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Evolution of the diatoms: V. Morphological and cytological support for the major clades and a taxonomic revision

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Phylogenetic reconstructions of the diatoms have been inferred with the 18S and 16S ribosomal RNA genes. Previous studies have shown that the group is divided into two major clades, with support coming initially from the arrangement of the Golgi bodies inside the cells in extant taxa. Features of extinct taxa that also support these clades can be found in the earliest fossil record of the diatoms and include the presence or absence of a central structure in the valve wall and the type of peripheral linking mechanisms between cells. Here we demonstrate that the general pattern of the auxospore expansion and the structure of their walls, the structure of the pyrenoid and the ultrastructure of the spermatozoid further support the molecular clades. Given the combined molecular and morphological support, we propose two new subdivisions (Coscinodiscophytina and Bacillariophytina), emend the classes Coscinodiscophyceae and Bacillariophyceae and propose a new class, the Mediophyceae for the bipolar centrics and the Thalassiosirales.

INTRODUCTION

With over 10,000 described species and potentially many more cryptic species (Mann 1999), the diatoms (Bacillariophyta) are one of the most successful microalgal groups in both aquatic and terrestrial habitats. They possess architecturally complex siliceous cell walls (valves and girdle bands) that are unique among the algae. They have an unusual pattern of reduction in cell size of one of the daughter cells following mitosis (see review in Mann & Marchant 1989); restoration of the maximum cell size normally occurs following sexual reproduction. Since the 19th century, their classification system has been based on the intricate designs of their cell wall. Before morphological structures can be used to reconstruct phylogeny, one has to clarify which morphological features are homologous; distinctly different structures may have a common origin, such as the small labiate process of the araphid diatoms and the raphe of the raphid diatoms, but also similar structures may have different origins. Common morphology does not necessarily imply common descent. Examples in the diatoms include the macro- and microlabiate processes and the fibulae. The fibulae (bars of silica bridging the valve beneath the raphe slit in some raphid diatoms) have evolved at least twice (Medlin *et al.* 2000). Not surprisingly, several recent molecular phylogenetic reappraisals of taxonomies in other algal groups have demonstrated numerous cases of parallelism and convergence [e.g. *Chlorella* Beijerinck, in Huss *et al.* (1989), the green macrophyte *Halimeda* Lamouroux, in Kooistra *et al.* (2002) and the unicellular red algae in Müller *et al.* (2001)]. This is particularly true for morphologically simple organisms or for those whose elaborate morphology is a function of just a few interactive processes.

The diatoms belong to the heterokont algae, i.e. algae with chlorophylls *a* and *c* and two heterodynamic flagella: the apically inserted flagellum bears tripartite mastigonemes, whereas the laterally inserted one is smooth. Only the spermatozoids of the oogamous genera are flagellated (Manton & von Stosch 1966), and although these cells lack the smooth posterior flagellum and the second basal body, they still belong to the heterokonts because of shared plastid ultrastructure and pigment composition.

Many workers have speculated on the origin of the diatoms. Diatoms may have been derived from a spherical uniformly scaled monad (Round 1981; Round & Crawford 1981, 1984) with an anterior flagellum (Cavalier-Smith 1986), or from a cyst-like form similar to the extant Parmales in the chrysophyte algae (Mann & Marchant 1989). A scaly ancestor may have existed at some point in their phylogeny because of the presence of scales on the reproductive cells of diatoms and scales on the reproductive stage of the Labyrinthuloides, which are earlier divergences in the heterokont lineage (Medlin *et al.* 1997). Recent phylogenies based on nuclear-encoded small-subunit ribosomal RNAs (SSU rRNAs) place the diatoms within the pigmented heterokont algal lineages (Bhattacharya *et al.* 1992; Leipe *et al.* 1994; Medlin *et al.* 1997) and closely related to a new algal class, the Bolidophyceae, picoplanktonic algae with a simplified cellular organization (Guillou *et al.* 1999). The diatoms and the bolidomonads share common pigments and two transverse plates at the base of each flagellum. A molecular clock based on four genes gave the average age of the diatoms as *c.* 165 Ma, with their earliest possible age as 240 Ma (Kooistra & Medlin 1996; Medlin *et al.* 1997). The first fossil record of a diatom occurs at 180 Ma (Rothpletz 1896) and this date occurs between our average and the earliest possible dates.

The central dogma of diatom taxonomy and phylogeny has

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° This paper is dedicated to Prof. G.A. Fryxell who has done much to inspire our interest in living diatoms, and to Mr Norman Hendey on the occasion of his 101st birthday.

been an assumption that the diatoms contain two groups, which can be distinguished by their mode of sexual reproduction and pattern centres or symmetry (e.g. Simonsen 1979; Round *et al.* 1990). The oogamous centric diatoms with radial organization of their valves and with numerous discoid plastids are distinct from the isogamous pennate diatoms that have bilaterally organized valves and fewer plate-like plastids. Both groups are familiar to most aquatic and cell biologists under these terms. Historically, the centric and pennate diatoms have been separated into two distinct orders based on these characters. Pennate diatoms undoubtedly evolved from the centric forms because they appear later in the fossil record (see references in Medlin *et al.* 1993). Simonsen (1979) presents an evolutionary tree based on morphology that consists of a grade of centric clades, which give rise to the pennate diatoms through the bipolar centric families. Recently, Round *et al.* (1990) gave the raphid pennate diatoms, i.e. those with a slit opening (raphe) in the cell wall for movement, equal taxonomic ranking with the araphid pennate diatoms (those without a raphe). Coscinodiscophyceae (centric diatoms), Fragilariophyceae (araphid pennate diatoms), and Bacillariophyceae (raphid pennate diatoms) are the three classes recognized in the current classification system of the diatoms.

The morphological features of the siliceous cell wall correspond best to the present classification system at the order level and below, and are the basis for diatom classification and species identification (Round *et al.* 1990). The most important features used to infer phylogenetic relationships at and below ordinal level are probably the raphe and the labiate or strutted processes through the cell wall (Simonsen 1979; Round *et al.* 1990). Centric diatom orders are defined by characters such as valve structure and symmetry, and the type, number and arrangement of tubular openings (labiate or strutted processes) through the valve.

The labiate process is a variable structure and may have multiple functions. It normally possesses elaborate lip-like internal extensions (macrolabiates), but some types of labiate processes are reduced to only a simple slit-like internal opening (microlabiates). The ontogenetic predecessors of the labiate processes in *Coscinodiscus granii* Gough may have been similar to those now seen in the slit scales in its auxospore wall (Schmid 1994b). Although in a restricted number of centric and pennate taxa, mucilage is secreted through the labiate process for movement in general, the function of the labiate process is not well established (Medlin *et al.* 1986; Pickett-Heaps *et al.* 1986). During interphase and new valve formation, the internal part of the tube has been hypothesized to be a cytological anchor for the nucleus (Schmid 1994a). Hasle (1974) hypothesized that the labiate process evolved into the raphe of the pennate diatoms. The raphe is the organelle for movement in the pennate diatoms.

The strutted processes through which chitin threads are secreted for chain formation and flotation are restricted to the centric order Thalassiosirales and are simple tubes with adjacent pores (Round *et al.* 1990, pp. 35–36). It quickly becomes apparent that the cytoplasmic organization beneath the strutted processes is more complicated than that beneath the labiate processes if one compares figures in Herth (1979) to fig. 11 in Schmid (1994a), although secretion vesicles were found also beneath the labiate process of *Actinocyclus subtilis* (Greville) Ralfs (Medlin *et al.* 1986).

The raphid and araphid taxa are morphologically separated from the centrics and from each other by a number of characteristics, such as their symmetry and the presence or absence of the raphe, and then they are further separated into orders, families and genera in the raphid taxa by raphe structure (e.g. the presence or absence of fibulae, the shape of the raphe endings, and the presence or absence of ribs, extra flaps of silica, etc.) and in the araphid taxa by features of the axial area and the apical pore field.

SSU rRNA sequence comparisons have proven to be a powerful alternative to morphology and ecology for inferring phylogenetic relationships at all taxonomic levels. This molecule is a functionally stable evolutionary marker that has evolved independently of morphology (Woese 1987; Bhattacharya *et al.* 1992). The rRNA genes are functionally conserved in all cells and there is no documented evidence of lateral gene transfer (Woese 1987). SSU rRNA sequence comparisons (Bhattacharya *et al.* 1992; Medlin *et al.* 1993) support a monophyletic origin of the diatoms within the pigmented protistan heterokont lineage. They have also identified the Bolidophyceae as the closest sister group of the diatoms from among all the heterokonts investigated to date (Guillou *et al.* 1999).

Within the diatoms, though, the situation was not so well defined, and rRNA sequence comparisons did not support a clear dichotomy between centrics and pennates. The first molecular evidence that suggested a paraphyly at the class level came from Medlin *et al.* (1993). In that study, the paraphyletic nature of the centrics was documented for the first time genetically. Two surprising facts emerged from that study: (1) the Thalassiosirales are the sister group of the pennates and (2) this family does not occupy a basal position in the phylogeny as postulated by Simonsen (1979). In the same study, araphid pennate diatoms were shown to be paraphyletic. Further, Sörhannus *et al.* (1995) showed that centric and araphid taxa were paraphyletic using an analysis of partial sequences from the 28S large-subunit (LSU) rRNA–coding region from eight diatoms. These initial data suggested that higher-level diatom systematics in current use do not reflect the evolutionary history of the diatoms. All subsequent analyses from several other genes have supported this finding [18S, 16S, TufA and RbcL in Medlin *et al.* (1996a, 2000); Cox 1 in Ehara *et al.* (2000); and RpoA in Fox & Sörhannus (2003)], and the diatoms have been divided into two groups: clade 1 containing the radial centrics and clade 2 containing the bipolar centrics, the radial Thalassiosirales and the pennates.

Despite these recent advances, relationships at higher taxonomic levels in the diatoms are still virtually unexplored with modern phylogenetic or morphology-based cladistic tools. This probably reflects the unique, highly diversified morphologies of the diatom orders, or perhaps a very rapid and recent radiation of the group because morphometric analyses have generally been restricted to genus level and lower (Cox & Williams 2000 and references therein). The purpose of this paper is to review and document all available cytological and morphological data supporting the major molecular clades of the diatoms, to place this evidence in the context of emerging molecular phylogeny and to propose a new, more comprehensive phylogeny including over 100 taxa representing all major orders of extant diatoms (Round *et al.* 1990). The first revision

of the current diatom classification at the class level is also presented based on these integrated data.

MATERIAL 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 in the slide collection of Medlin for examination of those isolates no longer in culture. SSU rRNA–coding regions were amplified using the polymerase chain reaction (PCR) (Saiki *et al.* 1988) and if necessary cloned as described in Medlin *et al.* (1988); plastid-encoded rRNA–coding regions were amplified as described in Kopp *et al.* (1997). Single-stranded templates were also produced for direct sequencing as described in Medlin *et al.* (2000).

Sequence analysis

Previously published and unpublished rRNA sequences from diatoms (Medlin *et al.* 1991, 1993, 1996a; Bhattacharya *et al.* 1992) and other chromophytes or oomycetes, dinoflagellates and prymnesiophytes (Neefs *et al.* 1991; Andersen *et al.* 1993; Medlin *et al.* 1996a) were used to align the 18S and 16S rRNA sequences using maximum primary and secondary structural similarity. Bases were aligned with one another based on their pairing across a helix. Our database is maintained in the ARB program (Technical University of Munich, Germany) and contains over 8600 eukaryotic and prokaryotic sequences. This program generates a maximum parsimony (MP) tree from all sequences and all positions in the database as its reference tree, using a filter based on 50% base frequency across all species. A subset of these sequences is downloaded for further analyses. For the 18S rRNA gene data set, we generated one data set of 281 taxa using one or two representatives from each major pro- and eukaryotic group that could be used in a bootstrap (BT) analysis using the 50% base frequency filter. Details of this data set can be obtained from the senior author. In addition, we used the taxa shown in Table 1 for an in-depth analysis of the branching order within the diatoms. The second eukaryotic data set contained 126 taxa. From these sequences, positional homology was assumed for 1764 positions out of a possible 1800; 764 of these were informative and were used in the MP analysis. We rooted the trees with three bolidophytes. For the 16S rRNA data set, the taxa used can also be found in Table 1 and positional homology was assumed for 1422 positions, of which 526 were used in the parsimony analysis. The final prokaryotic data set contained 37 taxa. These trees were rooted with *Escherichia coli* and *Agrobacterium tumefaciens* (Smith & Townsend) Conn.

