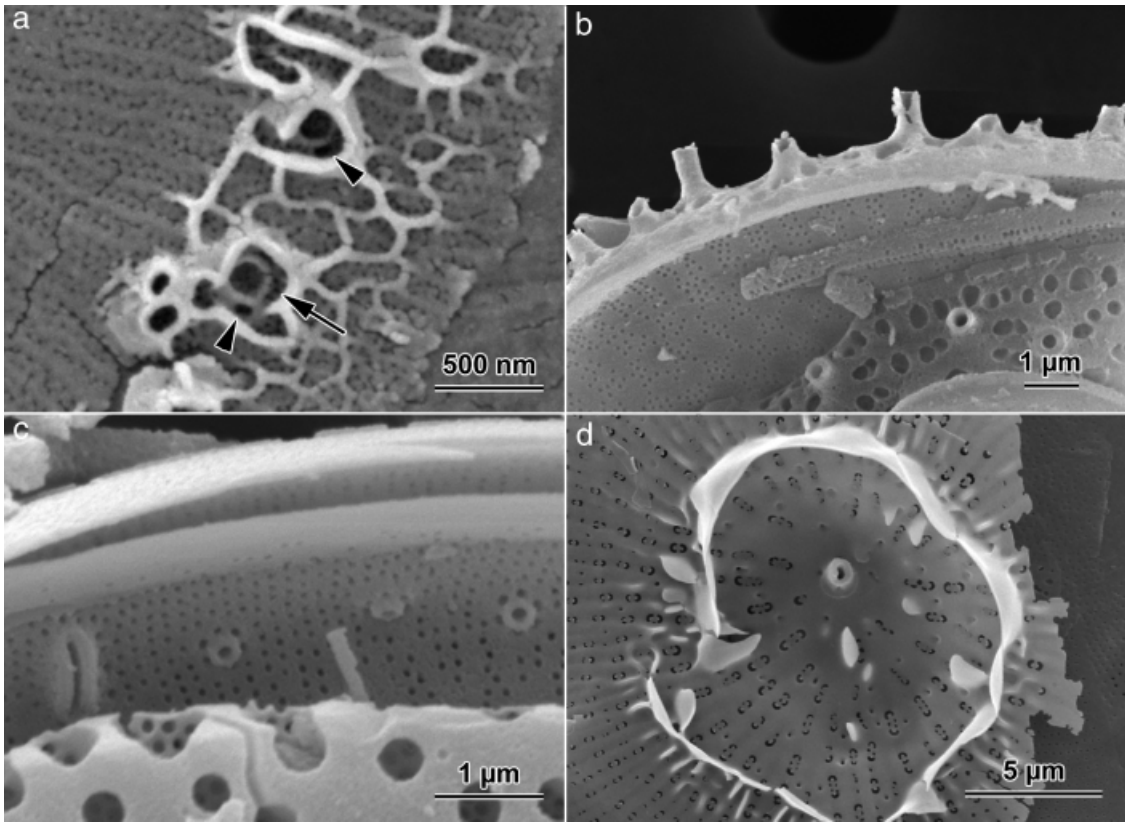

FIG. 4. Scanning electron microscopy showing (A) *Thalassiosira weissflogii* marginal rings of portulae. Note irregular cylinders and swirls enclosing groups of pores; note small pores (arrow) between the (larger) satellite pores (arrowheads). (B) A profile of external tubes of fultoportulae *s.l.* in an unnamed thalassiosiroid diatom from the Lower Oligocene. (C) Proximal endings of three fultoportulae *s.s.* and one rimoportula in the same extinct species shown in (B). (D) Fragmented marginal ridge and siliceous flaps in *Ditylum brightwellii*. Note fragments of the ridge and flaps semi-enclosing individual areolae.

just six of the strains. Two models were recovered in the model test analyses, with model two being only nearly significant, but values from each were incorporated into molecular analyses where appropriate and the resulting trees presented below as ML1, ML2, NJ1, or NJ2 (Fig. 6C–F, respectively).

TABLE 3. Summary of morphological features of genera from SSU rRNA sister clade to earliest divergences in Thalassiosirales.

	Non-fultoportulate genera				Fultoportulate genera	
	Ditylum brightwellii	*Lithodesmium undulatum*	*Streptotheca thamesis*	*Bellerochea malleus*	*Lauderia annulata*	*Porosira glacialis*
valve face perforation	radial rows areolae simple rota	radial rows areolae	scattered pores	radial mesh of open costa	radial costa irregular areolae	radial costa variable areolae
central structures	tube closed	tube open	scattered vein-like thickenings	annulus	annulus tubes	annulus
portula type	central bilabiate	central bilabiate	central bilabiate	central or marginal bilabiate	marginal labiate	Marginal labiate
marginal structures	variable ridge	continuous ridge	fimbriate ridge	delicate ridge	fultoportulae occluded processes	fultoportulae
copulae	numerous similar scale-like	few similar segments	half bands or segments similar	numerous similar scale-like	numerous similar open bands	numerous similar split bands
pervalvar axis	long cylinder	long cylinder	long cylinder	rectangular short cylinder	long cylinder	lens-shaped or short cylinder

