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In honour of
Professor T. V. Desikachary
on the occasion of
his seventy-fifth birthday

Evolution of the diatoms (Bacillariophyta): III. Molecular evidence for the origin of the Thalassiosirales

by

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With 5 figures and 1 table

Abstract. The Thalassiosirales are the only diatoms that possess strutted processes, specialized tubes through which chitan threads are extruded for chain formation, flotation, and protection from grazers. Comparisons of nuclear-encoded small-subunit ribosomal RNA sequences with maximum likelihood, weighted maximum parsimony and distance methods place *Streptotheca* and *Ditylum* as the taxa most closely related to the Thalassiosirales. This lineage is sister taxon to the pennate diatoms. One major clade of diatoms comprises the Thalassiosirales, the pennate diatoms, and the bipolar centrics with a central labiate process. A second major clade contains radial centric diatoms with peripheral rings of labiate processes. Alternative phylogenies reflecting current diatom systematics were constructed but were significantly worse when tested against the fitness of the best trees found with the maximum likelihood and maximum parsimony analyses. Cell wall structure of fossil taxa from Ocean Drilling Program (ODP) Leg 113, Site 693, Antarctica provides better morphological support for our molecular tree than cell wall structure of modern taxa. The presence of a central tube-like structure in the silica cell wall in the fossil taxa from this Lower Cretaceous deposit appears to be correlated with the clade in the molecular tree containing the bipolar centrics, the Thalassiosirales and the pennate diatoms. The central tube present in the fossil taxa may represent an ancestral structure from which the central labiate process in the bipolar centric taxa, the central strutted process of the order Thalassiosirales and probably the raphe of the pennate diatoms may have evolved. Ultrastructure evidence also supports the two clades recovered in the molecular tree. Molecular clock calibrations of the evolutionary distances between taxa regressed against first appearances in the fossil record set the average age of the Thalassiosirales between 79 and 108 Ma. The earliest possible date for the origin of the Thalassiosirales is at ca. 215 Ma. Modern Thalassiosiralean taxa probably evolved from taxa in the Lower Cretaceous.

Key words. Diatom, molecular phylogeny, 18S rRNA, Thalassiosirales, evolution.

Introduction

The order Thalassiosirales is a successful and diverse group of planktonic diatoms. *Skeletonema* and *Thalassiosira* spp. are, for example, dominant members of high-latitude coastal spring blooms. *Thalassiosira*, the largest genus in the order, has over 100 species. Many species of *Thalassiosira* were transferred from *Coscinodiscus* after Fryxell & Hasle (1972) demonstrated that the position of the velum, which occurs on the inside of the cell wall in *Thalassiosira* and on the outside in *Coscinodiscus*, could be used reliably to separate the two genera. This pattern and the presence of the strutted process, a tube through which chitan threads (McLachlan et al. 1965, Heath 1979) are extruded for chain formation, flotation and grazing deterrents, define the Thalassiosirales. Simonsen (1972) considered the strutted process to be such a unique character that he hypothesized the Thalassiosirales to have diverged early in diatom evolution. Although *Thalassiosira* spp. appear in the upper Eocene

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(Medlin et al. 1993), potential ancestors of the group, e.g. *Thalassiosiropsis*, are recorded from the Upper Cretaceous (Hasle & Syvertsen 1985) and *Praethalassiosiropsis* from as early as the Lower Cretaceous (Gersonde & Harwood 1990).

Initial 18S small-subunit (ssu) ribosomal RNA (rRNA) sequence analyses placed *Skeletonema* (order Thalassiosirales) as sister taxon to the pennate diatoms, with high bootstrap support (Medlin et al. 1993). The long branch leading to *Skeletonema* suggests that its position in the tree could be an artifact because of the apparent relatively high substitution rate in this lineage. *Skeletonema* does, however, emerge late in the tree after the other centric taxa rather than as one of the first divergences as predicted by Simonsen (1972). We present here additional 18S rRNA sequence data to suggest that although *Ditylum* and *Streptotheca* lie at the base of the Thalassiosirales, this lineage is sister taxon to the pennate diatoms. Further, the Thalassiosirales and the pennate diatoms are sister taxa to the bipolar (multipolar) centrics. These taxa constitute one of two major lineages of diatoms, which diverged early in the evolutionary history of the group. The average age of the Thalassiosirales has been estimated between 79 and 108 Ma, and the earliest possible origin of the Thalassiosirales at 215 Ma. Poor preservation of the diatom taxa in strata of this latter time period preclude the identification of clear ancestors of the group. However, *Praethalassiosiropsis* occurs within the time frame suggested by our calculations to be the average age of the Thalassiosirales and is thus the most likely candidate for a potential ancestor for the group.