The second 18S data set was subjected to the model test program v.3.06 to ascertain the appropriate model of evolution for our data set (Posada & Crandall 1998). Using this program, the general time reversal (GTR) model of evolution was selected and values for base substitution were implemented into a maximum likelihood (ML) and neighbour-joining (NJ) analysis using PAUP* (Swofford 2002).

In addition, the second 18S data set was analysed using Bayesian inference (BI) (Huelsenbeck *et al.* 2001). BI is, like ML, 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 BI are that it is relatively fast even when large data sets are used, and generates probabilistic measures of tree strength, which gives posterior probabilities (PP) for the phylogenetic stability. These values are more straightforward to interpret than 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. The analysis was done using MrBayes v.3.064, available at http://morphbank.ebc.uu.se/mrbayes/. We ran the Bayesian search using the GTR model with an undefined gamma distribution, during 500,000 generations, and saving every 100th tree. We discarded the first 1000 trees; the remaining 4000, all with higher PP, were used to construct a consensus tree. On this tree, 'credibility values' for each clade are shown, which represent the percentage of those 4000 trees having the corresponding clades.

MP analyses were implemented with the PAUP* program. 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 (TBR) branch-swapping option and a heuristic search with random additions of the taxa. The most parsimonious trees and the data matrix were loaded into the MacClade computer program (Maddison & Maddison 1992) to infer a weighted data set as described in Medlin *et al.* (1997) to generate weighted MP trees.

Distance analysis was performed using PAUP*. Dissimilarity values (Fitch & Margoliash 1967) based on pairwise comparisons of sequences were transformed into distances using models determined from the model test program. Branching-order stability was estimated by BT analysis as above. Stability of the branching order was estimated using BT analysis for 500 replicates for both the distance and the weighted MP trees because the data set was so large (Felsenstein 1985). Hillis & Bull (1993) have shown for computer-simulated data that BT values greater than 70% indicate a likelihood that the clade recovered has a 95% probability of being real; in data sets where there are a large number of characters (i.e. taxa), clades with rates of 50% are correct. The log det distance analysis was also used for the plastid data set to correct for base compositional bias (Lockhart *et al.* 1994) because the use of more traditional models of evolution is known to produce erroneous phylogenies in the 16S rDNA data set.

Electron microscopy

We have reproduced selected figures from earlier, published manuscripts to illustrate specific points of morphology. Methods used to obtain these data are described in Schmid (1988, 1989, 2001) and Kaczmarska *et al.* (2000).

RESULTS

Molecular data

Molecular data are presented in Figs 1–4. Nuclear-encoded SSU rRNA sequences for all taxa are approximately 1800

Table 1. List of strains and species of diatoms used in this analysis and their GenBank accession numbers.

Species	18s rRNA	16s rRNA	Culture source
Coscinodiscophyceae			
Actinocyclus curvatulus Janisch	X85401[1]		R. Crawford
Actinoptychus senarius (Ehrenberg) Héribaud	AJ535182		P. Hargraves
Aulacoseira ambigua (Grunow) Simonsen	X85404[1]	AJ536463	n/a[2]
A. baicalensis (K. Meyer) Simonsen	AJ535186		Y. Likhoshway
A. distans (Ehrenberg) Simonsen	X85403		n/a
A. islandica (O. Mueller) Simonsen	AJ535183		Y. Likhoshway
A. nyannensis (O. Mueller) Simonsen	AJ535187		Y. Likhoshway
A. skvortzowii Edlund, Stoermer & Taylor	AJ535184		n/a
A. subarctica (Grunow) Simonsen	AY121818		Y. Likhoshway
Corethron pennatum (Grunow) Ostenfeld = *C. criophilum*	X85400[1,3]	AJ536466	R. Crawford
C. hystrix Hensen	AJ535179		R. Crawford
C. inerme Karsten	AJ535180		R. Crawford
Coscinodiscus radiatus Ehrenberg	X77705[1]	AJ536462	CCAP[5]
Guinardia delicatula (Cleve) Hasle	AJ535192		R. Crawford
G. flaccida (Castracane) H. Peragallo	AJ535191		R. Crawford
Leptocylindrus danicus Cleve	AJ535175		P. Hargraves
L. minimum Gran	AJ535176		P. Hargraves
Melosira varians C. Agardh	X85402[1]; AJ243065	AJ536464	n/a
Paralia sol (Ehrenberg) Crawford	AJ535174		n/a
Proboscia alata (Brightwell) Sundstrum	AJ535181		R. Crawford
Rhizosolenia imbricata Brightwell	AJ535178		R. Crawford
R. setigera Brightwell	M87329	AJ536461	n/a
R. similoides Cleve-Euler	AJ535177		R. Crawford
Stephanopyxis nipponica Gran & Yendo	M87330[3]	AJ536465	CCMP[4]
Mediophyceae			
Bellerochea malleus (Brightwell) Van Heurck	AF525670		CCAP
Biddulphiopsis titiana (Grunow) von Stosch & Simonsen	AF525669		A.-M. Schmid
Chaetoceros didymus Ehrenberg	X85392[1]		CCAP
Chaetoceros sp.	X85390		n/a
C. rostratus Lauder	X85391[1]		CCAP
Cyclotella meneghiniana Kützing	AJ535172		D. Mann
Cymatosira belgica Grunow in Van Heurck	X85387	AJ536456	CCAP
Detonula confervacea (Castracane) Gran	AF525672		R. Crawford
Ditylum brightwellii (West) Grunow	X85386[1]	AJ536460	CCMP
Eucampia antarctica (Castracane) Mangin	X85389		R. Crawford
Helicotheca tamesis (Shrubsole) Ricard	X85385[3]		CCMP
Lampriscus kittonii Schmidt	AF525667		A.-M. Schmid
Lauderia annulata (= *borealis*) Gran	X85399[3]	AJ536459	CCAP
Lithodesmium undulatum Ehrenberg	Y10569		CCMP
Odontella sinensis (Greville) Grunow	Y10570	AJ536457	K. Kowallik
Papiliocellulus elegans Hasle, von Stosch & Syvertsen	X85388		CCAP
Phaeoceros sp.	AJ535167		n/a
Planktoniella sol (Wallich) Schütt	AJ535173		A.-M. Schmid
Pleurosira cf. *laevis* (Ehrenberg) Compère	AJ535188		P. Snoeijs
Porosira pseudodenticulata (Hustedt) Jousé	X85398[1,3]		n/a
Skeletonema costatum (Greville) Cleve	X52006; X85395		CCAP
S. pseudocostatum Medlin	X85393; X85394		CCAP
S. menzelii Guillard, Carpenter & Reimer	AJ535168		CCMP
Skeletonema sp. CCMP 1009	AJ535165		CCMP
S. subsalsum (A. Cleve) Bethge	AJ535166		CCMP
Thalassiosira eccentrica (Ehrenberg) Cleve	X85396	AJ536458	CCAP
T. profunda (Hendey) Hasle	from S. Douglas		n/a
T. pseudonana Hasle & Heimdal	AJ535169		n/a
T. rotula Meunier	X85397		CCAP
Bacillariophyceae			
Araphids			
Asterionella formosa Hassall	AF525657		D. Czarnecki
Asterionellopsis glacialis (Castracane) Round	X77701	AJ536455	CCAP
Asteroplanus kariana (Grunow) Gardner & Crawford	Y10568		CCAP
Cyclophora tenuis Castracane	AJ535142		n/a
Diatoma tenue C. Agardh	AJ535143		D. Czarnecki
Fragilaria crotonensis Kitton	AF525662		D. Czarnecki
F. islandica Grunow in Van Heurck	AJ535190		n/a
Fragilaria sp.	AJ535141		n/a
F. striatula Lyngbye	X77704	AJ536453	CCAP
Fragilariforma virescens (Ralfs) Round & Williams	AJ535137		D. Czarnecki

Table 1. Continued

Species	18s rRNA	16s rRNA	Culture source
Grammatophora oceanica Ehrenberg	AF525655		n/a
Hyalosira delicatula Kützing	AF525654		n/a
Rhabdonema arcuatum (C. Agardh) Kützing	AF525660		A.-M. Schmid
Rhaphoneis cf. *belgica* (Grunow) Grunow	X77703		n/a
Staurosira construens Ehrenberg	AF525659		D. Czarnecki
Striatella unipunctata (Lyngbye) C. Agardh	AF525666		n/a
Synedra sp. (marine)	AJ535138		L. Medlin
S. ulna (Nitzsche) Ehrenberg	AJ535139		D. Czarnecki
Thalassionema nitzschioides (Grunow) Van Heurck	X77702[1]	AJ536454	CCAP
Thalassionema sp.	AJ535140		R. Crawford
Raphids			
Achnanthes bongranii (M. Peragallo) A. Mann	AJ535150		n/a
Achnanthes sp. 1	AJ535151		n/a
Planothidium lanceolatum (Brébisson) Round & Bukhtiyarova	AJ535189		D. Czarnecki
Amphora cf. *capitellata* Frenguelli	AJ535158		n/a
Amphora cf. *proteus* Gregory	AJ535147		n/a
A. montana Kronberg	AF243061		n/a
Anomoeoneis sphaerophora (Kützing) Pfitzer	AJ535153		D. Czarnecki
Bacillaria paxillifer (Müller) Hendey	M87325	AJ536452	n/a
Campylodiscus ralfsii C. Agardh	AJ535162		n/a
Cocconeis cf. *molesta* Kützing	AJ535148		n/a
Cylindrotheca closterium (Ehrenberg) Reimer & Lewin	M87326		CCAP
Cymbella cymbiformis W. Smith	AJ535156		D. Czarnecki
Encyonema triangulatum (Ehrenberg) Kützing	AJ535157		D. Czarnecki
Entomoneis cf. *alata* (Ehrenberg) Ehrenberg	AJ535160		n/a
Eolimna minuta (Grunow) Lange-Bertalot	AJ243063		n/a
E. subminutissima (Manguin) Moses	AJ243064		n/a
Eunotia pectinalis f. *minor* (Kützing) Rabenhorst	AJ535146		n/a
Eunotia sp.	AJ535145		D. Mann
Fragilariopsis sublineata Hasle	AF525665		R. Crawford
Gomphonema parvulum (Kützing) Kützing	AJ243062		D. Czarnecki
Lyrella sp.	AJ535149		D. Mann
Navicula diserta Hustedt	AJ535159		n/a
Nitzschia cf. *frustulum* (Kützing) Grunow	AJ535164		n/a
Peridinium balticum endosymbiont	Y10567		CCMP
P. foliaceum endosymbiont	Y10566		CCMP
Phaeodactylum tricornutum Bohlin	AJ269501		n/a
Pinnularia sp.	AJ535154		D. Mann
Pleurosigma sp.	AF525664		n/a
Pseudogomphonema sp. 1	AF525663		n/a
Pseudogomphonema sp. 2	AJ535152		n/a
Pseudo-nitzschia multiseries (Hasle) Hasle	from S. Douglas		S. Douglas
P. australis Frenguelli	from S. Douglas		S. Douglas
Sellaphora pupula v. *capitata* (Kützing) Mereschowsky	AJ535155		D. Mann
Surirella fastuosa v. *cuneata* A. Schmidt	AJ535161		n/a
Tryblionella apiculata (Gregory) D. Mann	M87334[3]		n/a
Undatella sp.	AJ535163		L. Medlin

[1] Sequence has been corrected in GenBank since its original deposition.
[2] Culture no longer available but voucher specimens available upon request; applies to all n/a entries.
[3] The name has been changed from that originally deposited in GenBank.
[4] Provasoli–Guillard National Center for Culture of Marine Phytoplankton.
[5] Culture collection of Algae and Protozoa.