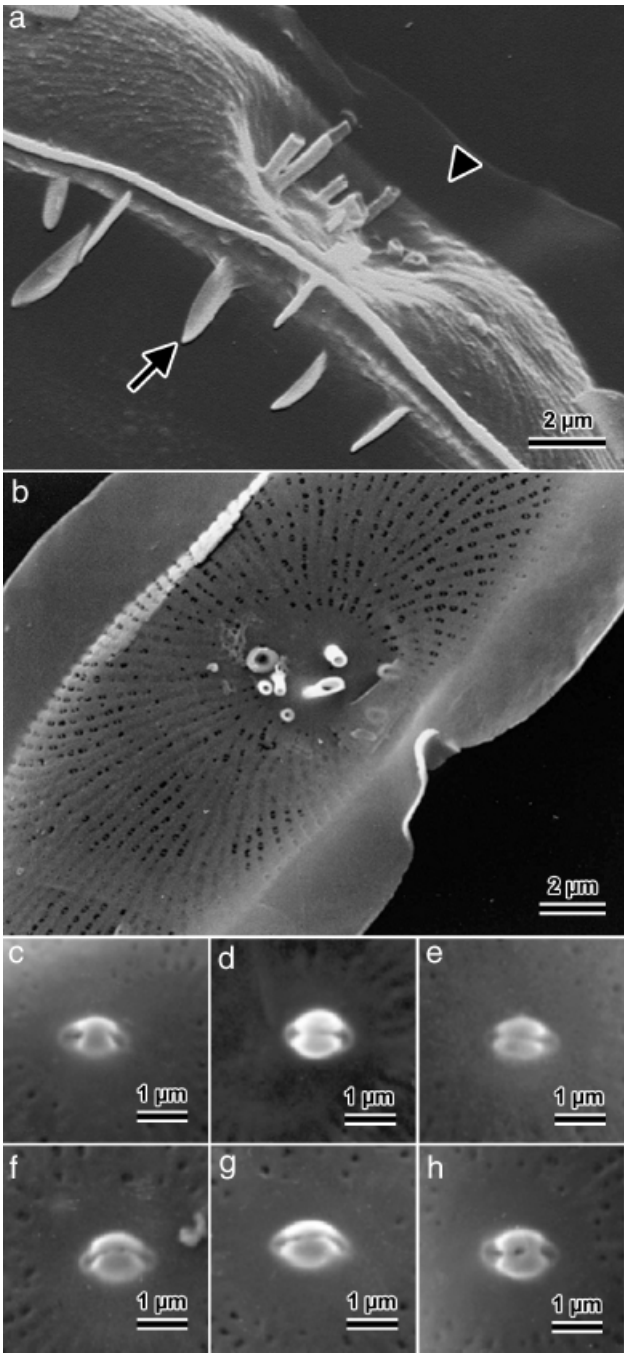

FIG. 5. Scanning electron microscopy showing external structure of the new member of clade A, *Mediopyxis* sp. (a,b). (A) Marginal ridges (arrowhead) and siliceous flaps (arrow). (B) Tube processes, with the external tube of the rimoportula more or less in the center. A range of the internal endings of rimoportulae (c–h) from a bilabiate (c, d) to microlabiate form (e–g) and a form with an interrupted distal part of the lip (h).

The topologies of the trees produced from all analyses were similar overall. *Lampriscus kittonii* was used to root the tree. In other analyses, it was a member of the clade that was the closest outgroup to the clade containing the Lithodesmiales and the Thalassiosirales (Medlin and Kaczmarska 2004). Five or six clades

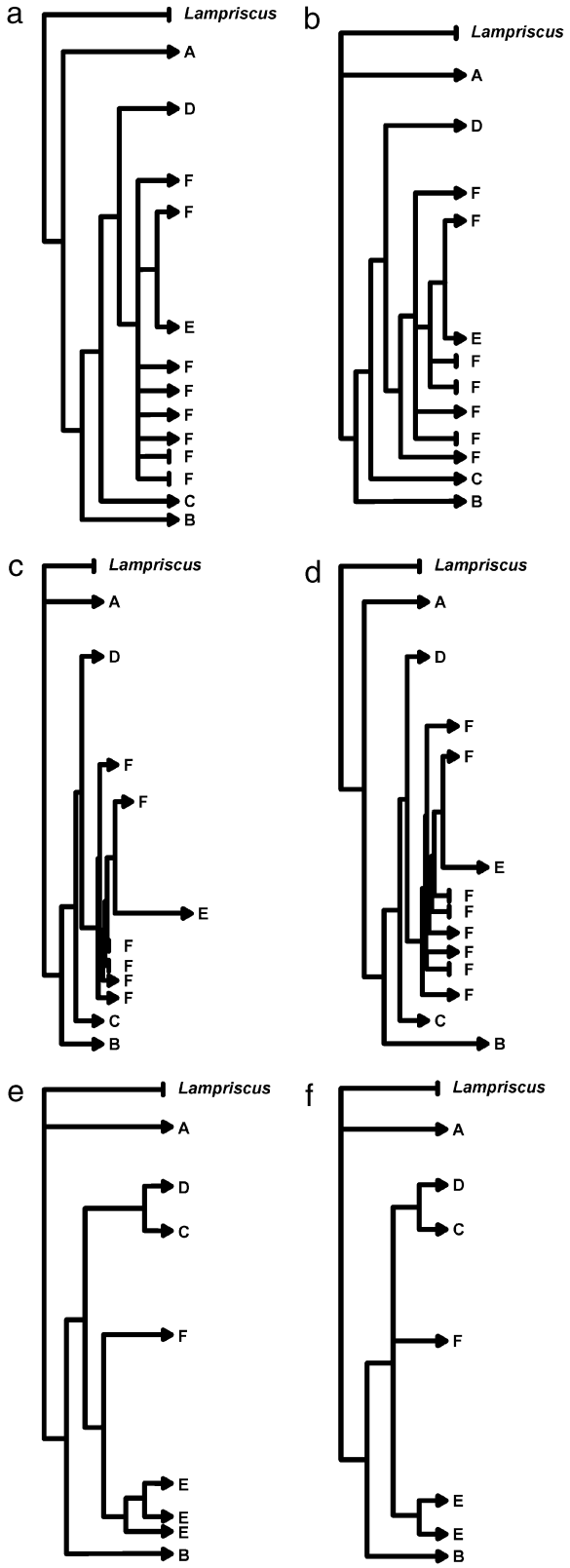

FIG. 6. Schematic representations of the trees recovered in each of the molecular analyses (A) BI; (B) MP; (C) ML1; (D) ML2; (E) NJ1; and (F) NJ2, to show differences in deep-branching topology of clades A–F. BI, Bayesian inference; MP, Maximum Parsimony; ML, Maximum Likelihood; NJ, Neighbor Joining.