Materials and methods

DNA Methods. Nucleic acids were extracted as described in Medlin et al. (1988) or with a 3% CTAB (hexadecyltrimethylammonium bromide) procedure (Doyle & Doyle 1990) from cultures (Medlin et al. 1986) representing the three classes of diatoms and most major orders of centric taxa (Table 1). Voucher slides are available for examination of those isolates no longer in culture. Small-subunit rRNA coding regions were amplified using the polymerase chain reaction (PCR; Saiki et al. 1988) and cloned as described in Medlin et al. (1988). Single-stranded templates were also produced for direct sequencing using biotin-labelled eukaryote-specific primers (Dynabeads M-280 Streptavidin, DYNAL A.S. Oslo, Norway). No fewer than 6 PCR reactions in each orientation were pooled for direct sequencing of the single-stranded templates. Both coding and non-coding strands were completely sequenced (Sanger et al. 1977, Elwood et al. 1985).

Sequence analysis. Previously published rRNA sequences from diatoms (Bhattacharya et al. 1992, Medlin et al. 1991, 1993) and other chromophytes/oomycetes, dinoflagellates, and prymnesiophytes (Medlin et al. 1994, Neefs et al. 1991, Andersen et al. 1993) were used to align the ssu rRNA sequences using maximum primary and secondary structural similarity. Positional homology was assumed for 1739 positions; 528 of these were informative and used in the maximum parsimony analysis. The final data set contained 34 taxa (Table 1). We rooted the trees with three oomycetes and one pelagophyte because the use of multiple outgroups improves the analysis. The oomycetes, which lie at the base of the pigmented heterokont lineage (Saunders et al. 1995), are not included in our presentation, but trees with these taxa included can be obtained from the authors.

Maximum parsimony analyses were implemented with the PAUP program (Swofford 1993). Introduced gaps were treated as missing data, and informative characters were treated as multistate and unordered. Unweighted maximum parsimony trees were obtained using the tree-bisection-reconnection (TBR) branch swapping option and a heuristic search with random additions of the taxa. The most parsimonious trees (MPT) and the data matrix were entered into MacClade (Maddison & Maddison 1992) to produce a weighted data set in which the frequency of nucleotide substitutions was inversely related to the number of changes at that position (scale 1–100). The type of substitution at each position was also weighted (scale 1–100). These weightings greatly enhance the ability of the maximum parsimony analyses to recover the correct tree when multiple substitutions have occurred (Hillis et al. 1994). These constraints were used to generate weighted maximum parsimony trees. Stability of the branching order was estimated using bootstrap analysis for 100 replicates (Felsenstein 1985).

Distance analysis was performed using PHYLIP (Felsenstein 1993). Dissimilarity values (Fitch & Margoliash 1967), based on pairwise comparisons of sequences, were transformed into distances using the Kimura-two-parameter-model (Kimura 1980). Distance matrices were converted into trees using the neighbor-joining option in PHYLIP. Branching order stability was estimated by bootstrap analysis as above.

Table 1. Sources of small subunit rRNA sequences used in this study.

Taxa	Study	Cloned or Dynabeads
OOMYCETES		
Lagenidium giganteum Couch	Neefs et al. (1991)	
Phytophthora megasperma Drech.	Neefs et al. (1991)	
Achlya bisexualis Coker	Neefs et al. (1991)	
PELAGOPHYCEAE		
Pelagomonas calceolata And. & Saund.	Andersen et al. (1993)	
BACILLARIOPHYCEAE		
Centric diatoms		
Stephanopyxis cf. *broschii* Grun.	Bhattacharya et al. (1992)	Cloned
Rhizosolenia setigera Brightw.	Bhattacharya et al. (1992)	Cloned
Coscinodiscus radiatus Ehrenb.	Medlin et al. (1993)	Cloned
Corethron criophilum Castracane	This study, n/a	Cloned
Actinocyclus curvatulus Janisch	This study, AWI	Dynabeads
Aulacoseira distans (Ehrenb.) Sim.	This study, n/a	Cloned
Aulacoseira ambigua (Grun.) Sim.	This study, n/a	Cloned
Melosira varians C. Ag.	This study, n/a	Cloned
Chaetoceros sp.	This study, n/a	Cloned
Chaetoceros didymus Ehrenb.	This study, n/a	Cloned
Chaetoceros rostratus Lauder	This study, n/a	Cloned
Cymatosira belgica Grun. in Van Heurck	This study, CCAP 1018/1	Cloned
Papiliocellulus elegans Hasle, v. Stosch & Syv.	This study, n/a	Cloned
Eucampia antarctica (Castr.) Mangin	This study, AWI	Dynabeads
Streptotheca thamesis Shrubsole	This study, CCAP 1076/1	Dynabeads
Ditylum brightwellii (West) Grunow	This study, CCAP 1022/2	Dynabeads
Thalassiosira eccentrica (Ehrenb.) Cl.	This study, n/a	Cloned
Thalassiosira rotula Meunier	This study, CCAP 1085/4	Cloned
Skeletonema costatum (Grev.) Cl. (two clones)	Medlin et al. (1991)	Cloned
Skeletonema pseudocostatum Medlin (two clones)	Medlin et al. (1991)	Cloned
Porosira glacialis (Grun.) Jørgensen	This study, AWI	Dynabeads
Lauderia borealis Gran	This study, CCAP 1044/1	Cloned
Araphid pennate diatoms		
Asterionellopsis glacialis (Castr.) Round	Medlin et al. (1993)	Cloned
Fragilaria striatula Lyngb.	Medlin et al. (1993)	Cloned
Thalassionema nitzschioides (Grun.) V.H.	Medlin et al. (1993)	Cloned
Rhaphoneis cf. *belgica* (Grun.) Grun.	Medlin et al. (1993)	cloned
Raphid pennate diatoms		
Nitzschia apiculata (Greg.) Grun.	Bhattacharya et al. (1992)	Cloned
Bacillaria paxillifer (Müll.) Hend.	Bhattacharya et al. (1992)	Cloned
Cylindrotheca closterium (Ehrenb.) Reim. & Lewin	Bhattacharya et al. (1992)	Cloned