nucleotides in length, with the exception of *Cymatosira belgica* and *Aulacoseira ambigua,* which were longer with several inserted helices (see Medlin *et al.* 1996b). Plastid-encoded SSU rRNA sequences for all taxa are approximately 1400 nucleotides in length. We present in Fig. 1 a subset of the ARB MP tree showing the heterokont lineage. In this lineage, the diatoms comprise three monophyletic clades: clade 1 containing the radial centrics, clade 2a the bipolar centrics and the radial Thalassiosirales and clade 2b the pennate diatoms (Table 1). It is not possible to perform BT analyses on all 8600 sequences in the ARB database, but we have done a BT analysis with the first 18S rRNA data set with 281 taxa selected for analysis and these values are shown in Figs 1, 3 along with the BT values for the second 18S data set from the NJ analysis. Nevertheless, producing a tree from so many taxa has distinct merit. Bollback (2002) noted that by increasing the number of sites (in this case species) from 1000 to 5000, the probability of committing a type II error drops from 95% to 10%. With more taxa, the number of informative sites dramatically increases, thus providing better resolution in the tree. The effect of increasing taxa and characters on the phylogenetic signal in a data set has been critically evaluated following Hillis & Huelsenbeck (1992). Therefore, we believe that a data set with 8600 sequences can produce a fully robust

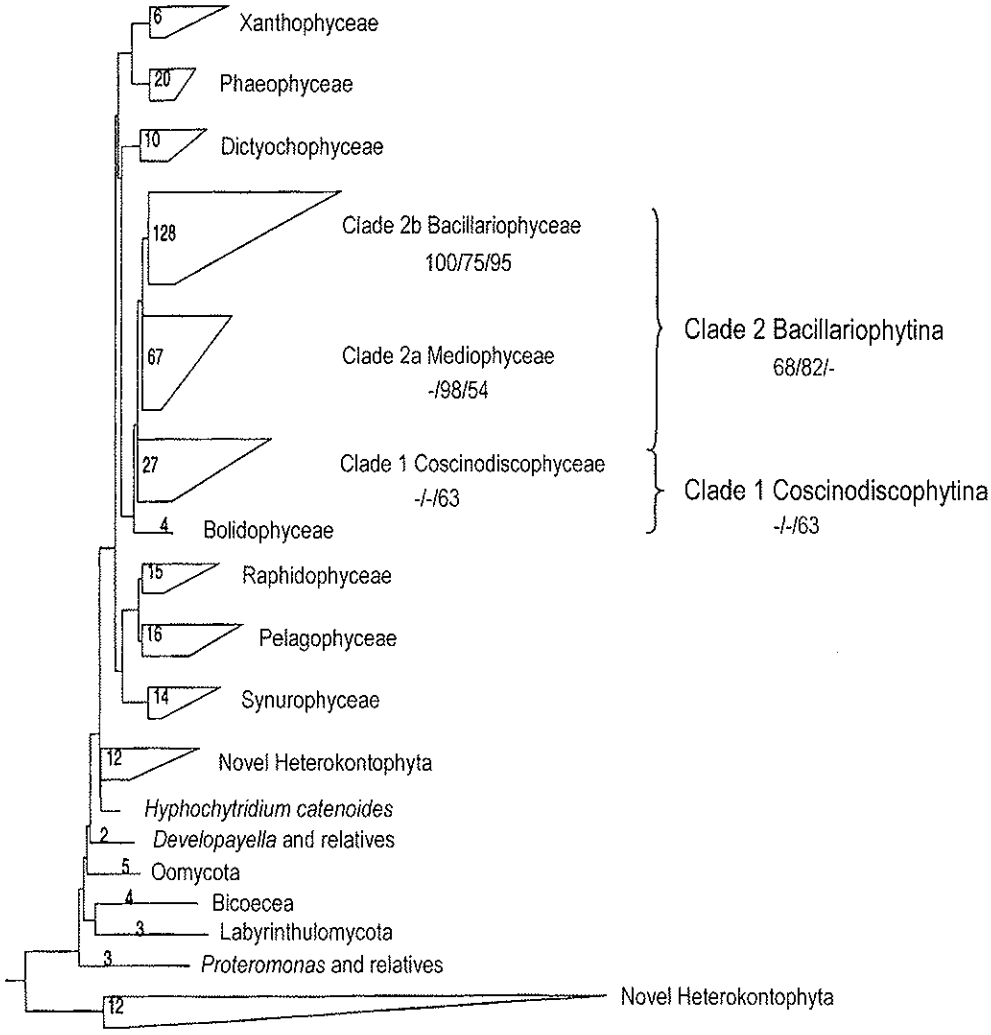


Fig. 1. Phylogeny for the Heterokonta produced from the ARB program using an MP analysis of 8600 sequences. The species are collapsed into triangles for clarity. The numbers inside each triangle refer to the number of sequences collapsed to make the triangle. The BT support on this tree comes from an MP analysis of 281 taxa and the BI and NJ analyses of the data set used for Fig. 3 (BI/MP/NJ).

tree, even though that tree cannot be bootstrapped. We present this tree from our larger data set to contrast with the analysis of the smaller data set, in which the monophyly of these three clades is not retained in all analyses because of the use of too few out-groups distantly related to the in-group. Computer constraints preclude us from including many more distant out-groups, and this obviously affects the monophyly of these three clades. The ML analysis for the second 18S data set alone ran for over three months. Despite this, clade 1 had BT support of 63% in the NJ analysis and clade 2a had 54% support. Clade 2b had the strongest support, being recovered 100%, 75%, and 95% of the time in the Bayesian, MP and NJ analyses, respectively. The Bayesian analysis also recovered clade 2a and clade 2b as a monophyletic grouping (PP = 68), whereas the MP analysis gave a BT value of 82% for clade 2. In the tree produced by the ARB program, the divergence of clade 1 from clade 2 diatoms occurs first, then clade 2 splits into 2a and 2b. A trichotomy of the three clades at the base of the diatoms is absent from this tree.

In our more detailed analysis, we used the three closest out-groups and 1764 positions out of 1800 for the analyses. A phylogeny of the diatoms inferred from the nuclear gene using the Bayesian method is presented in Fig. 2, one using the ML method in Fig. 3 and one from the plastid gene using the log det method in Fig. 4. In Figs 2, 3 the clades recovered correspond well to existing orders and families of diatoms and the support for these clades is seen best in the Bayesian tree (Fig. 2). Values for PP greater than 50% in the Bayesian tree are placed at the nodes in Fig. 2. BT values greater than 50% supporting the recovered branches in either the log det distance or the weighted MP–NJ trees are placed at the major internal nodes of the trees in Figs 3, 4.

In the Bayesian tree, we have consolidated the major clades into triangles at the order level for clarity (Fig. 2) and to show the high PP for each of the major clades. In this analysis, the first divergence among the diatoms is that of *Paralia* Heiberg from all other diatoms. Next, the radial centric diatoms diverge into two major clades. The bipolar centrics, the Thalassiosirales and the pennate diatoms form the next series of clades. Each clade has a high PP. The grade of clades encompassing the radial centrics forms the clade referred to as clade 1 in Medlin *et al.* (1995, 1997, 2000), whereas the grade of

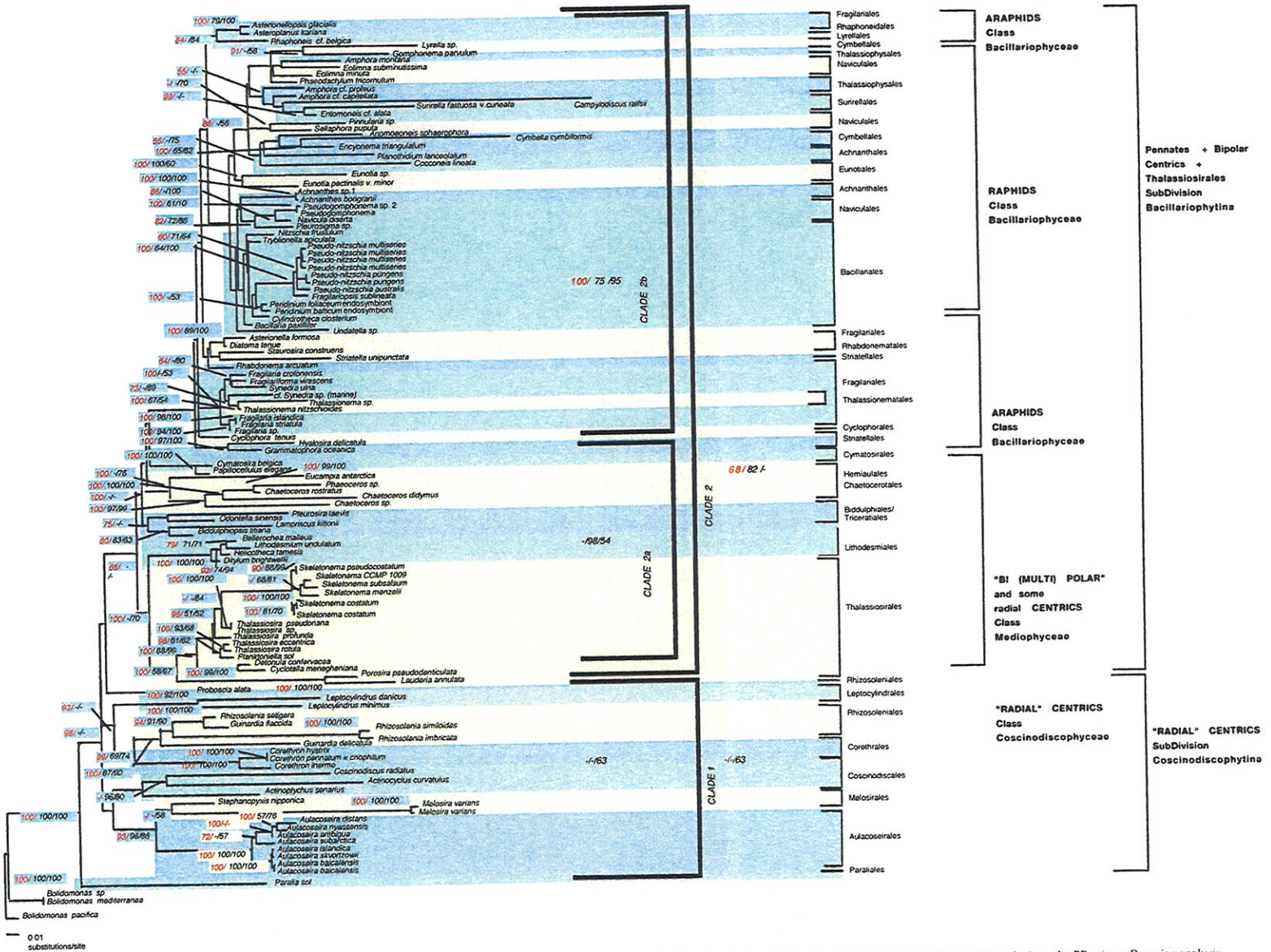


Fig. 3. Phylogeny inferred with the ML analysis using the 18S rRNA gene using 126 taxa. The numbers at the nodes are the BT values obtained from 500 replications of the data set for the BT analysis or the PP using a Bayesian analysis. Numbers are from the following analyses: Bayesian (red)/MP/NJ (Felsenstein 1985). Diatom orders are bracketed in the tree as well as the major clades, classes and subdivisions recovered in the analysis.