were easily distinguished depending on the method used (Figs. 6A–F and 7). The basic difference between them was that clade E emerged from within clade F in the ML, MP, and BI analyses (Figs. 6 and 7), whereas in the NJ1 analysis, clades E and F were sister groups, and in NJ2 they were members of an unresolved polytomy including E, F, and C + D. The composition within each clade (or assemblage) was consistent for all analyses, and strong BT support and high PPs were obtained for all major clades in all analyses except for clade F (Fig. 7). We will use the term "clade/assemblage F" to indicate that in some analyses member taxa of this grouping form a polytomy and do not meet the strict criteria for clade status. In BI and ML2, the first clade to diverge was clade A followed by clade B and then C and D (Figs. 6 and 7). In the MP, ML1, and NJ1-2 analyses, clade A was not resolved from the outgroup species *Lampriscus*, but in analysis of all diatoms, *Lampriscus* is well separated from the members of clade A (Kooistra et al. 2003, Medlin and Kaczmarska 2004). The two remaining clades/assemblages (E and F) exhibited various branching orders and sister group relationships with one another depending on the algorithm and the model of evolution. In the ML analyses (Figs. 6C, D) using both models of evolution, clade D was sister to a clade composed of clade E and F taxa. Clade E containing all *Skeletonema* species was embedded in clade F among many strains of *Thalassiosira* species in all other analyses. The BI analysis had similar overall results except that the composite clade E and F was an unresolved polytomy (Figs. 6A and 7). The results of the NJ analyses using the two models of evolution show clade E and F as separate clades and sister to one another (Fig. 6E) or as an unresolved polytomy with clade (C + D) (Fig. 6F). The MP analysis (Fig. 6B) also presents clade E as emerging from within clade/assemblage F, which was itself sister to clade D as in the BI and ML trees. Bootstrap or PPs for these analyses (except for the ML analysis) can be found in detail on the BI tree in Fig. 7 (BI/MP/NJ1/NJ2). Each of the clades shown is strongly supported in all analyses, except for clade/assemblage F + E. In the NJ2 analysis, clades E and F were reduced to polytomy. However, in bootstrap analysis of this method, both E and F were highly supported at 100/100 (data not shown) and 87/98, respectively.

We will use the full-detail BI tree (Fig. 7) to designate major clades within the thalassiosiroid lineage and to discuss the relationships between different groups of taxa. The BI is currently the most robust analysis available. Clade A was the first to diverge and was strongly supported in all methods. It includes members of orders Lithodesmiales (*Ditylum brightwellii, Lithodesmium undulatum*) and Hemiaulales (*Bellerochea malleus* and *Streptotheca* [=*Helicotheca*] *thamesis* [Ricard 1987, Round et al. 1990]). Lithodesmiales, as presently defined, is paraphyletic in all of our trees. All members of this clade lack fultoportulae but share other valve characters that support their clustering as a natural grouping (Table 3). Interestingly, clade A also exhibited an unusual bias in frequency of transition substitutions. C to T and T to C substitutions in this clade occurred two to three times more frequently compared with the alternative transition changes (A to G and G to A). In contrast to lithodesmioid diatoms, the rates of the two transition types (C/T and A/G) were more homogenous in the five fultoportulate clades.

All members of the remaining five clades (B–F) possess fultoportulae within an external tube (*Lauderia*, *Porosira*, *Thalassiosira*, *Stephanodiscus*, *Cyclotella*, *Planktoniella*, *Minidiscus*, and *Skeletonema)*. Among fultoportulate diatoms, clade B, with 100% BT and 99% PP support (Fig. 7), includes two species, each from a different family (based on the classification proposed by Round et al. 1990 and Nikolaev et al. 2001). *Lauderia annulata* represents the sole genus of the family Lauderiaceae, whereas *Porosira glacialis* is a member of the much larger family Thalassiosiraceae, several genera of which are present in other clades (Fig. 7). These members of the two genera differ from all other diatoms included in our analysis by having fultoportulae scattered throughout the valve (except for the very center of the valve face), and radial interstriae between the rows of pores in the basal silica layer.

Two small brackish species, *Thalassiosira weissflogii* and *T. fluviatilis*, both members of the family Thalassiosiraceae, combined to form the small, strongly supported clade C in all analyses (Fig. 7). Among clade C taxa, the basal silica layer also shows radial interstriae and rows of small pores, as in species of clade B. Similarly, these taxa do not have portulae in the very center of the valve face. We have kept *T. fluviatilis* and *T. weissflogii* as distinct species even though they have been synonomized by Fryxell and Hasle (1977) because we feel that the amount of variation between the two strains (eight nucleotide substitutions) is too great for one species. Based on the sequencing of many strains of a single species, one to four base substitutions within a single species are acceptable, depending on their location in the secondary structure model of the rRNA (L. K. Medlin, unpublished results). Five or more base substitutions provide evidence for a species complex, which should be investigated with more variable gene coding or non-coding regions.

Clade D includes a distinct and tight group of taxa (Fig. 7) with two well-supported sub-clades in all analyses. Within each subgroup, fewer than 15 substitutions among strain pairs were found. *Cyclotella meneghiniana* in the family Stephanodiscaceae likely represents a species complex because the strains sequenced here range from freshwater to marine habitats. A more in-depth analysis of this species has been presented elsewhere (Beszteri et al. 2005). The second sub-clade included species with similar, pseudoloculate areolae, *Detonula confervacea* (family Skeletonemaceae), *Thalassiosira oceanica*, and two of the three strains of *T. pseudonana* (both members of the family Thalassiosiraceae) sequenced for this study.