Maximum likelihood analyses were performed using the fastDNAml program (v. 1.0 Larsen et al. 1993). The resulting tree was used to construct user-defined trees (RETREE, PHYLIP) to constrain maximum parsimony (PAUP) and maximum likelihood (DNAML, PHYLIP) analyses. Three alternative hypotheses of relationships were tested according to Kishino & Hasegawa (1989).

Molecular clock calibrations. Dates of first appearances of lineages in the fossil record were regressed against their measured branch lengths from the maximum likelihood and neighbor-joining trees according to Hillis & Moritz (1990). Lineages within each clade were identified as fast, median or slow, based on their branch lengths. The average age of a clade was estimated by multiplying the length of its median lineage with the regression coefficient. The earliest possible age of a clade is the date corresponding to the point on the upper 95% confidence limit given the length of the

median lineage in that clade. Base changes along each long branch in the distance tree were recorded by comparing the nucleotide sequence of the long branch to that of its nearest and next nearest neighbor to calculate an observed distribution of base changes according to Van de Peer et al. (1993). This observed distribution was tested against an expected distribution using a chi-square test to identify taxa with an higher than expected distribution of changes in four variability classes of the 18S rRNA gene. These taxa were eliminated from the molecular clock calculations and the age calculations for the Thalassiosirales.

Results

Small subunit rRNA sequences for 14 taxa were approximately 1800 nucleotides in length. However, *Aulacoseira ambigua* and *Cymatosira belgica* had insertions making their ssu rRNA coding regions 1847 and 2317 nucleotides long, respectively, including amplification primers. Secondary structure models of these two taxa plus one from the Thalassiosirales are presented in Figures 1–3. The increased length of the gene in *Aulacoseira ambigua* was due to one insertion, whereas *Cymatosira belgica* had three different insertions. A search of the database against each insertion reveals no homology to Group I introns.

A phylogeny of the diatoms inferred from the maximum likelihood method is presented in Fig. 4A. Bootstrap values greater than 60% supporting the recovered branches in either the distance or the weighted maximum parsimony trees are placed at the internal nodes of the tree. In all analyses, the diatoms diverge initially into two clades (Fig. 4A). This division does not correspond to centrics or pennates (see discussion in Simonsen 1972, Mann & Marchant 1989) or to the three classes of diatoms in Round et al. (1990). Beyond this initial separation, the relationships inferred from our analysis correspond best to ordinal level in current diatom systematics.

Members in the Coscinodiscales, Rhizosoleniales, Corethrales, and Melosirales were found in the first clade recovered in each of the three analyses (Fig. 4). In general these taxa have labiate processes located peripherally around the cell wall. Exceptions to this do occur, e.g. *Azpeitia*, but we believe the position of the labiate process in the valve center of this genus may have occurred after the evolution of the lineage because *Azpeitia* first appears in the Eocene, although other members of the Stellarimaceae and the closely related Benetoraceae occur in the lower Cretaceous (Nikolaev & Harwood 1994, Sims 1994). Clearly, extant members of the Stellarimaceae should be targeted for rRNA analysis to investigate this hypothesis. Also, in many *Rhizosolenia* species, the labiate process appears to be centrally located but this may be a misinterpretation because of the distortion of the valve.

Within this first clade are two lineages (Fig. 4A). Taxa with large, elaborate labiate processes (Hasle & Sims 1986) belong to the first lineage (orders Coscinodiscales and Rhizosoleniales). A close relationship between *Rhizosolenia* and *Corethron* was suggested by Fryxell & Hasle (1977) because of similar girdle band architecture; however Round et al. (1990) erected the order Corethrales because *Corethron* lacks any kind of processes. Taxa in the second lineage (order Melosirales) have only small labiate processes (Round et al. 1990).