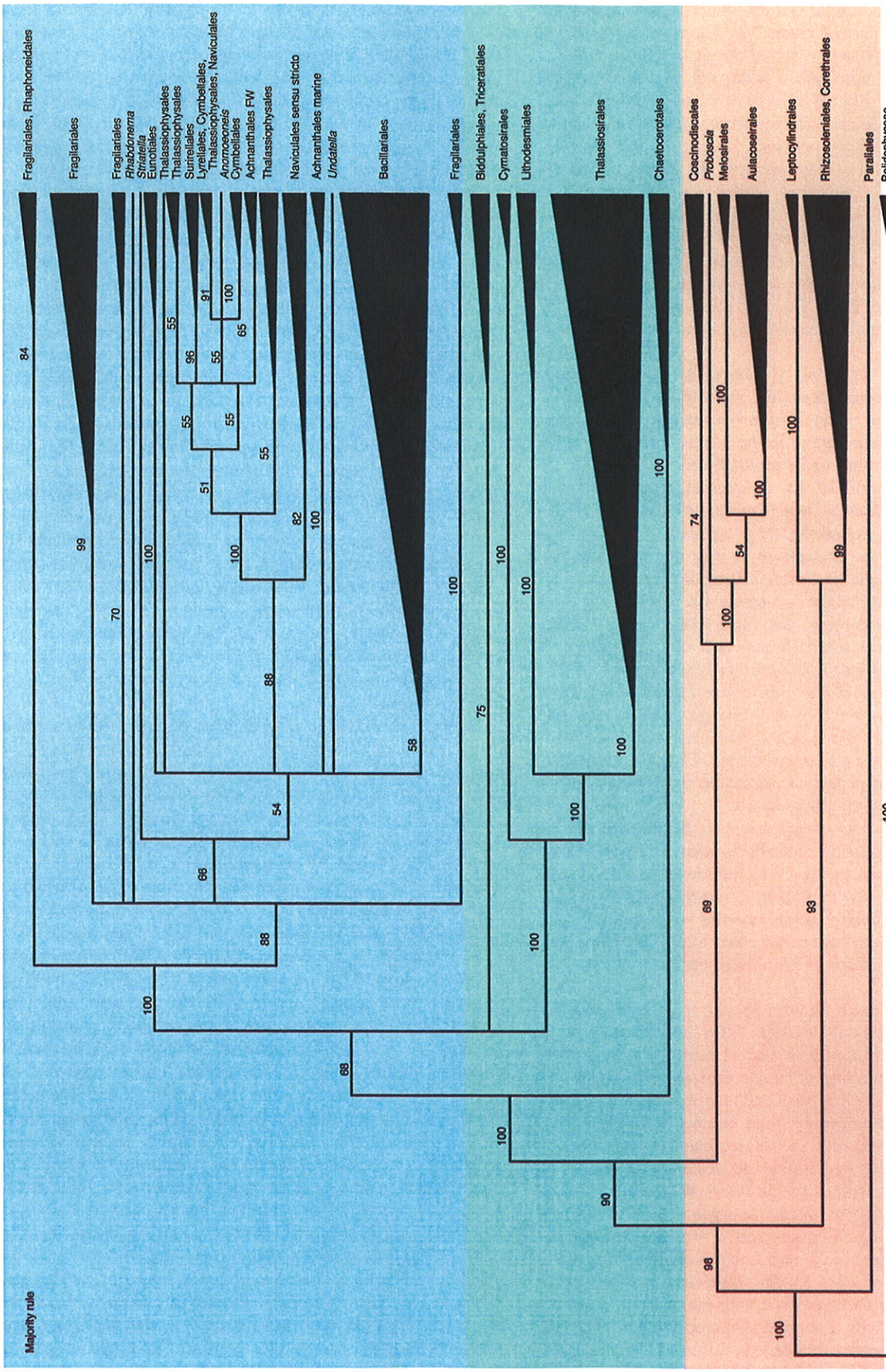


Fig. 2. Phylogeny inferred with the Bayesian analysis. Major clades are collapsed into triangles for clarity. PP > 50% are placed at each node. Pink, clade 1; green, clade 2a; blue, clade 2b.

clades containing the bipolar centrics, the Thalassiosirales and the pennates forms part of clade 2 as described in these papers. Here we will present a set of previously unappreciated characteristics (morphological, cytological, reproductive and fossil) that can be attributed to each of these grades of clades supporting the contention that they form a cohesive group of taxa and will be referred to as clade 1, clade 2a and clade 2b, hereafter, for clarity. The phylogeny of the diatoms does not recover the traditional dichotomy of the group into centrics or pennates (but see discussion in Simonsen 1972, 1979) or to the three classes of diatoms in Round *et al.* (1990). In the ML and log det analyses, all the taxa analysed are displayed in full detail. Again, the diatoms are essentially divided into a grade of clades (Figs 3, 4). Each of these minor clades corresponds best to ordinal or family level in current diatom systematics (Figs 3, 4).

Members of the Paraliales, Coscinodiscales, Rhizosoleniales, Corethrales, Leptocylindrales, Melosirales and Aulacoseirales were found in clade 1 from the nuclear analysis and most of them were represented in the plastid analysis (Figs 3, 4). This clade was found to be monophyletic in the NJ analysis with a BT support of 63. In general, these taxa have labiate processes; when present (Corethrales and Leptocylindrales do not have processes), they are located peripherally around the valve face. Exceptions to this do occur, for example (1) in large cells of *Coscinodiscus wailesii* Gran there are at least 50 to 100 labiate processes on the valve face in addition to the marginal ones, and (2) in modern *Azpeitia* Peragallo there is a single central labiate process in addition to a marginal ring of labiate processes. In the latter case we believe that one of the marginal labiate processes moved to the valve centre after the emergence of this lineage. Sims (1994) has stated that *Azpeitia* probably arose from *Actinocyclus* Ehrenberg, which only has marginal processes. Clearly, extant genera with only central processes should be targeted for rRNA analysis to investigate this hypothesis.

Also, in many species of *Rhizosolenia* Brightwell, the labiate process appears to be centrally located. Van den Meene (2002) has shown that during valve morphogenesis in *Rhizosolenia* (e.g. *R. setigera*) the labiate process rotates from near the periphery to its final position near the valve centre. Thus, we suggest that modern taxa in the grade of clades comprising clade 1 that have a central process have acquired this feature secondarily.

Paralia roots the entire diatom lineage in all analyses, but has strong morphological ties with other centric diatoms in clade 1, so we have placed it in clade 1 rather than in its own clade. It has a marginal ring of labiate processes and its closest morphological relative, *Ellerbeckia* Crawford, has a G–ER–M unit for its Golgi arrangement (see below). It is on a long branch and its basal position in our tree may be an artefact of long-branch attraction, which tends to place long branches at the base of the tree (Stiller & Hall 1999). Within clade 1, the ML analysis collapsed two major lineages (1 = Coscinodiscales–Melosirales–Aulacoseirales and 2 = Leptocylindrales–Rhizosoleniales–Corethrales) and could not determine their branching order. However, in the Bayesian analysis, their branching order was well resolved with the taxa that have an elongated pervalvar axis diverging first (see lineage 2 above). There was no BT support for these two lineages within clade 1 in the ML tree, but there was moderate to strong support in the Bayesian analysis (from 93 to 69, Fig. 2). Within these two lineages there are morphologically cohesive subclades with BT and PP support and they correspond to existing diatom orders (Round *et al.* 1990). Taxa with large, elaborate macrolabiate processes (Hasle & Sims 1990) belong to both these lineages. In the first lineage of the ML tree and in the second lineage of the BI are the orders Coscinodiscales and Melosirales, sister to the Aulacoseirales. In the second lineage of the ML tree are the orders containing diatoms with deep pervalvar axes, i.e. the Corethrales and Rhizosoleniales, sister to the Leptocylindrales. Fryxell & Hasle (1977) suggested a close relationship between *Rhizosolenia* and *Corethron* Castracane because of similar girdle band architecture. Round *et al.* (1990) erected the order Corethrales because *Corethron* lacks any kind of processes. Within the Rhizosoleniales, the genus *Rhizosolenia* is not monophyletic and species of *Guinardia* Peragallo root each of two lineages in this clade. Further investigations into the relationship between *Guinardia* and *Rhizosolenia*, as shown in the molecular analyses, are warranted. Some taxa in the second lineage (order Melosirales) have only small microlabiate processes (Round *et al.* 1990). These two major lineages of clade 1 are followed by the divergence of a third lineage in the ML tree represented by the single sequence of *Proboscia alata*. The Bayesian analysis placed *Proboscia* Sündstrom in the clade with Coscinodiscales as sister to the Melosirales and Aulacoseirales (PP = 100%). *Proboscia alata* is not affiliated with the Rhizosoleniales, a separation that is supported by their different morphology and certain details of the cytoplasm during the cell cycle (van den Meene 2002), and a new order should be erected for this genus.

Clade 2a contains the second series of clades with the radial centrics of the Thalassiosirales and the bipolar centrics. Again these grades can be clustered into systematically (to imply relationships) and taxonomically (to mean describable) meaningful groups at the order level in the diatoms. The Lithodesmiales and the Thalassiosirales root clade 2a in the ML tree with strong BT and PP support (100%), followed by the sequential unsupported divergences of other bipolar centrics, such as the Biddulphiales, the Chaetocerotales followed by the Cymatosirales (all of clade 2a), and finally the pennate diatoms (clade 2b). In the Bayesian tree, the Chaetocerotales root clade 2a followed by the same orders as in the ML tree, but this time with strong support (100 PP) for their branching order. Clade 2a is recovered as a monophyletic group in the NJ analysis with a BT support of 54% and 98% in the MP analysis. Medlin *et al.* (1996a) already documented that the Lithodes-miales were the true sister group of the Thalassiosirales and that removal of this group as its sister group yielded a less robust tree. Most of the diatoms in clade 2 *sensu lato* have a distinct structure in the middle of the valve. The order Thalassiosirales has (usually) one large marginal labiate process but one or more central strutted processes. The bi- or multipolar centric diatoms of the orders Lithodesmiales, Cymatosirales, Biddulphiales and Chaetocerotales have one–two small but centrally located labiate processes or an annulus (see examples in Cymatosiraceae), although the latter is also found in clade 1. The pennate diatoms have a central rib or raphe, although in some species this is moved to a lateral position during valve morphogenesis. The labiate processes of the ar-