Clade E contains all *Skeletonema* species (family Skeletonemaceae) from the present analysis. This clade was

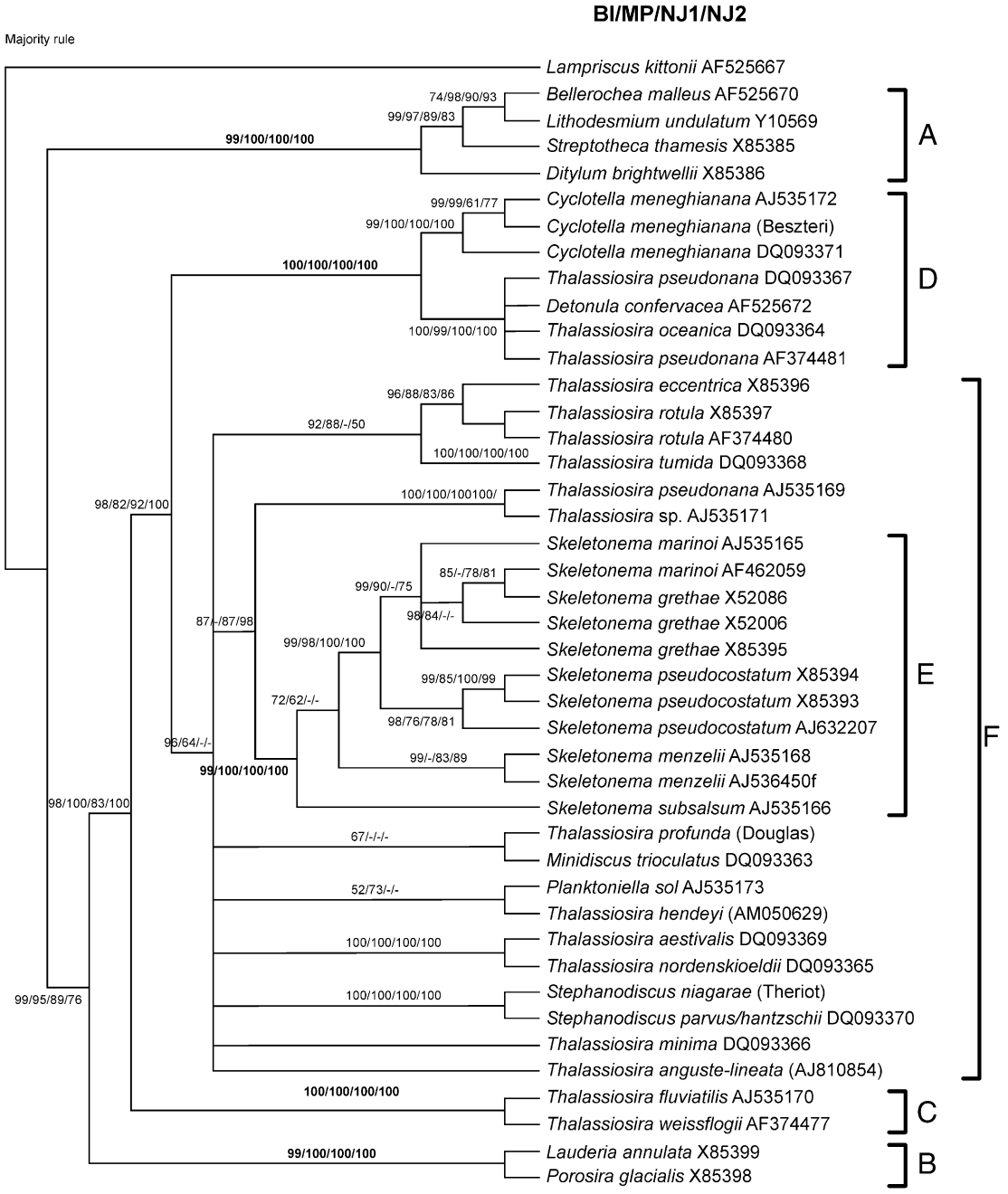

FIG. 7. Fifty percent majority rule Bayesian analysis tree based on 900 phylogenetic tress. Posterior probabilities and bootstrap values above 50 are shown on the nodes that were recovered in each of the molecular analyses (BI/MP/NJ1/NJ2). BI, Bayesian inference; MP, Maximum Parsimony; NJ, Neighbor Joining.

well supported in all analyses (Fig. 7), grouping only members of *Skeletonema*. It confirms the monophyletic origin of this genus but not the family to which it belongs (Round et al. 1990, Table 1) because *Detonula confervacea* was found in clade D. Clade E either arises from within assemblage F (Figs. 6A–D) or is sister to clade F (Figs. 6E, F). *Skeletonema subsalsum* rooted clade E in BI and ML analyses (Figs. 6C, D and 7). Two strains of *Skeletonema menzelii*, one a coastal/estuarine strain and the other an open ocean strain, are very different in SSU rRNA sequence composition and likely represent different species. An in-depth study of global *Skeletonema* strains show that none of the living species named as *Skeletonema costatum* corresponds to the type material of *S. costatum*, and that the genetic diversity of the strains is so extensive worldwide that four new species have been described (Sarno et al. 2005, Zingone et al. 2005).