In all analyses, the second clade comprises three groups. The bi-(multi) polar centric diatoms of the orders Hemiaulales, Lithodesmiales, Cymatosirales, and Chaetocerotales are the first group. These diatoms have a small, but centrally located labiate process. The order Thalassiosirales, with (usually) one large marginal labiate process but one to several central strutted processes, form the second group. The pennate diatoms comprise the third lineage. The labiate processes of the araphid taxa are usually small and commonly located at the apices. Low bootstrap values indicated weak support for the branching order within this major second diatom clade, which may be evidence for a rapid diversification within this clade.

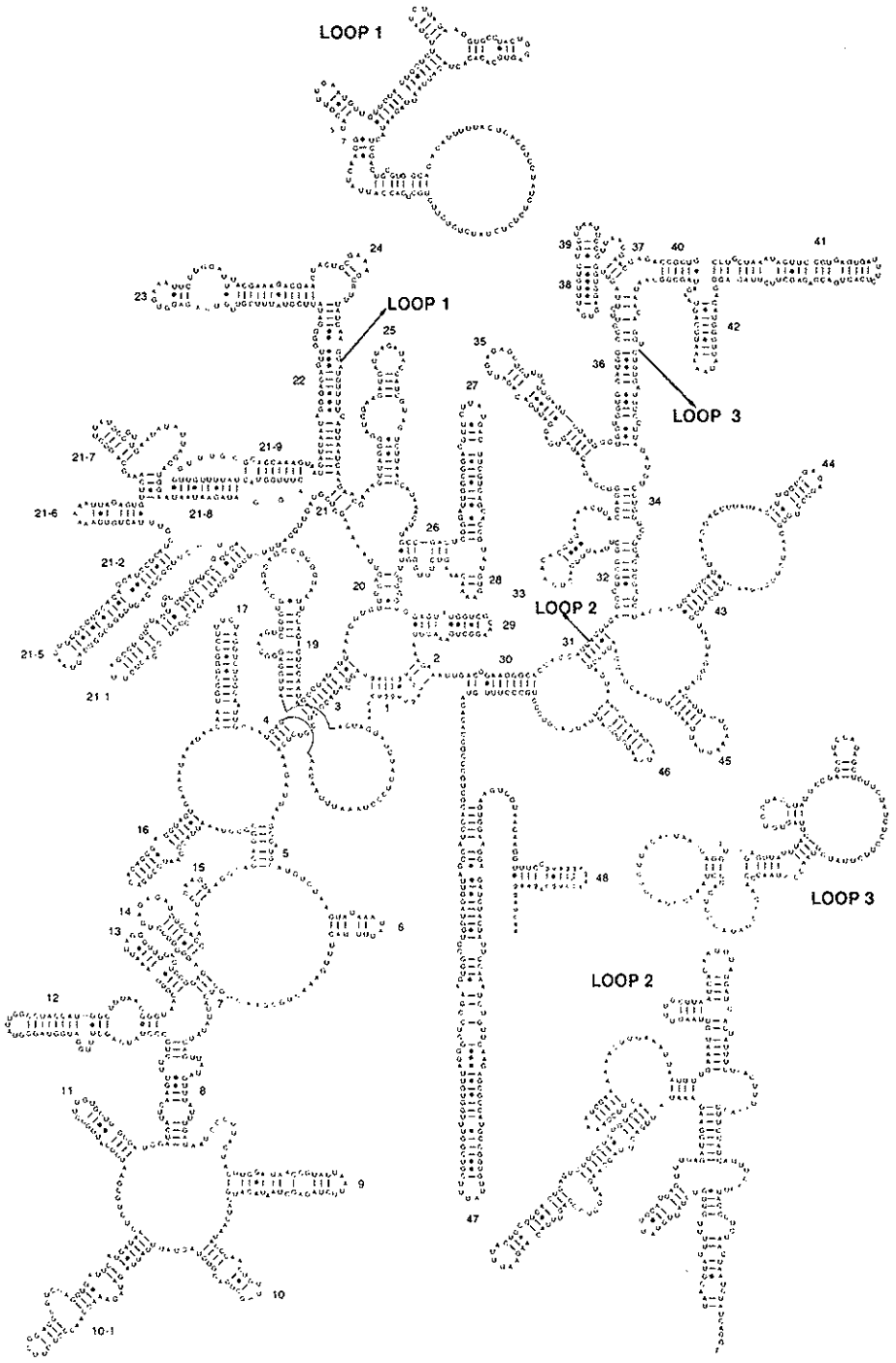


Fig. 1. Secondary structure model of *Cymatosira belgica*.