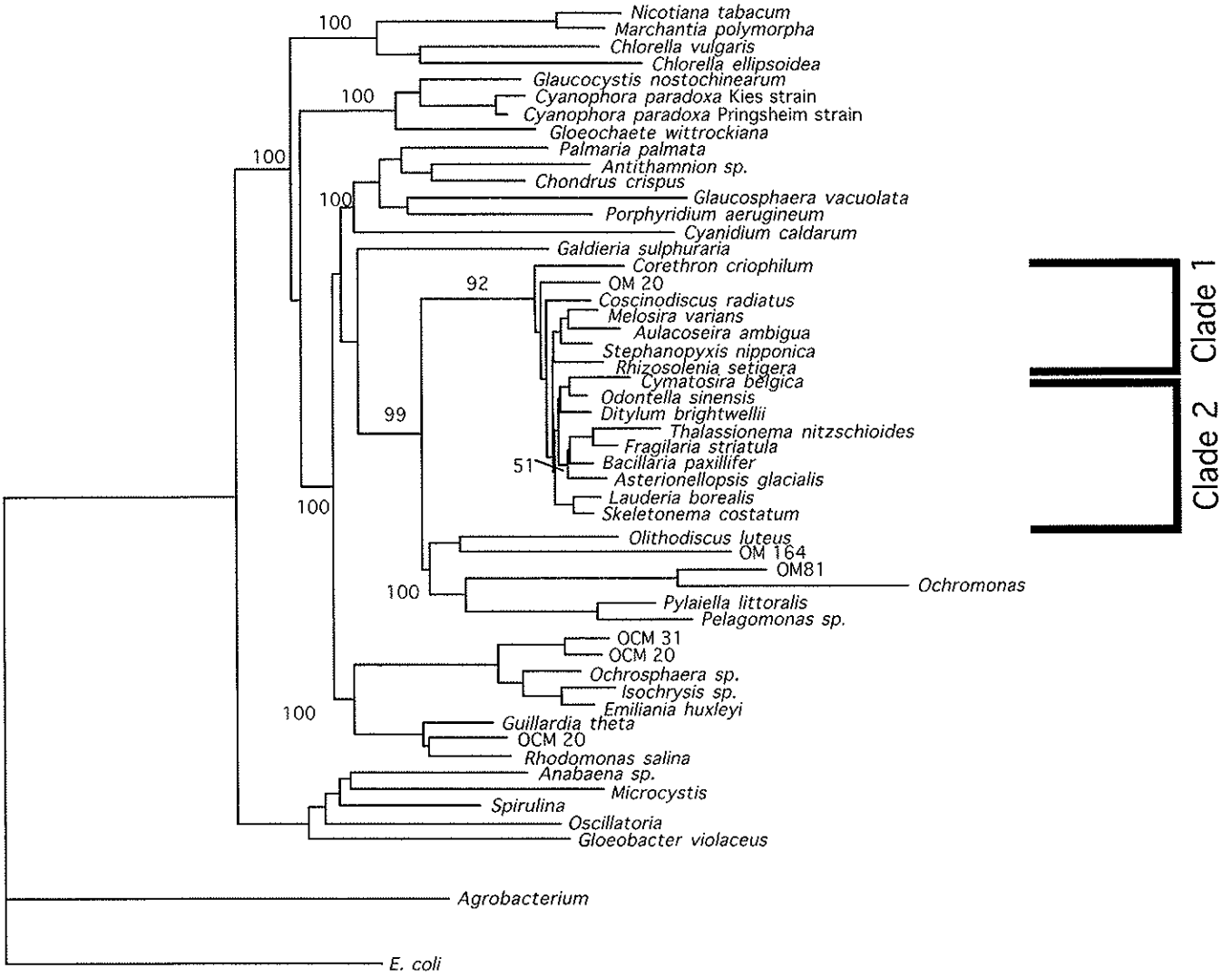


Fig. 4. Phylogeny of the diatoms inferred with the log det distance method using the 16s rRNA gene. The numbers at the nodes are the BT values obtained from 500 replications of the data set.

aphid taxa are usually small and are commonly located at the apical ends of a central sternum.

The pennate diatoms form clade 2b. Among them, the araphid taxa are paraphyletic. The first divergence in the araphid taxa in the ML tree are the genera *Grammatophora* Ehrenberg and *Hyalosira* Kützing with very strong BT and PP support (100/98/100), and then *Cyclophora* Castracane diverging with the Fragilariales. The early divergence of *Grammatophora* and *Hyalosira* is of particular interest because of the macro- and microamoeboid gametes found in *Grammatophora* (see references in Drebes 1977b), which may represent an intermediate between true oogamy (characteristic of centrics) and isogamy (common in pennates). *Rhabdonema* Kützing, with its macro- and microgametes, is a later divergence among the araphids and appears to have retained this transitional feature in the sexual reproduction of the diatoms. The freshwater and marine species of *Fragilaria* Lyngbye do not form a monophyletic group and each has strong BT and PP support as a separate clade (Fig. 3). *Fragilaria sensu stricto* is marine (Hasle & Syvertsen 1981), whereas *Fragilaria crotenensis* and *Fragilariforma* Williams & Round are freshwater (Williams & Round 1986) and should be kept in distinct genera as shown here by our molecular analysis (see discussion in Williams & Round 1986). Round *et al.* (1990) have suggested that *Fragilaria* as a genus should be conserved and only contain the freshwater species, although none of the freshwater species assigned to *Fragilaria* (Williams & Round 1986) were included in the original circumscription of *Fragilaria* (see discussion in Hasle & Syvertsen 1981). Many of the new genera founded by Round & Williams (Round *et al.* 1990) are distinctly different from *Fragilaria* spp. *sensu stricto.*

The araphid taxa bearing septa are not grouped together because *Grammatophora–Hyalosira* and *Rhabdonema* and *Striatella* C. Agardh are in separate clades. The other septate genera, *Pteroncola* Holmes & Croll, *Tabellaria* Ehrenberg and *Tetracyclus* Ralfs have not been sequenced, but we would predict that the marine *Pteroncola* would fall with *Grammatophora,* and the freshwater genera would form their own clades as the freshwater and marine *Fragilaria–Synedra* Ehrenberg spp. have segregated. *Asterionellopsis* Round and *Rhaphoneis* Ehrenberg are the araphids most closely related to the raphid diatoms, despite the early appearance of *Rha-*

phoneis in the fossil record, although this position in the ML tree (Fig. 3) does not have any BT support. In the Bayesian tree (Fig. 2) their position is more strongly supported because they are the first to diverge, followed by an unresolved branching order to all other raphid and araphid taxa.

In all trees, the raphid diatoms are monophyletic with very strong BT and PP support (Figs 1–4). In the ML tree they diverge simultaneously into a naviculoid-like and nitzschioid–naviculoid *sensu stricto* lineage, although this is not supported by the BT analysis. The naviculoids *sensu stricto* are the sister group to the nitzschioid diatoms. Eunotiaceae falls unsupported at the base of all other naviculoid diatoms. In an earlier analysis with only a single *Eunotia* sequence, the Eunotiales also fell at the base of all raphid diatoms (Medlin *et al.* 2000).

Among the other naviculoid diatoms, we find a more or less simultaneous divergence of a lineage of cymbelloid taxa rooted by monoraphid diatoms, one lineage of amphoroid taxa, one lineage of *Pinnularia* Ehrenberg and *Sellaphora* Mereschowsky, and *Gomphonema* Ehrenberg mixed with *Lyrella* Karayeva and *Eolima* Lange-Bertalot, whose species were formerly placed in *Navicula* Bory, as well as one species of *Amphora* Ehrenberg and *Phaeodactylum* Bohlin. The branching order of these clades has mixed support, with the first divergence of the cymbelloid taxa rooted by monoraphid taxa receiving the highest support (Fig. 3). The nitzschioid diatoms are monophyletic. Fibulae have arisen at least twice, e.g. in the Bacillariaceae and in *Entomoneis* Ehrenberg and in the Surirellales, the last two groups found in the other naviculoid lineage and not in the *Navicula sensu stricto* lineage. The relationships recovered from the rRNA tree suggest that the unique raphe system of the surirelloid diatoms is likely to have its origins in the amphoroid diatoms and not in the nitzschioid lineage. The genus *Nitzschia* Hassall is represented by only one species and so we do not know yet if it is monophyletic or not. The monoraphids have evolved at least twice. The marine *Achnanthes* Bory roots the naviculoid *sensu stricto* lineage, whereas the freshwater *Planothidium* Round & Bukhtiyarova and *Cocconeis* Ehrenberg are sister to the cymbelloid lineage, including *Anomoeoneis* Pfitzer.

In the Bayesian tree (Fig. 2), all of the naviculoid taxa form a monophyletic lineage with strong PP support, and similar relationships are found to those in the ML tree (Fig. 3). One major exception is that *Undatella* Paddock & Sims falls outside the Bacillariales.

In the plastid tree (Fig. 4), similar divergence patterns to the nuclear 18S SSU tree are recovered. *Corethron,* an unidentified diatom plastid sequence from a clone library taken from near shore waters (Rappé *et al.* 1997), and then *Coscinodiscus* Ehrenberg form a continuous grade of divergences, and then the diatoms divide into two groups. The first group contains the remaining clade 1 diatoms as found in the 18S SSU tree, whereas the second group contains the clade 2 diatoms of the bipolar centrics plus the Thalassiosirales and the pennate diatoms. Similar relationships are found in the lineages comprising clade 2 diatoms. An initial divergence of the Thalassiosiroid diatoms is followed by a divergence of the bipolar centrics and then the pennate diatoms (Fig. 4). In all trees, the bipolar centrics are the last centrics to diverge before the pennate diatoms.

Cytoplasmic support for molecular data

GOLGI ARRANGEMENT: Next we analysed the internal structure of the cytoplasm to support the phylogeny recovered in our tree. Among the diatoms, there appear to be at least three, possibly four types of arrangement of Golgi apparatus (Figs 5–11; Medlin *et al.* 2000, fig. 2). The centric genera of clade 1 studied to date have their Golgi bodies associated with a cisterna of endoplasmic reticulum (ER) and a mitochondrion, e.g. *Coscinodiscus* (Schmid 1984a, 1988), *Stephanopyxis* (Ehrenberg) Ehrenberg (see reference in Medlin *et al.* 2000) and *Ellerbeckia* (similar to *Paralia*) (Schmid & Crawford 2001), which we call type 1. In the cortical cytoplasm, the mitochondrion faces the plasmalemma (Fig. 5), but near the nucleus, the mitochondrion faces the nucleus (Fig. 6). This association, termed the G–ER–M unit, is also found in the oomycetes and the red algae (Schmid 1988) and may be an archaic character inherited from ancestors common to oomycetes and diatoms. Because this feature is also found in the red algae, it could be a feature brought into the lineage with the plastid endosymbiosis. In the kleptoplastidy of *Euglena* Ehrenberg by the ciliate *Perispira ovum,* both its plastid and mitochondrion are retained in this modern-day repeatable endosymbiosis (Johnson *et al.* 1995). One exception to the distribution of the G–ER–M unit in the rRNA tree can be found in the Aulacoseirales, the last lineage in the Bayesian tree to diverge before the clade 2 diatoms, which have another type of Golgi arrangement (type 2).

Most pennates, the Thalassiosirales and the bipolar centrics, which all belong to clade 2, have type 2 Golgi arrangement. This involves the Golgi bodies encircling the nucleus to form a perinuclear shell or ring in profile (Fig. 7) with the forming face of the Golgi against the nuclear membrane. The Labyrinthuloides have only two Golgi bodies and they are positioned

→

Figs 5–11. Illustrations of various arrangements of Golgi bodies. Abbreviations (not all relevant, because the figures are reproduced from elsewhere): Av, vacuole; cPl, plasmalemma; D (G in Fig. 10), dictyosome (golgi); DP, dark particle; ER, endoplasmic reticulum; M, mitochondria; N, nucleus; NP, nuclear pore; SDV, silica deposition vesicle; Si, silica wall; T, nuclear tentacle (in Fig. 8, microtubules).

Fig. 5. Type 1: G–ER–M unit of *Coscinodiscus wailesii* in cortical protoplasm. Reproduced from Schmid (1988), courtesy of Springer-Verlag.

Fig. 6. Type 1: G–ER–M unit of *C. wailesii* spermatocyte near nucleus. Reproduced from Schmid (1988), courtesy of Springer-Verlag.

Fig. 7. Type 2: perinuclear shell of Golgi bodies in *Stephanodiscus niagarae* Ehrenberg. Reproduced from Drum *et al.* (1966), courtesy of Koeltz.

Figs 8, 9. Type 2.1: arrangement of Golgi bodies in *Synedra.* Reproduced from Schmid (1989), courtesy of Springer-Verlag.