Clade/assemblage F encompassed 16 strains representing 15 species from four genera. Support for the assemblage F was 96/64% in the BI and MP analysis when it included clade E (Figs. 6A, B and 7) and 78/87% when it was a monophyletic clade in the NJ1 and NJ2 analyses (Figs. 6E, F). This assemblage includes mainly members of the family Thalassiosiraceae (*Thalassiosira*, *Planktoniella*, and *Minidiscus*). Unexpectedly, freshwater members of the family Stephanodiscaceae (Gleser et al. 1988, Round et al. 1990, Nikolaev et al. 2001), *Stephanodiscus* "*hantzschii/parvus*" and *S. niagarae*, were also included in the assemblage as one of the unresolved polytomies in BI and NJ trees (Figs. 6A, E, F and 7). Marine species sequenced in this clade/assemblage represent a wide range of morphologies and ecological affinities. The two lineate species in our data set, *T. hendeyi* and *T. anguste-lineata* (differing by 44 nt), do not cluster together. Extrapolating from this, we also predict that the species possessing longer fultoportula extensions do not represent a natural group. The generitype, *T. nordenskioeldii* and *T. aestivalis*, clustered together in clade/assemblage F as did *T. rotula*, *T. tumida*, and *T. eccentrica* with strong to moderate support. *T. pseudonana* (one of three strains sequenced that were given this name) and *Thalassiosira* sp. formed a strongly supported cluster but *T. profunda* and *Minidiscus trioculatus* weakly cluster together. The organization within clade/assemblage F likely reflects the fact that there are very few base changes defining this node and more variable regions of other genes may be required to resolve relationships among these taxa. We must also begin to search for other characters, organismal and/or ecological, that link these taxa together. The overall position of the clade (or assemblage) F in all trees was among later emergences. It was either sister to clade E (NJ1; Fig. 6E) or to clade D with clade E emerging from within F (BI, MP, ML1, ML2; Figs. 6A–D) or part of a polytomy with clades D + C and E (NJ2; Fig. 6F). Clade/assemblage F was never directly associated with clades C or B.

Finally, all trees indicate that some culture and strain identifications require critical re-evaluation. For example, the three strains of *T. pseudonana* included in our analyses never clustered as a single unit. Two strains falling in clade D were more similar to each other (five-nucleotide substitution), whereas the one in clade/assemblage F differed from these strains by 79 and 84 nt. Of the two strains of *T. oceanica*, one grouped together in the clade D species, whereas the other was found in another clade (data not shown because its identity could not be verified). Sufficient variation was found between *T. weissflogii* and *T. fluviatilis* to suggest that these taxa may not be synonymous.

DISCUSSION

Although the emergence of the fultoportula is central to the evolution of thalassiosiroid diatoms and the process is a defining character of the lineage, the sequence of events leading to its formation is not known either from the fossil record or ontogenetically. It appears quite suddenly in the Miocene with no known precursors (Hasle 1985). Thus, the question of how the fultoportula evolved and which diatom lineage(s) are the closest relatives of the Thalassiosirales remains open. We used nuclear SSU rRNA sequence comparisons to address this question. The approach to phylogenetic inference adopted here is based primarily on molecular data. Using this morphology-independent indicator of relatedness, we then focused our attention on the structures present in closely related taxa and examined their characteristics in search of previously unappreciated features supporting similarities in SSU sequences. In extant, closely related species included in this analysis, we identified characters whose simple modification could illustrate the origin of the fultoportula.

Sister Clades: Lithodesmioids and Thalassiosiroids. The SSU rRNA analysis has consistently recovered thalassiosiroids as a sister clade to lithodesmioid species (Medlin et al. 1993, 1996a, b, 2000, Kooistra et al. 2003, Medlin and Kaczmarska 2004), although one recent report suggests that *Odontella* may also be included in this group (Sinninghe-Damsté et al. 2004). A sister relationship between lithodesmioids and thalassiosiroids is also supported by analysis of mitochondrial cytochrome c oxidase subunit I (*cox*I) sequence similarities (Ehara et al. 2000). In analyses based on plastid gene sequences, thalassiosiroid diatoms root the bi(multi)polar lineage but diverge separately, immediately preceding lithodesmioids (Medlin and Kaczmarska 2004). Therefore, all these analyses indicate that both lineages are closely related.

Morphological analysis of the two SSU rRNA sister clade members revealed more similarities than anticipated (Table 3). The overall valve shape among the lithodesmioids ranges from elongated to bi- or quadriangular, but in some species circular or ellipsoidal centers are superimposed on these more complex outlines (Round et al. 1990). All of the non-fultoportulate extant diatoms of this lineage possess an unusual bilabiate process (von Stosch 1977, Hargraves 1984). Such a process is not known outside Lithodesmiaceae and Bellerochaceae (von Stosch 1977, Round et. al 1990), supporting the molecular grouping of these diatoms as a natural clade, even though they are currently placed in separate orders (Round et al. 1990).

Valve face micro-architecture in lithodesmioid *Bellerochea–Lithodesmium* and *Lauderia–Porosira* rooting thalassiosiroids is also quite similar. All show radially organized rows of perforations and interstriae expanding from the valve center. The valve face center is occupied by an annulus, a portula, or both (compare *Bellerochea*, *Lithodesmium*, *Porosira*, and *Lauderia* in von Stosch 1977, 1986, Syvertsen and Hasle 1982, Syvertsen and Lange 1990). All of these genera carry a ring of specialized structures along the margin of the valve face, ridges in lithodesmioids, and portulae in thalassiosiroids, which are involved in linking cells of a colony. The labiate processes in both groups are known to occur in either a central or lateral position. However,

the majority of lithodesmioids carry labiate processes at the valve face center, while most thalassiosiroids bear them at the valve margin. The exceptions include *Bellerochea malleus*, two species of the genus *Neostreptotheca*, all species of the genus *Minidiscus*, and some species of *Skeletonema*. It suggests that the position of the rimoportula in the common ancestor of these lineages may have been variable or intermediate between the margin and valve center.

In light of the structural and molecular similarities outlined above and summarized in Table 3, we speculate that the ancestral stock of fultoportulate diatoms may have carried a combination of features now present in clade A and B species. It might have resembled these diatoms in cell proportion (elongated cylinder with numerous, scale-like copulae), in valve face ornamentation (interstriae radiating from an annulus with rays of small pores between them), and valve face architecture (carried ridges or corona of fimbria located off valve center). The position of the rimoportula in the common ancestor of these lineages may have not yet been fixed. Our putative ancestor differs from the ancestral thalassiosiroid proposed earlier (Makarova 1981, Hasle and Syvertsen 1985).