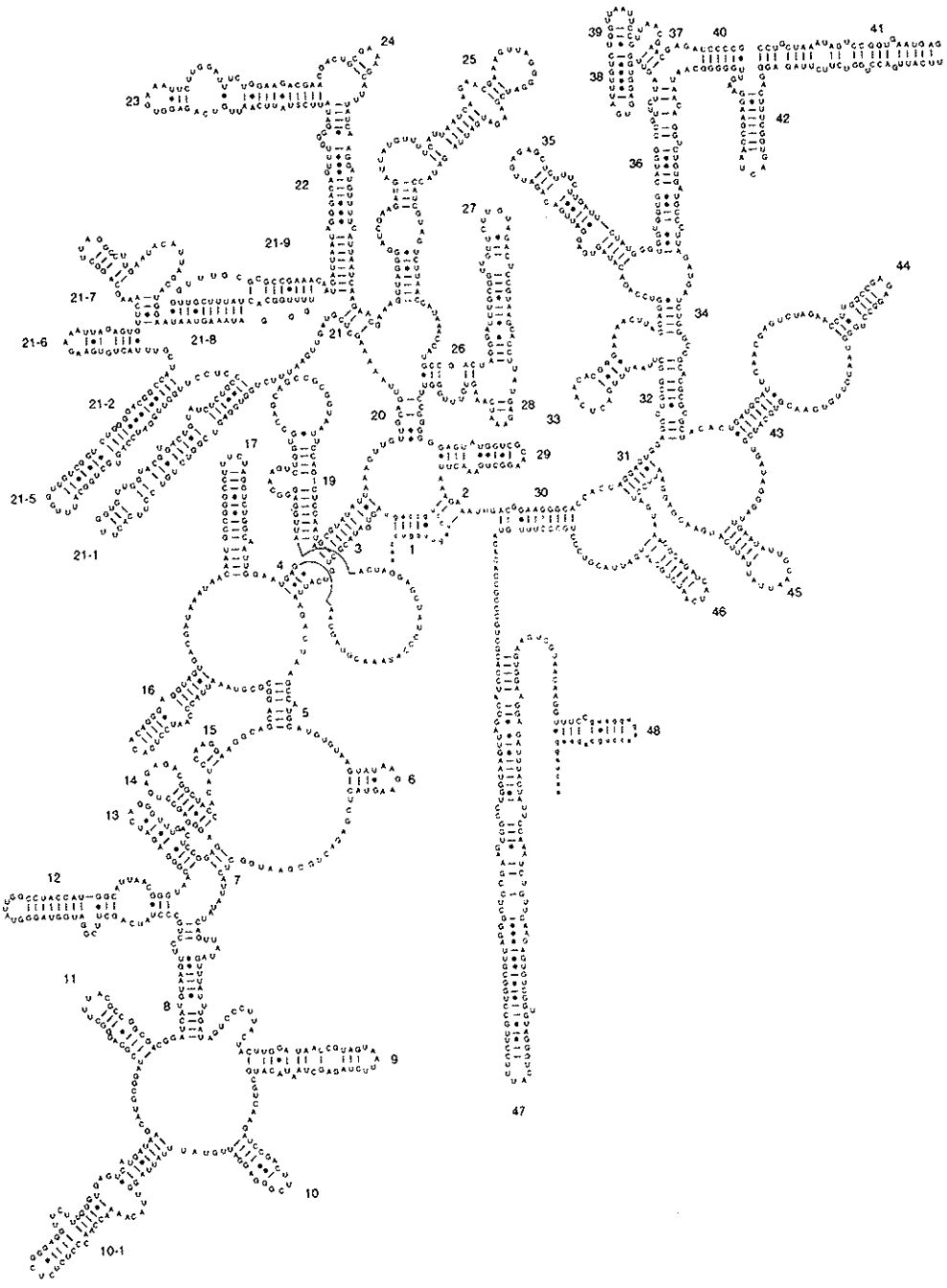