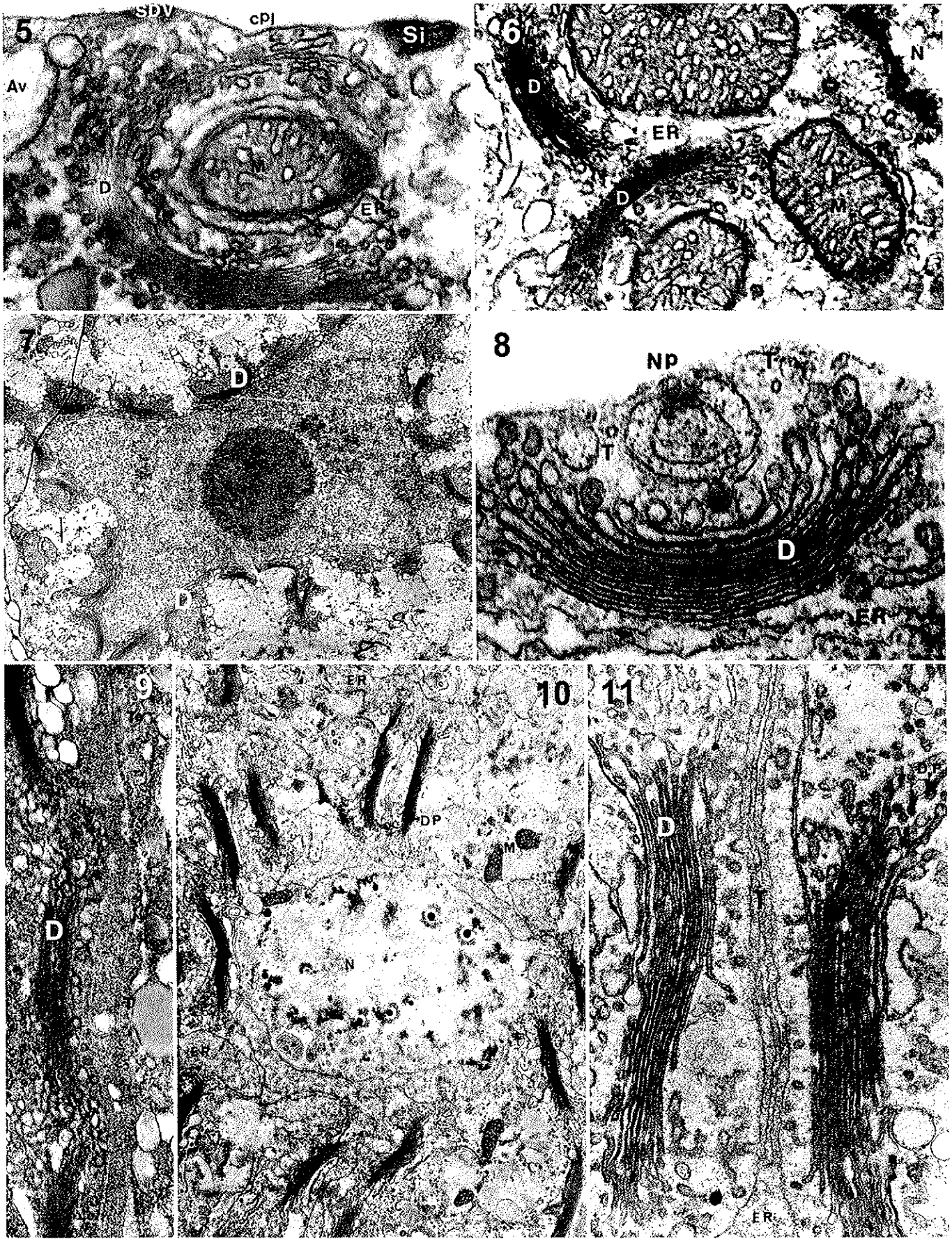
Fig. 8. Golgi positioned along the nuclear tentacle.

Fig. 9. Longitudinal section along the nuclear tentacle.

Figs 10, 11. Type 2.2: arrangement of Golgi bodies in *Pinnularia.* Reproduced from Drum (1966), courtesy of Academic Press.

Fig. 10. Paired Golgi ('Doppelplätchen') along nuclear tentacle in cross-section.

Fig. 11. Paired Golgi ('Doppelplätchen') along nuclear tentacle in longitudinal section.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next to the nucleus with their forming face against the nucleus (Porter 1989). The taxa possessing this trait can be found plotted on the tree shown in fig. 1 of Medlin *et al.* (2000). Detailed literature references to the type of Golgi structure shown in Medlin *et al.* (2000) can be requested from A.-M. Schmid (Annemarie.Schmid@sbg.ac.at). One modification of the perinuclear shell (termed Golgi type 2.1) occurs in *Synedra ulna,* in which the nuclear envelope, together with nuclear matrix and RNA, but no DNA, is pulled out into two long tentacles reaching the cell poles (Schmid 1989). Golgi bodies are aligned along one side of the tubes, i.e. the 'Plattenband' *sensu* Geitler (Schmid 1989), whereas nuclear pores lie on the other side of the tentacle (Figs 8, 9). Schmid (1989) speculated that this arrangement probably became necessary to cope with a space problem in a diatom 8 by 12 μm wide and 500 μm long. Golgi type 2.2 (Figs 10, 11) can be found in *Pinnularia,* where nuclear tentacles are 'pulled out', not just in two directions, but also in multipolar directions, because of more available space around the nucleus than in *S. ulna.* In this case, the tentacles are much narrower where they exit the nucleus, leaving enough space on the nuclear envelope for pores, and thin out distally into ER cisternae. Golgi bodies are paired along the nuclear extensions ('Doppelplättchen'), i.e. on both sides of the tentacle.

Finally, in type 3 Golgi, found in *Biddulphiopsis titiana,* only the outer membrane of the nuclear envelope forms filose tentacles along which Golgi bodies are paired, whereas the remaining envelope around the large nucleus bears a porous pattern of the same density as that seen in *Coscinodiscus* (A.-M. Schmid, unpublished observations). Although the G–ER–M unit of *Coscinodiscus* can also be found along ER cisternae in the cortical cytoplasm, the type 3 Golgi arrangement has been found only along radial ER cisternae (see references in Medlin *et al.* 2000). If these four variations are plotted onto the rRNA tree, then it quickly becomes apparent that they are well correlated with certain clades in the tree (see Medlin *et al.* 2000, fig. 1). Type 1 (Schmid 1989) is found primarily in clade 1, with the exception of *Odontella sinensis* in clade 2, whereas type 2 dominates clade 2. Interestingly, other members of *Odontella* C. Agardh investigated possess the type 2 arrangement (Pickett-Heaps *et al.* 1986; Schmid 1988), and it can be suggested that *O. sinensis* has retained the ancestral state. Type 2.1 is found in only *S. ulna,* type 2.2 in *Pinnularia,* and type 3 in *Biddulphiopsis* von Stosch & Simonsen. Because Golgi arrangements 2.1, 2.2, and 3 are restricted in their distribution and are embedded in clade 2, it is then possible that they should all be considered a variant of Golgi type 2. Thus, we may have only two types of Golgi arrangement: (1) the G–ER–M unit and (2) the perinuclear shell, correlated with clades 1 and 2, respectively. One point that should be further investigated is the Golgi arrangement in other bipolar centrics, to see if they are similar to type 3 or if this arrangement is a one-off exception. The variations in the second type may be adaptations to cope with larger cells with extra cellular space. It also seems likely that whatever Golgi secretions are made in the diatoms of clade 1, they require a great deal of energy always to be associated with a mitochondrion for a constant supply of adenosine triphosphate.

AUXOSPORE DEVELOPMENT: The main function of the diatom auxospore is to restore maximum cell size and ensure the continued vegetative propagation of the population. In view of the strongly conserved nature of reproductive structures and zygotes in a great number and variety of biota, we regard the auxospore as an entity that is strongly linked to the evolutionary transformation from the ancestors into the diatoms proper (Round 1981; Round & Crawford 1981). The auxospore is thus well positioned to record major evolutionary divergences within diatoms (Kaczmarska *et al.* 2001).

Formation of the auxospore, in reality not a spore, is usually associated with the sexual phase of the diatom life cycle and is best documented as a result of fertilization (Round *et al.* 1990; Mann 1993). In modern diatoms, it undergoes a sequence of developmental stages (ontogeny); some of these are common to all auxospores, whereas others are characteristic of specific groups (von Stosch 1982; Kaczmarska *et al.* 2000, 2001). Most auxospores whose fine structure and developmental history have been documented using transmission or scanning electron microscopy have cell walls made of two components: a basic, expanding organic matrix and, embedded within it, siliceous scales or bands (Crawford 1974; von Stosch 1982; Round *et al.* 1990; Kaczmarska *et al.* 2000). The auxospores of species producing gametes free of parental investments (e.g. thecae, mucilaginous envelopes) begin their development with a globular form. Auxospores that are extensively attached to the parental theca [some centric diatoms, such as species of *Lampriscus* A. Schmidt (Idei & Nagumo 2002)] or confined to specialized structures (theca, papillae, mucilaginous envelope – many benthic pennates) modify their shape to fit this confinement or to facilitate attachment. At later stages of development, auxospores will follow one of the two distinct developmental pathways: isodiametric or nonisodiametric. The feature best defining the two types of growth is the presence or absence of bands. When bands are absent, the auxospore expands equally (or nearly so) in all directions. Such expansion results in globular auxospores (isometric *sensu* Drebes 1977b; Kaczmarska *et al.* 2001). Siliceous bands restrict auxospore growth to one or a few directions and lead to the production of nonisometric auxospores, either properizonial (complex shapes, anisometric; von Stosch 1982; Kaczmarska *et al.* 2001) or perizonial (tubular shape, bilateral; von Stosch 1982; Kaczmarska *et al.* 2001).

Pliable organic walls fortified with siliceous scales facilitate the isometric swelling and generally lead to an initial cell with strongly convex (dome-shaped) valves. These valves are formed beneath the auxospore wall and follow its contours. In contrast, the uni- or multidirectional expansion facilitated together by a system of scales and bands allows the auxospore to expand or swell only in a specific number of directions (two, three, etc.), which correspond to the elongated, triangular, etc., shape and the fairly flat (often following plasmolysis) future initial valve. There is a great deal of variation in the exact details of specific auxospore development, and not all stages are equally well known (Kaczmarska *et al.* 2001), phylogenetically informative (Mann 1993) or documented.

This is particularly true when available knowledge is based exclusively on light microscopy, a methodology that is inadequate for examining the minute, thin or lightly silicified structures such as scales or thin bands. Scales in the auxospore walls were first shown with the transmission electron microscope by Reimann (1960). Thus, by necessity, this summary is limited to auxospore wall fine structure and development

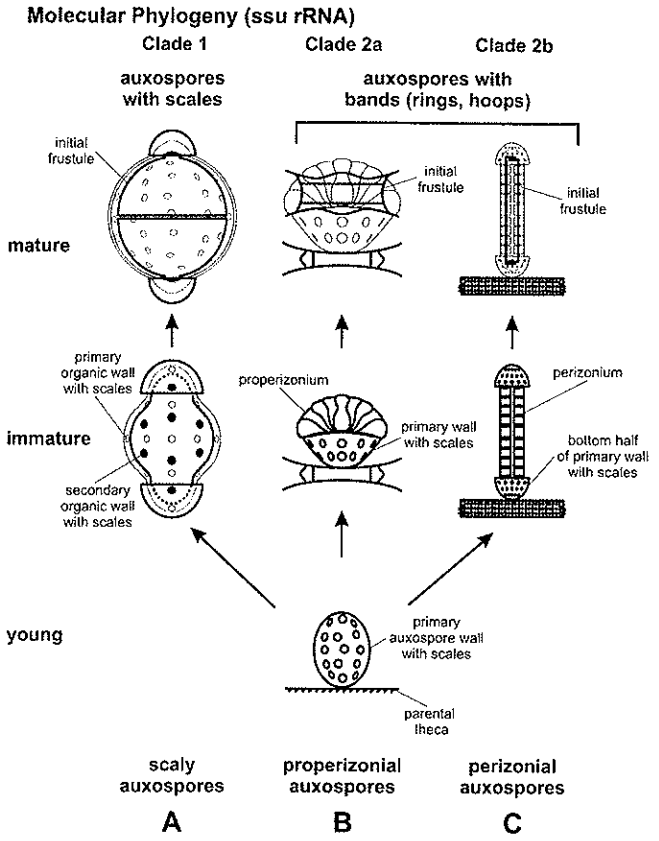


Fig. 12. Diagrammatic summary of the types of sexual auxospore in diatoms and their relationship to classical and molecular phylogenies.

and the general form of initial valves for species where these characters have been documented using electron microscopy. An exception is made for the Corethrales and the Rhizosoleniales, which form a substantial molecular sister clade to other radial centrics, for which only data from light microscopy are available to date. Most of the specific terminology used here follows the definitions in Kaczmarska *et al.* (2001), except for the names of the auxospore types.