Origin of the fultoportula: morphological considerations. Logically expanding this hypothesis, we propose that fultoportulae may have evolved from a ring of fragmented projections: *the tubular origin of the fultoportula*. The fimbria or siliceous flaps would loop and fuse around a cluster of pores along the valve face periphery, possibly similar to those that now can be seen in *Ditylum* (Round et al. 1990, p. 293, Fig. g), or in *Thalassiosira weissflogii* (Fig. 4A) to produce a corona of more or less regular cylinders/tubes along the valve face margin. This essential step in fultoportula evolution would set apart clusters of pores from all other valve perforations, thereby allowing some of them to specialize for novel functions, e.g. developing stronger control over the accumulation and direction of cell exudates. This could involve differentiation in the pore number, size, and arrangement within the cylindrical enclosure, or a pre-fultoportula *s.l.* Initially, cylinders remained relatively simple (open, e.g. some occluded processes in *Lauderia*, or wide-open fultoportula in *Minidiscus*). Then, cylinder walls grew in height, diminished in diameter, and developed spines, skirts, etc. The cylinder base integrated into a network of areolae to become a fultoportula *s.s.* within a tube, or fultoportula *s.l.* Such a fultoportula eventually also transferred to a central position on the valve face. Some of the forms similar to hypothesized intermediate stages in the evolution of the fultoportula within the external tube might have resembled an occluded process with a perforated velum currently seen in *Lauderia annulata* (=*L. borealis,* Hasle 1973b).

Syvertsen and Hasle (1982) explicitly, but not forcefully, considered the possibility of a phylogenetic relationship between occluded processes and fultoportulae, and documented five different types of portulae. A set of sequential steps illustrating the evolution of the fultoportula from an occluded process is shown in Syvertsen and Hasle (1982, their text Figs. 6A–D) but the origin of the occluded process itself was not addressed. It is significant that genera with occluded processes lie at the base of the Thalassiosirales in our rRNA tree and that occluded processes are present in *Mediopyxis*, a member of a sister clade to that order. We note that both the occluded and strutted processes retained a colony-linking function, which may be inherited from ancestors common to lithodesmioids and thalassiosiroids. "Occluded processes" without an external tube are infrequent, and their number/presence on a valve can be variable in both *Mediopyxis* and in *Lauderia* (Syvertsen and Hasle 1982).

In an alternative scenario, *the areolar origin of the fultoportula*, it is conceivable that the fultoportula *s.s.* evolved through a transformation of a loculate areola with internal cribra (Syvertsen and Hasle 1982, Round and Crawford 1984) initially into a fultoportula within an areola. Here as well, the reduction in the number of pores in a cribrum and a selective change in the pore size could lead to a perforation pattern seen in present-day fultoportulae. Then, an upward extension of the areola side-walls could secondarily produce a fultoportula within an external tube. Initially, a single centrally located areola and then a ring of the areolae underwent such changes. This emergence of marginal fultoportulae might have been under the control of a mechanism dependent on a signal(s) gradient diffusing from the center of the valve. Concentration (diffusion)-dependent developmental control is known in a diverse group of organisms and could be postulated here as well (Driever and Nüsslein-Volhard 1988, Schaller et al. 1989, Wolpert 1989). *Thalassiosira mala*, the species that possesses all fultoportulae within the areola and without an external tube, is such a species and should be targeted for future molecular examination as soon as it is available in culture.

Although no extant member of the Lithodesmiales possesses loculate areolae and they are absent among the clade B and C species rooting the Thalassiosirales, both of which bear pseudoloculate or poroid areolae, the presence of an ancestor with such valve micro-architecture cannot be disregarded. Loculate areolae are already present in the Lower Cretaceous diatoms. Loculate areolae patterning may be simulated in a simple model by repeated phase separations during valve formation (Sumper 2002). The simplicity of such patterning may explain the presence of seemingly complex, loculate areolae among the earliest diatoms and their re-appearance in several younger distantly related lineages.

A third scenario is also possible. The central strutted process could be derived from the annular process (Medlin et al. 2000), which we call here *annular origin of the fultoportula*, whereas the marginal strutted processes could be derived from the marginal fringe. The homology of the central and marginal fultoportula has not been fully substantiated. The overall structural sim-

ilarity is indeed strong, but subtle differences between the marginal and central processes are not uncommon. The presence of a range of variation in the appearance of the central structure of the new diatom genus shown in results provides support for the third scenario. Here, the external tube of the bilabiate process in modern Lithodesmiales may be derived from the central tube of the fossil genus *Archepyrgus*. The structures in Figs. 5C–G have been arranged to show a possible evolutionary sequence to derive a microlabiate process from the bilabiate process. Considering this sequence (Figs. 5E–H), the microlabiate process could be evolving into a central strutted process, possibly through an intermediate stage involving an occluded process. At this time, we have no indication of how the occluded processes seen in *Mediopyxis* (Figs. 5A, B) could have been formed. We also do not know what the central, external tube in the bilabiate process represents. The displacement and loss of the central microlabiate process could accompany the third scenario. Hypothetical relationships between the annular process and fultoportula have already been considered earlier (Hasle and Syvertsen 1985, Medlin and Kaczmarska 2004, see also discussion in the next section), and further details are advanced by Kühn et al. (2006).