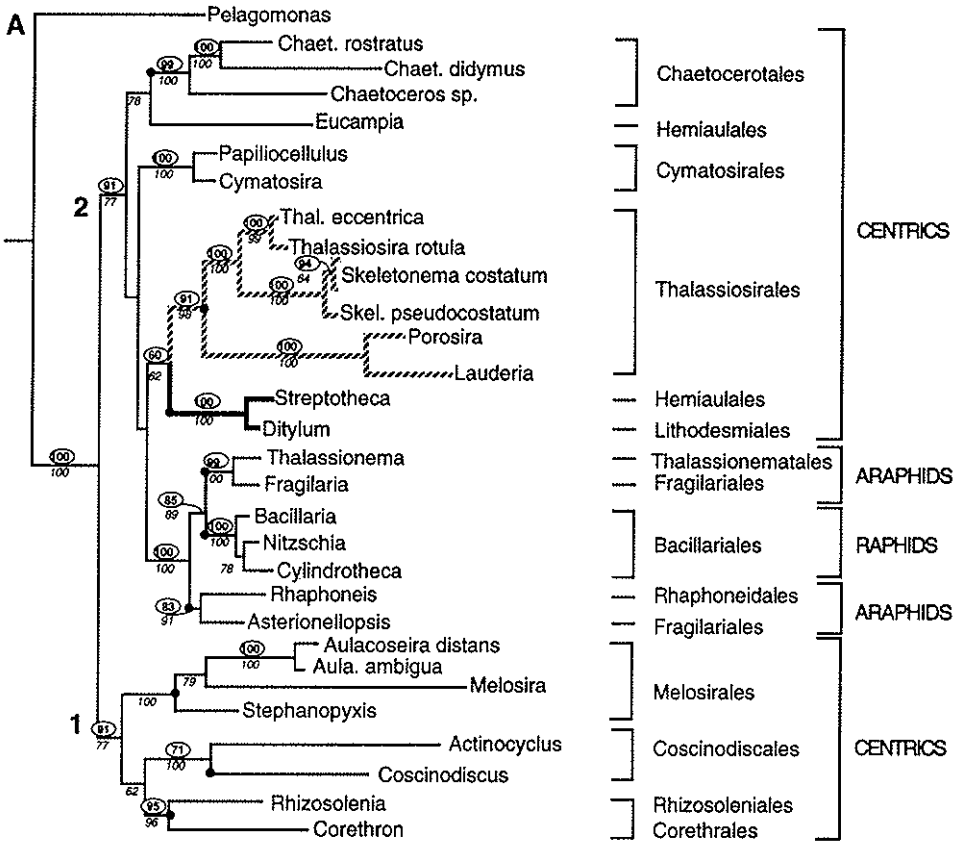