Scaly auxospores: Auxospores with only scaly walls (isometric auxospores *sensu* Drebes 1977b; Kaczmarska *et al.* 2001; Fig. 12A) remain globular or nearly so throughout auxospore development (e.g. Crawford 1974, 1975; Schmid 1994b, c). The auxospore wall of this type consists of organic matter and scales, and is found in genera with circular valves, such as *Melosira* C. Agardh, *Ellerbeckia, Stephanopyxis, Coscinodiscus,* etc. (Table 2). Their enlargement is facilitated by a dilatable organic matrix carrying (presumably) siliceous scales integrated into a continuous envelope, and by the addition of scales into the innermost (youngest) layers of the walls. This mode of development results in relatively uniformly distributed scaly walls surrounding the entire auxospore protoplast. Species utilizing the parental theca as partial cover for the auxospore may not produce scales underneath the parental theca (e.g. some species of *Melosira*; Round *et al.* 1990, p. 98, fig. 63a). This architecture does not detract from the overall globular shape of the auxospore. When mature, these auxospores contain strongly convex initial valves (*Aulacoseira* Thwaites, *Melosira, Stephanopyxis*) mimicking the globular shape of their auxospores. These nearly globular initial cells will eventually produce two conventional valves after mitosis and cell division. Each of the two daughter cells will thus have one conventional valve and one initial valve, but both cells will then produce a conventional cell in addition to a copy of itself at subsequent divisions. This type of auxospore is common among radial centric species with processes positioned close to the valve face periphery (e.g. *Stephanopyxis, Actinocyclus*; Table 2). These diatoms are also members of clade 1 of the molecular phylogenetic tree based on 18S rRNA shown here (Figs 1–3) and in Medlin *et al.* (1997). Exceptions to this phylogenetic grouping are diatoms from the Order Thalassiosirales from clade 2a (e.g. *Thalassiosira* Cleve, *Skeletonema* Greville and *Cyclotella* Kützing). No scales were seen on the auxospores of *Stephanodiscus* Ehrenberg (Round 1982, fig. 4a–d). Radial symmetry and scaly auxospore walls are similar to those seen among members of clade 1, but may represent ancestral characters retained by the taxa derived from the lineage with nonisometric auxospores (e.g. *Lithodesmium* Ehrenberg; Figs 2–4).

Auxospores of *Rhizosolenia* and *Corethron,* representing one of the major lineages within clade 1, have not yet been exhaustively examined with electron microscopy, nor with respect to their ontogeny and wall structure. The sexual process has not been fully documented in these genera and it would seem that the tubular 'auxospores' depicted in the literature represent a previously unrecognized mechanism of cell enlargement. However, the sequence of figures shown by Cupp (1943) and unpublished observations by R. Crawford on *Corethron hystrix* and *C. pennatum* indicate that Cupp's fig. 34C (parts c and e) are indeed the first stages of expansion of the fertilized female cell. The next stage of *C. hystrix* auxospore development illustrated by Cupp (1943) is fig. 34C (part d). Crawford has established that the fully expanded sphere forms spineless hemispherical initial valves within the auxospore wall (in which scales have not yet been found). This initial cell then expands in the pervalvar axis to form a long cylinder. This stage must be accompanied by multiple girdle band formation in one of the cingula, like all vegetative cells do without the need for the protection given by an auxospore wall. On completion of this stage, in *C. pennatum* (R. Crawford, unpublished observations) the cytoplasm then retracts again to form a small sphere that leaves a void within the wall of the initial cell. In other genera, a conventional mitosis would then follow, resulting in cytokinesis and the formation of two conventional valves. In *C. pennatum,* however, only one valve is formed into the void, and because valve formation takes place only after nuclear division, it is supposed that a depauperate mitosis occurs here (R. Crawford, unpublished observations). The new valve regularly has both types of spines, thus preserving the polarity of the mother cell or female gametangium in *C. pennatum* and in *C. hystrix* [Cupp 1943, fig. 34C (part b)]. This elaborate expansion and contraction of the initial cell is clearly to accommodate the formation of the spines of the new valve within the protection of the cingulum. It results in a heterovalvar cell that has one initial valve and one conventional valve that is still attached to one of the valves of the mother cell. The contours of the initial cell may project some distance into the mother-cell valve and, in the case of the cell illustrated by Drebes (1974, fig. 11d), completely so.

In *Rhizosolenia,* the process is outwardly very different be-

Table 2. Summary of molecular phylogeny and the structure of sexual auxospores in diatoms. Only the taxa that had been examined using both light microscopy and electron microscopy are included. Numbers in parentheses following the names of diatoms indicate sources: 1, M. Idei, unpublished observations; 2, Crawford (1975); 3, Schmid & Crawford (2001); 4, Crawford (1981); 5, von Stosch (1954, 1982); 6, Schmid (1994c); 7, Hargraves (1990); 8, Hoops & Floyd (1979); 9, Schmid (1984b); 10, von Stosch *et al.* (1973); 11, Drebes (1977a); 12, Drebes (1972); 13, Idei & Nagumo (2002); 14, von Stosch (1962); 15, Mann (1982a); 16, Mann (1984); 17, Mann (1989); 18, Cohn *et al.* (1989); 19, Mann & Stickle (1989); 20, Mann & Stickle (1995); 21, Kaczmarska *et al.* (2000); 22, Mann (1987, unpublished observations); 23, Mizuno (1994); 24, Mizuno (1998); 25, I. Kaczmarska, unpublished observations.[1]

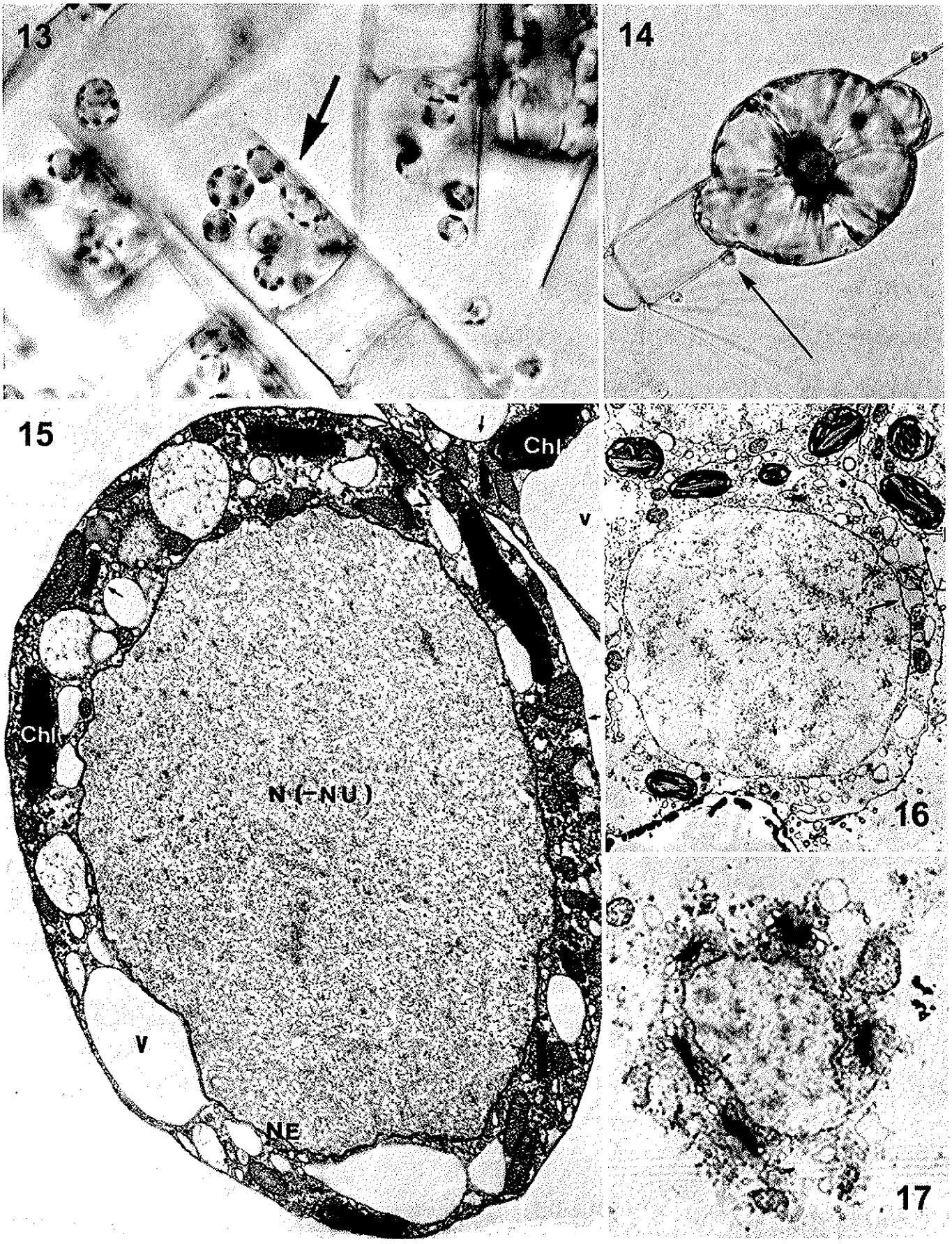
Symmetry	Genus	Mucilaginous matrix	Auxospore shape/ growth	Auxospore walls: Primary: Scales	Auxospore walls: Secondary: Properizonium	Auxospore walls: Secondary: Perizonium	Molecular clade
Radial	*Actinocyclus* (1)	–	G/Is	+	–	–	1
	Melosira (2)	–	G/Is	+	–	–	1
	Ellerbeckia (3)	–	G/Is	+	–	–	nd
	Orthoseira (4)	–	G/Is	+	–	–	nd
	Stephanopyxis (5)	–	G/Is	+	–	–	1
	Actinoptychus (5)	–	G/Is	+	–	–	1
	Coscinodiscus (6)	–	G/Is	+	–	–	1
	Leptocylindrus (7, 25)	–	G/Is	+	–	–	1
	Cyclotella (8)	–	G/Is	+	–	–	2(a)
	Thalassiosira (9)	–	G/Is	+	–	–	2(a)
	Skeletonema (25)	–	G/Is	+	–	–	2(a)
Multipolar	*Bellerochea* (5)	–	BMP/N	+	+	–	2(a)
	Lithodesmium (5)	–	BMP/N	+	+	–	2(a)
	Biddulphia (5)	–	BMP/N	+	+	–	nd
	Odontella (5)	–	BMP/N	+	+	–	2(a)
	Chaetoceros (5, 10)	–	BMP/N	+	+	–	2(a)
	Attheya (11)	–	BMP/N	+	+	–	nd
	Amphitetras (5)	–	BMP/N	+	+	–	nd
	Bacteriastrum (12)	–	Tu/N	nd	+*	–	nd
	Lampriscus (13)	–	BMP/N	+	+	–	2(a)
Bilateral	*Rhabdonema* (14)	+	E/B	+	–	+	2(b)
	Rhoicosphenia (15)	+	E/B	nd	–	+	nd
	Neidium (16)	+	E/B	+	–	+	2(b)
	Diploneis (1)	–	E/B	+	–	+	2(b)**
	Caloneis (17)	+	E/B	nd	–	+	2(b)
	Craticula (18)	+	E/B	nd	–	+	nd
	Navicula (19)	+/–	E/B	nd	–	+	2(b)
	Placoneis (20)	+	E/B	nd	–	+	nd
	Nitzschia (25)	–	E/B	+	–	+	2(b)
	Pseudo-nitzschia (21)	–	E/B	+	–	+	2(b)
	Surirella (22)	+	E/U/B	nd	–	+	2(b)
	Achnanthes (23)	–	E/B	nd	–	+	2(b)
	Cocconeis (24)	+	E/B	nd	–	+	2(b)