None of the three hypotheses (tubular, areolar, or annular origin of the fultoportula) can be fully supported or refuted at this time. However, the first hypothesis explains more similarities between the two seemingly very different types of diatoms, lithodesmioids and thalassiosiroids, than do the other two. First, the most frequent position of fultoportulae is at the valve face margin, where the ridges are now present in non-fultoportulate lithodesmioids and some hemiauloids. Second, the marginal ridges in these species, the occluded processes in *Lauderia*, and fultoportulae in the majority of thalassiosiroids examined here serve a similar function, which is to facilitate linking cells into colonies. Third, the structures similar to forms hypothesized as transition from ridges to fultoportulae may be seen in the extant thalassiosiroid species in nature and in culture. Fourth, the marginal fultoportulae are present in nearly all species, whereas the central fultoportulae are less frequent (including diatoms rooting thalassiosiroids), suggesting that the central location is secondarily derived. We also observe that the occluded processes are most frequent at the margin of the valve face except for *Mediopyxis*. The areolar hypothesis of fultoportula origin necessitates the simplest of the modifications to existing structures. However, it is difficult to explain the mechanisms that selected the areola(e) evolving into the fultoportula from all the others in order to proceed with this evolutionary transformation. The third hypothesis, postulating the independent origin of central and marginal fultoportulae, gains support with the finding of the new genus *Mediopyxis*. This scenario (annular origin of the fultoportula) basically deals with the origin of the central processes (annular, bilabiate, or strutted) and assumes that the marginal processes could be formed either by scenario one or two. Thus, one has to determine first and foremost whether or not the central and marginal processes are homologous. Here, a study of the morphogenesis of *Mediopyxis* will be informative.

Fultoportula evolution: fossil considerations. The fossil record provides no evidence of fultoportula emergence nor does it indicate an obvious pre-fultoportulate ancestor. The earliest known fultoportulae documented by SEM come from Miocene sediments (Hasle 1985, Gleser et al. 1988). Miocene fultoportulae are already fully developed and present in both marginal and in central positions. A report of fultoportulate species in sediments earlier than the Miocene, based on LM examination (Gleser et al. 1988), awaits verification by electron microscopy because fultoportulae in *T. eccentrica* are unresolvable in LM. Re-examination of archival SEM-based documentation of *Thalassiosira* cf. *baltica* (Kaczmarska 1982, pl II, Fig. 15) from the older, Lower Oligocene Polish Karpathian Flysch (approximately 35 Myr) shows two to three rings of marginal fultoportulae (Figs. 4B, C) and the absence of the central fultoportula *s.l.* in a likely new diatom genus (Kaczmarska, unpublished results).

An annular process has been considered a precursor of the modern strutted process (Hasle and Syvertsen 1985), although the fossil evidence in support of this relationship is still absent. The annular process is a tubular invagination of the inner siliceous layer into the cell interior. The process wall is perforated by lateral slits opening into the tube lumen. Externally, this process opens into a chamber with one foramen or a spine-like tube (Sims 1994, Nikolaev and Harwood 1997). This process, initially known only from Paleocene *Thalassiosiropsis wittiana* (a diatom with a nearly linear pattern of areolae, Hasle and Syvertsen 1985, approximately 55 Myr, Sims 1994), is now known in three other more ancient diatoms. *Gladiopsis ellipsoidea*, *G. speciosa* (Sims 1994), and *G. modica* (Nikolaev and Harwood 1997), known from the Lower Cretaceous (Aptian/Albian, approximately 110 Myr, Nikolaev and Harwood 1997), also carry a central annular process. Deriving the fultoportula from the annular process would only require a relatively simple topological transformation: moving the tube slits onto the valve face and transforming them into pores, or losing slits altogether to form an occluded process, as suggested in scenario 3 above. In this scenario, the central fultoportula in Thalassiosirales would be a homologue of a rimoportula in Lithodesmiales and other members of Mediophyceae (Medlin et al. 1996a) or has replaced it. If thalassiosiroid diatoms emerged from such a lineage, their rimoportula would have to be reacquired. It thus might be that the annular process represents an ancient, now extinct type of process and evolutionary dead-end, similar to several other unusual Cretaceous and Paleocene processes. Nonetheless, a more in-depth study of fultoportulae is called for in order to clarify the homologous nature of the central and marginal processes.

Fultoportulae vs. molecular segregation. Our molecular data concur with the morphological delineation of the order Thalassiosirales, as a monophyletic fultoportula-bearing group of diatoms (Hasle 1973a, b, Gleser et al. 1988, Round et al. 1990). However, unexpectedly, SSU rRNA-based clades B–F within the Thalassiosirales do not correspond well with characters used to circumscribe several presently recognized families, genera, and intra-generic groupings of species (Hasle 1973b, Hasle and Fryxell 1977b, Fryxell and Hasle 1979a, b, Gleser et al. 1988, Round et al. 1990, Nikolaev et al. 2001). There is considerable variation in the design of the internal (proximal) exits of the fultoportula *s.s.*, and in the structure of the external superstructure of the fultoportula *s.l.* These variations do not immediately correlate with either the molecular clades B–E or the present generic definitions, clearly indicating that more work is needed in this area before new genera are circumscribed (or old ones are re-defined) from these paraphyletic groups. For example, the generitype *T. nordenskioeldii* is embedded within a large, weakly supported cluster of other *Thalassiosira*, *Minidiscus*, *Planktoniella*, and *Stephanodiscus* species. The molecular analyses confirm that the genus *Skeletonema* is monophyletic but apparently not closely related to *Detonula confervacea* with which it shares a colony-linking function of the marginal fultoportulae. *Porosira* clusters strongly with *Lauderia*, each presently in different families, Thalassiosiraceae and Lauderiaceae, respectively. New phylogenetically informative characters should be sought among these clades.

In this initial review, a few associations emerge when the number and the distribution of the fultoportulae are considered in light of the molecular relationships between the species examined here. We emphasize that the trends presented below should be regarded as tentative because evolutionary relatedness between clades C, D, E, and F is only weakly supported by most of our analyses. The number of fultoportulae appears to decrease in the more derived clades. Similarly, the colony-linking method changes in species that are colonial. The earliest divergences in Thalassiosirales produce weakly joined colonies. Stronger chitinous threads connecting sibling valves via central fultoportulae (chain-forming thalassiosiroids), fused, or intertwining of the external tube endings (skeletonemoid species) are present in more derived members.