Fig. 2. Secondary structure model of *Aulacoseira ambigua*.

A



B

Tree	Log-Likelihood	Difference in Log-Likelihood	Standard Deviation	Significantly Worse	Number of steps in MPT
1. Best tree	14443.799	-	-	-	2258
2. Monophyly of the Centrics	14479.662	-35.862	17.877	Yes	2270
3. * plus 3 centric clades	14482.788	-38.989	18.980	Yes	2269
4. Thalassiosirales and Pennates as sister clades	14469.000	-25.201	10.723	Yes	2268

C

Lineage (•)	First appearance in the fossil record in Ma.
1. Chaetoceros	70
2. Thalassiosira / Skeletonema	40
3. Ditylum	70
4. Thalassionema	40
Fragilaria	45
5. Nitzschia	45
6. Rhaphoneis	70
7. Aulacoseira / Stephanopyxis	70
8. Coscinodiscus	70
9. Rhizosolenia / Corethron	70

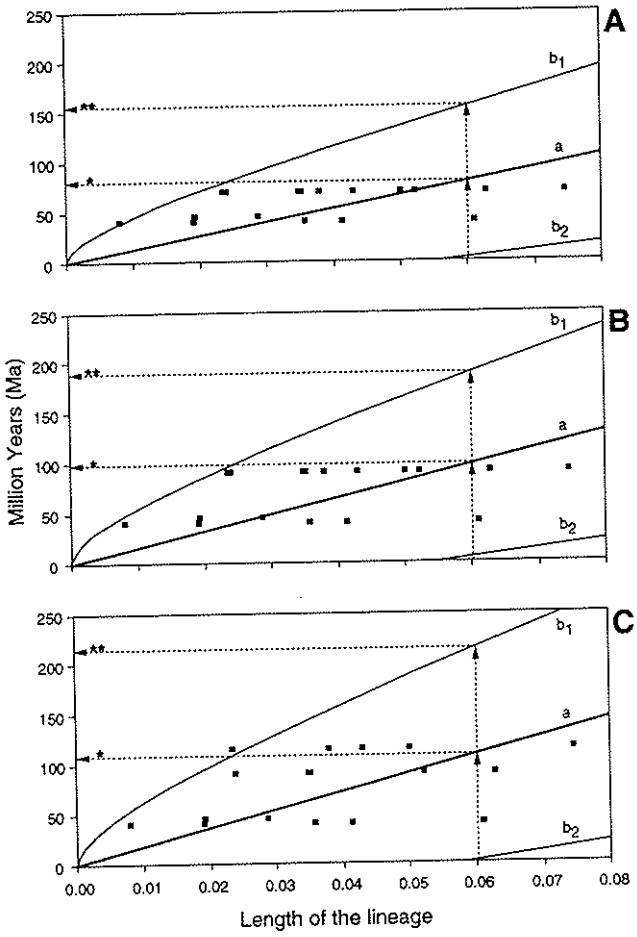


Fig. 5. Results of molecular clock calibrations in which dates of first appearances of lineages in the fossil record are regressed against their measured branch lengths from the maximum likelihood tree. The straight line (a) is the regression line, forced through zero; the upper and lower curves (b_1 and b_2) are the 95% confidence limits for a new estimate of time. Lower confidence limits, lower than zero, are reset at zero. The average age of the Thalassiosirales (*) is the length of their median lineage multiplied by the regression coefficient. The earliest possible age of the group (**) is the date corresponding to the point on the upper 95% confidence limit given the length of the median lineage of the group. These calculations are made with three different first appearance dates. – A. All first appearance dates in the fossil record taken as time of origin. B. First appearance dates of 70 Ma reset to 90 Ma. – C. Taxa with a first appearance date of 70 Ma are hypothesized to emerge at 115 Ma if they can be linked to fossil sister taxa that lack labiate processes.

Using these three hypotheses (Fig. 5A, B, C), we calculated the average and earliest possible age for the origin of the Thalassiosirales based on the nucleotide substitution rate in the lineage by multiplying the length of the median lineage in the Thalassiosirales (i.e. *Skeletonema pseudocostatum*) by the regression coefficient from each of the three models. The average age of the Thalassiosirales ranges ca. 79 to 108 Ma (Fig. 5A, B, C), but the earliest possible origin for the Thalassiosirales is ca. 215 Ma (Fig. 5C). *Praethalassiosiropsis* occurs within the time frame suggested by our calculations to be the average age of the Thalassiosirales. A regression of branch lengths from the neighbor-joining tree yields slightly younger estimates for the origin of the Thalassiosirales (data not shown).

Discussion

Our rRNA analysis reveals a dichotomy in the diatoms that is difficult to explain if morphological features, such as pattern centers, reproduction and plastid morphology, which currently define diatom classes, are used to characterize the molecular tree. Furthermore, valve structures and tubes, such as labiate and strutted processes used to characterise many diatom

orders, also fail to describe the two clades recovered in our molecular tree. However, these structures can describe younger branches in the molecular tree.

If morphological features present in the exceptionally well-preserved Lower Cretaceous material from Ocean Drilling Program (ODP) Leg 113, Site 693, Antarctica deposit are used to characterise the molecular tree, then we can recover the clades in our molecular analysis (Medlin et al. 1996). Diatoms in this deposit were placed into four groups by their valve structure and type of linking apparatus (Gersonde & Harwood 1990). None of the diatoms in this fossil deposit possess labiate or strutted processes, as currently defined but instead possess other types of processes. If we use the presence or absence of a central invagination that develops into a central tube in the valve face of the diatoms of this deposit and a reduction in the elaborate linking structures between cells to support our rRNA tree, then three of the four groups defined from this fossil deposit can be correlated with the two clades recovered in our rRNA tree (Medlin et al. 1996).

The absence of a central structure/tube and the presence of robust linking structures are characteristic of Group 1 and Group 4 taxa of Gersonde & Harwood (1990), and we have proposed that these are the features characteristic for the ancestral stock of clade one of our molecular tree (Medlin et al. 1996). *Stephanopyxis* has linking structures similar to those of Group 1 taxa, which are formed from part of the areolar wall, whereas in *Aulacoseira*, the linking structures are formed from the main part of the wall like the Group 4 taxa of Gersonde & Harwood (1990). Significantly, modern taxa from clade one lack, in general, any central structure or tube in the valve but do possess one of two types of peripherally located tubes, the labiate processes. These labiate processes must have evolved since the initial divergence of the two clades because no labiate processes or tubes can be found in the presumed ancestral stock for clade one from the Lower Cretaceous deposit.