Members of the earliest divergence within the Thalassiosiroid lineage in our molecular trees (clade B) exhibit morphological similarities: patterns of perforation of the base silica layer, radial interstriae, structure of the labiate process, numerous cingulae, and a prominent central annulus (Table 3). They both carry a large number of the fultoportulae dispersed throughout most of the valve face with no apparent pattern other than being present in greatest concentration around the valve margin and absent at the valve center. These fultoportulae are thought to extrude a mass of an "unknown resin" holding cells into colonies, solely in *Porosira* (Joergensen 1905, Gran 1908, Hasle 1973b, Syvertsen and Lange 1990), but with reinforcement from the occluded processes in *Lauderia* (Syvertsen and Hasle 1982).

The remaining clades possess fewer fultoportulae concentrated in discrete regions of the valve. A small cluster of two species with two rings of fultoportulae, marginal and sub-central, may represent an intermediate stage of the reduction of fultoportulae number. Faint radial interstriae patterning of the basal silica layer, a prominent rimoportula, and pseudolocular areolae are characters shared with sequenced members of clade B and with some members of clade D: *T. pseudonana*, *T. oceanica*, and *Detonula confervacea*. In *Cyclotella meneghiniana*, basal silica layer patterning is also radial, although the interstriae are restricted to the marginal area of the valve face and the rimoportula is small. Most of the species clustered in clades D and F carry one or just a few fultoportulae outside the valve periphery (except *T. tumida*, *T. rotula*, and *T. angustelineata*).

Representatives of the genus *Skeletonema* (clade E) also possess very few fultoportulae when compared with most of the species examined here. They lack central fultoportulae altogether, but the marginal portulae participate in a complex and strong colony-linking system. The ends of the external tubes are dilated horizontally and are often fused between the sibling valves to link cells, somewhat similar to that of *Detonula confervacea* (clade D).

Members of clades D and F are the most morphologically diverse with respect to colony formation. Clade D and F species may be solitary, form colonies in a manner reminiscent of *Skeletonema*, or may form aggregates rather than colonies, by entangling cells into a mat of fibrous extrusions from the marginal fultoportulae (e.g. *Cyclotella*, Herth and Zugenmaier 1977). Many species from clade/assemblage F also use threads extruded from the (near) central fultoportulae to string cells into a filamentous colony, with the threads from the central fultoportula of one cell continuing to the fultoportula of the sibling valve. However, we note that *Stephanodiscus* (if colonial) and *Planktoniella* do not form colonies in this way.

In conclusion, we present results in support of the need for revision of the order Thalassiosirales, but the formal revision of this speciose order requires more in-depth analyses of a greater number of species using more variable gene regions. Nonetheless, all our sequence analyses demonstrate the paraphyletic nature of the order Lithodesmiales, families Thalassiosiraceae, Skeletonemaceae, and Stephanodiscaceae, and the genus *Thalassiosira*, as defined presently (Gleser and Makarova 1986, Gleser et al. 1988, Round et al. 1990, Gleser et al. 1992, Nikolaev et al. 2001). Members of Thalassiosiraceae and *Thalassiosira* are present in three different clades. Our results indicate that some species of *Thalassiosira* may be closer relatives of *Stephanodiscus*, whereas others are more closely related to some species presently assigned to the genus *Cyclotella*

(both from the family Stephanodiscaceae), as compared with other species of the genus *Thalassiosira*. Strains from two of the six currently recognized genera of the family Stephanodiscaceae (*Cyclotella* and *Stephanodiscus*) are included in the analysis and are members of two divergent clades (D and F). Similar relationships were also obtained by Alverson and Theriot (2002) and Sinninghe Damsté et al. (2004).

TAXONOMIC REVISIONS

Order Lithodesmiales (Round and Crawford in Round et al. 1990) Kaczmarska and Medlin, comb. nov.

Diagnosis. Valves with a bilabiate portula. Culture variations also include interruption, partitioning the two lips or a microlabiate. Occluded processes may be present. Valve face periphery equipped with specialized structures, ridges, fimbria, or fimbriate thickenings.

Extant genera included are *Mediopyxis*, *Lithodesmium*, *Ditylum*, *Bellerochea*, and *Streptotheca*, and probably *Lithodesmioides* and *Neostreptotheca* when their sequencing becomes possible.

Members of the SSU rRNA clade A present an amazing diversity of valve form and perforation patterns, suggesting a considerable antiquity of this lineage. *Mediopyxis* (Fig. 5) expands the range of morphological diversity still further, and we include descriptions in our emendment to accommodate this new genus. Four genera studied here (Fig. 7, Table 3) are presently included in two orders: the Lithodesmiales and Hemiaulales (Round et al. 1990). Despite the differences in valve form and ornamentation pattern, they do share two distinct characteristics: the marginal ridges (or thickenings) and unusual, bilabiate portulae (von Stosch 1977, 1986, Hargraves 1984, Round et al. 1990), supporting their clustering as a natural grouping. Such an association was already suggested although at the level of the family Lithodesmiaceae erected by H. & M. Peragallo (Simonsen 1979). With both a central process and peripheral colony-linking apparatus, members of this order combine some features of both Coscinodicophyceae (Clade 1) and Mediphyceae (Clade 2a). Their basal position in several phylogenetic trees published earlier (Medlin et al. 1996b, 2000, Ehara et al. 2000, Medlin and Kaczmarska 2004) is supported by such intermediate frustule morphology.

Family Lauderiaceae (Schütt) emended Medlin and Kaczmarska

Description. Valve faces fairly flat, gently curving into low not well-marked mantles, valves with a central annulus. Fultoportulae numerous, dispersed over the face but concentrated at the margins, central fultoportula absent. Single, large, widely open rimoportula mounted on short neck oriented perpendicular to the valve margin. Similar cingulae, each perforated with several rows of areolae.

Extant genera include: *Lauderia* and *Porosira*.

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