The presence of a central tube-like structure and a reduction in linking mechanisms are characteristic of Group 2 taxa of Gersonde & Harwood (1990). We have suggested that this group forms the ancestral stock for clade two of our rRNA tree (Medlin et al. 1996). Morphological changes in the valve center of these Lower Cretaceous diatoms can be hypothesized to have occurred from a valve with an invagination (e.g. the uvular process of *Archaegladiopsis*, see Nikolaev & Harwood 1996) to a valve with a central tube whose internal opening is covered with a perforate plate (e.g. the perforate process of *Praethalassiosiropsis*; Gersonde & Harwood 1990). The next step is for the perforate plate to be lost, then the annular process or multi-strutted processes of *Gladiopsis speciosa* (Schulz) Gers. & Harw. (Sims 1994) and *Thalassiosiropsis* (Hasle & Syvertsen 1985) are formed as the pores in the wall of the central tube coalease. If the perforations in the wall of the central tube are further reduced, then the sipho-shaped process (*Gladiopsis ellipsoidea* Gers. & Harw.) is formed. Finally a valve with a open central tube without any perforations in the wall (e.g. the rhyncho-shaped process of *Rhynchopyxis* (Gersonde & Harwood 1990, Nikolaev & Harwood 1996, and Harwood, pers. comm.) is produced. As originally defined, *Gladiopsis* contains taxa with at least three or more types of central valve structures/tubes: the uvular process, the annular or multi-strutted process, and the sipho-shaped process (Nikolaev & Harwood 1996). Nikolaev & Harwood (1996) have proposed one new genus, *Archaegladiopsis*, for those taxa formerly placed in *Gladiopsis* with a uvular process. The line of development from the perforate process of *Praethalassiosiropsis* into the multi-strutted (annular) process of *Thalassiosiropsis* (Harwood, pers. comm.) appears to stop at the Palaeocene (Sims 1994). Although true *Thalassiosira* spp. do not appear until the Eocene, a search for more direct ancestors spanning the gap from when the last *Thalassiosiropsis* spp. existed to modern Thalassiorsirales should perhaps be directed toward taxa with a central

structure/tube more closely resembling that of *Ditylum* or *Rhynchopyxis*. Such evolutionary sequences are supported by the phylogenetic relationships inferred from the rRNA tree. The removal of *Ditylum* from the base of the Thalassiosirales in our rRNA tree yields a significantly less robust tree and also implies that the morphological changes inferred from the fossil material have merit. This suggests that the satellite pores of true Thalassiosirales may be a later addition to the central tube or strutted process in this lineage. Significantly, in an analysis of partial large subunit (28S) rRNA sequences, Sörhannus et al. (1995) found the Thalassiosirales to be outgroup to *Lithodesmium* and the pennates. Clearly, there is a close association between these three taxa.

From our rRNA analysis, it can be shown that *Coscinodiscus* is not related to *Thalassiosira*. Any scheme that tries to reconstruct a recent evolutionary history between the orders to which these taxa belong would be totally incorrect from several lines of evidence. Not only do they belong to two entirely different clades of diatoms as shown by our rRNA tree, but they also have completely different valve structures and tube processes (Fryxell & Hasle 1972) and have different responses to microtubule poisons (Schmid 1994). In addition, *Coscinodiscus*, *Ellerbeckia* and *Stephanopyxis* (clade one taxa) have their Golgi bodies associated with a mitochondrion within cisternae of the endoplasmic reticulum like the Oomycetes (Schmid, unpubl.), whereas Thalassiosiraceae and most, if not all, pennates have their Golgi bodies either as a perinuclear shell or in a "Plattenband" (see references in Schmid 1989).

Our analysis suggests that the central valve structures/tubes presumed to be representative of the ancestral stock for clade two of the molecular tree also evolved into the central labiate process of the bipolar centrics and into the labiate processes or raphe of the pennate diatoms. If so, then these valve structures would be homologous to the central strutted process of the Thalassiosirales. This interpretation can be supported by the cytological evidence for the function of the labiate process as a cytological anchor for the nucleus during interphase and when new valves are formed (Schmid 1994). In the Thalassiosirales, cytoplasmic threads are attached from the nucleus to the strutted processes (Schmid 1984) and in the bipolar centrics to the small central labiate processes (Schmid 1994). In the pennates, cytoplasm is attached to the raphe ribs (Höfler 1940). In contrast, clade one taxa have cytoplasmic strands attached to the large peripheral labiate processes (Schmid 1994). Thus, the large, peripheral labiate process of the Thalassiosirales appear to be another structure. It is often oriented radially in the wall and is morphologically distinct from the small central labiate process of the bipolar centrics and from the peripheral labiate processes of clade one radial centrics.

Our calculations indicate that the average age for the Thalassiosirales based on nucleotide substitution rates ranges from 79–108 Ma. Estimates of the age of the Thalassiosirales are influenced not only by the algorithm from which the branch lengths have been derived but also by the first appearance dates in the fossil record. Our estimate is inferred from a regression of maximum likelihood branch lengths against first appearance dates, corrected for times in the fossil record where there is poor preservation. We have assumed fossil predecessors of extant taxa for this calculation based on similarities in valve structure and linking mechanisms and not on the presence of labiate or strutted processes. *Praethalassiosiropsis* from the Lower Cretaceous deposit at 115–100 Ma (Gersonde & Harwood 1990) can be viewed as a likely progenitor of the Thalassiosirales or at least a representative of the stock from which the Thalassiosirales are derived. Our estimate of the average age of the Thalassiosirales (79–108 Ma) as inferred from our calculations agrees with this hypothesis. The hypothesized earliest possible age of the Thalassiosirales (215 Ma) cannot be confirmed,

however, because of the lack of any recognizable diatom fossils at this time (Gersonde & Harwood 1990).

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