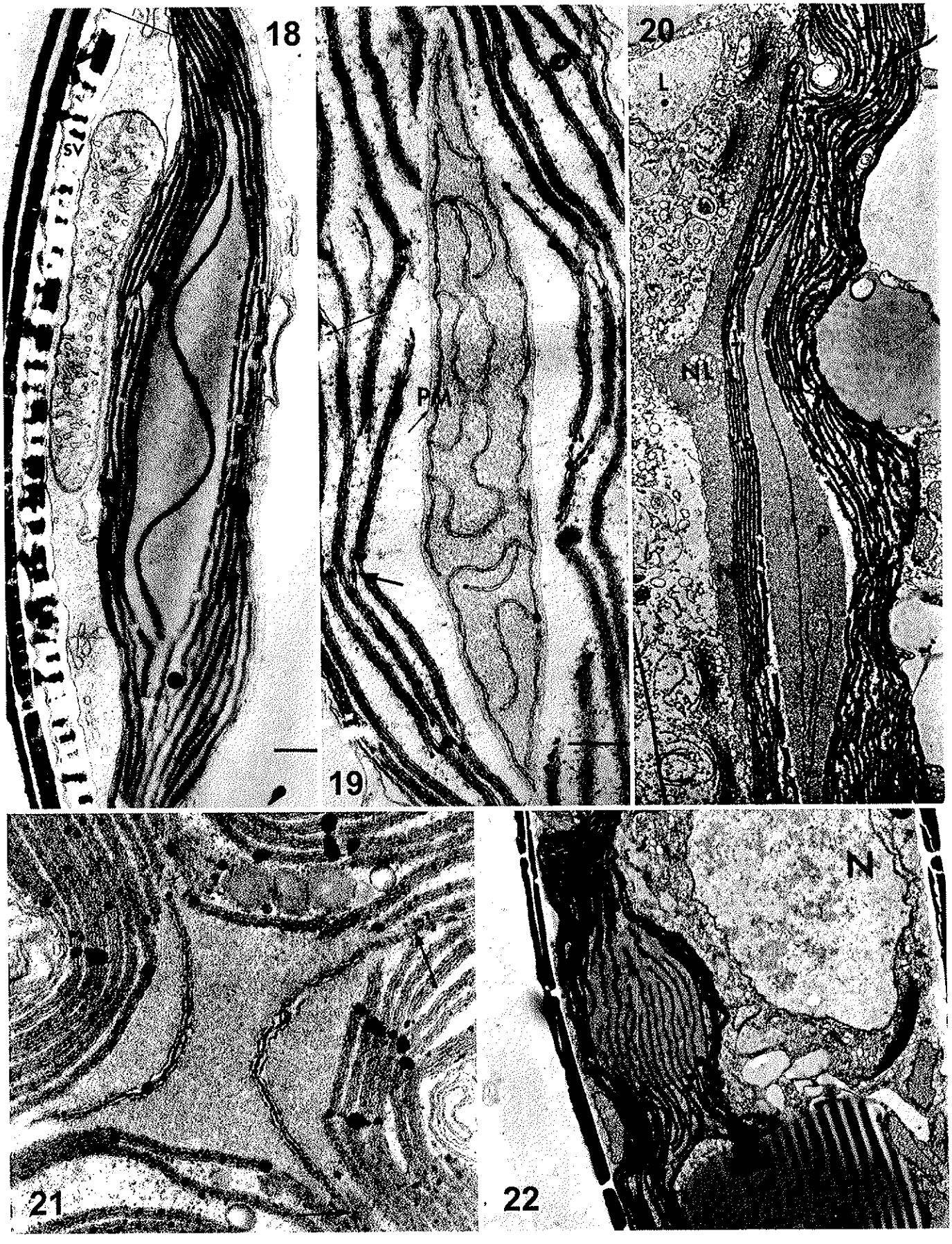
[1] +, present; –, absent; G/Is, globular/isometric; BMP/N, bi- or multipolar/nonisometric; Tu/N, tureen-shaped/nonisometric; E/B, elongated/bipolar; E/U, elongated/unidirectional; E/U/B, E/B or E/U; +*, unusual properizonium of *Bacteriastrum hyalinum*;** unpublished sequence data (L.K.M.); nd, no data.

cause the 'auxospore' is developed perpendicular to the mother cell (see illustrations in Drebes 1974). However, Drebes' fig. 37b shows continuity of the cytoplasm within the mother cell even after the initial cell has developed further (fig. 37d). An earlier stage than Drebes' fig. 37b might well be spherical, but expansion along the pervalvar axis [possibly through addition of cingulae, similar to *Proboscia alata* (Mangin 1915)] ends with the formation of an initial valve distally (Drebes' fig. 37b, d). Presumably after some contraction a conventional valve is formed complete with long, tapering process (Drebes' fig. 37d). That illustration appears to show the spine of the process projecting through the initial valve and this is difficult to explain. So too is the formation of a conventional valve at the other end of this cell (Drebes' fig. 38). Presumably both valves are formed after a depauperizing nuclear division. Also unique is the 'initial valve' at the bottom of the cell in Drebes' fig. 38 and numerous other reports (e.g. Cupp 1943, fig. 43c, f, g; Berard-Therriault *et al.* 1999). It appears to be continuous

→

Figs 13–17. Spermatozoids (-cytes) (arrows in Figs 13, 14). Chl, chloroplast; NE, nuclear envelope; N (NU), nucleus; V, vacuole.
Fig. 13. Hologenous spermatozoa of *Lithodesmium*. Reproduced from Manton *et al.* (1970), courtesy of Company of Biologists.
Fig. 14. Merogenous spermatozoa of *Corethron*. Courtesy of R. Crawford.
Fig. 15. Elongated nucleus of *Coscinodiscus* spermatocyte. Reproduced from Schmid (1988), courtesy of Springer-Verlag.
Fig. 16. Rounded nucleus of *Lithodesmium*. Reproduced from Manton *et al.* (1969b), courtesy of Company of Biologists.
Fig. 17. Perinuclear arrangement of Golgi bodies in spermatozoa of *Lithodesmium*. Reproduced from Manton *et al.* (1969a), courtesy of Blackwell.


13
14
15
Chl
V
Chl
N (-NU)
V
NE
16
17


18
SV
19
PM
20
L
NL
21
22
N

with the mother cell valve or, alternatively, it may represent gametangial structure similar to that seen in *Ellerbeckia* (Schmid & Crawford 2001).

Although more information is needed for both genera, it seems that *Rhizosolenia* and *Corethron* have more in common than first appears, because they both involve elaborate protection for the developing valves. Their similarity to other genera in clade 1 for which information is available ends after the formation of the expanded spherical zygote and hemispherical initial valves, at least in *Corethron.* The last member of the same lineage, *Leptocylindrus,* possesses scaly, globular auxospores (Hargraves 1990; I.K., unpublished observations), but it produces a resting spore instead of vegetative cells. Thus, it appears that each member of this lineage evolved its own modification to the formation of the initial cell, further substantiating its distinct position in clade 1 of the rRNA tree (Fig. 1).

Nonisometric auxospores expand unequally in one, two or more directions and are of two varieties: properizonial (anisometric; von Stosch 1982; Kaczmarska *et al.* 2001) and perizonial (bilateral, tubular; Kaczmarska *et al.* 2001). If they are not constrained by parental thecae or other specialized structures initially, they are globular, but change shape later in their development (von Stosch 1982). At the globular stage, auxospore (primary) walls may contain scales embedded in organic matrices (von Stosch 1982; Kaczmarska *et al.* 2000, 2001). Further development of the nonisometric auxospore follows one of two pathways, depending on species.

Properizonial auxospores: In properizonial auxospores (Fig. 12B), the wall of the early-stage, globular auxospore splits and new, secondary structural elements (bands, loops or hoops) are added and integrated to facilitate later auxospore expansion into more complex (nonspherical) shapes. Bands, hoops, etc., complement but do not replace the primary, scaly wall (von Stosch 1982). Depending on species, the relative proportion of the surface area of the auxospore protoplast covered by either primary or secondary wall varies (von Stosch 1982). Among all auxospores, this type contains the greatest variety of elements that facilitate nonisodiametric expansion, and include scales, bands of varying length, loops, hoops and cups collectively called the properizonium (Drebes 1972, 1977a; von Stosch 1982). When mature, this type of auxospore will produce an initial frustule whose flattened shape is similar to that of the vegetative cell (von Stosch *et al.* 1973), often through the contraction of the protoplast within the auxopsore. This type of auxospore is known from *Chaetoceros* Ehrenberg, *Odontella,* etc. (Table 2; Kaczmarska *et al.* 2001). These diatoms are called bi- or multipolar centrics and most have a radial pattern of valve ornamentation and various tubular structures (bi-, macro- and microlabiate processes) located near the centre of the valve. They cluster loosely in clade 2a of the phylogenetic tree based on 18S and 16S SSU (Figs 1–4; Medlin *et al.* 1997). Only the Thalassiosirales do not conform to the bi- or multipolar valve morphology or to the properizonial type of auxospore described above. Phylogenetic relatedness between Thalassiosirales and Lithodesmiales in the context of molecular and morphological evidence is discussed in a separate report (L.M., I.K., M. Beaton and A.C. Benoit, unpublished observations).

Perizonial auxospores: The development of perizonial auxospores, the second type of nonisometric auxospores, often begins with a spherical stage (Fig. 12C; most of the diatoms listed in Table 2) covered by primary walls. This is particularly evident in the species producing gametes free of restraints of parental theca, papilla or mucilaginous envelope. The presence of scales is now documented in primary walls of pennate taxa as diverse as *Rhabdonema, Neidium* Pfitzer, *Diploneis* Ehrenberg, *Pseudo-nitzschia* Peragallo (Table 2; Kaczmarska *et al.* 2001) and possibly *Biremis* (Mann 1993, 2003, unpublished observations), and suggest that scales in primary walls of pennate auxospores may turn out to be more common when electron microscopy is used to investigate auxospore structure as routinely as it is now used to examine valve structure. Early auxospores confined to the parental theca (e.g. *Sellaphora pupula*), papilla [e.g. *Eunotia flexuosa* (Brébisson) Kützing] or mucilaginous envelopes surrounding sexual cells (of mainly benthic pennates) demonstrate shapes that fit their confinement (see Round *et al.* 1990 and references therein for other examples). Later, the primary wall of the young auxospore splits and separates into two nearly equal halves (Mann 1984; Round *et al.* 1990; Kaczmarska *et al.* 2000). The primary walls split when a new structure, the perizonium, develops underneath it and outgrows the size of the young auxospore. The halves of the primary wall may be retained by the older auxospore as apical caps and may or may not remain as an integral part of the expanding cell. The perizonium is tubular and consists of a set of longitudinal or transverse (or both) bands of varying width. When bands of both orientations are present, the longitudinal bands follow the formation of transverse elements (D. Mann, unpublished observations). In most species, such perizonia undergo bipolar expansion by the sequential addition of new bands at the ends of the auxospore (Mann 1982a, b, 1987, 1988, 1989; Kaczmarska *et al.* 2000). An interesting modification is shown by *Cocconeis pellucida* Hantzsch (Mizuno 1998), but the uniqueness of this type of perizonium precludes any speculation as to its evolutionary significance. Perizonial auxospores are similar to properizonial auxospores in that they also produce initial frustules that are not always dome-shaped, but are more similar to vegetative frustules than those produced by the globular scaly auxospores of clade 1 species. Perizonial auxospores are known from genera such as *Licmophora* C.

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Figs 18–22. Pyrenoids in diatoms. All figures reproduced from Drum *et al.* (1966), courtesy of Koeltz. Abbreviations (not all relevant, because the figures are reproduced from elsewhere): D, disk bands or membranes; L, leucosin body; N, nucleus; NL, nucleolus; P or PM, pyrenoid (membrane); SV, silica deposition vesicle.

Fig. 18. Clade 1: embedded pyrenoid in *Melosira varians.*

Fig. 19. Clade 2a: embedded pyrenoid in *Stephanodiscus niagarae.* Arrows, thylakoid membrane.

Fig. 20. Clade 2b: embedded pyrenoid in *Surirella ovalis* Brébisson.

Fig. 21. Clade 2b: embedded pyrenoid in *Mastogloia grevillei* W. Smith. Arrows, thylakoid membrane.

Fig. 22. Clade 2b: protruding pyrenoid in *Gomphonema parvulum.*

Agardh, *Craticula* Grunow and *Cymatopleura* W. Smith (Table 2). These species are pennate in valve architecture and fall in clade 2b of the phylogenetic tree based on sequence similarities of 18S rRNA (Medlin *et al.* 1997).

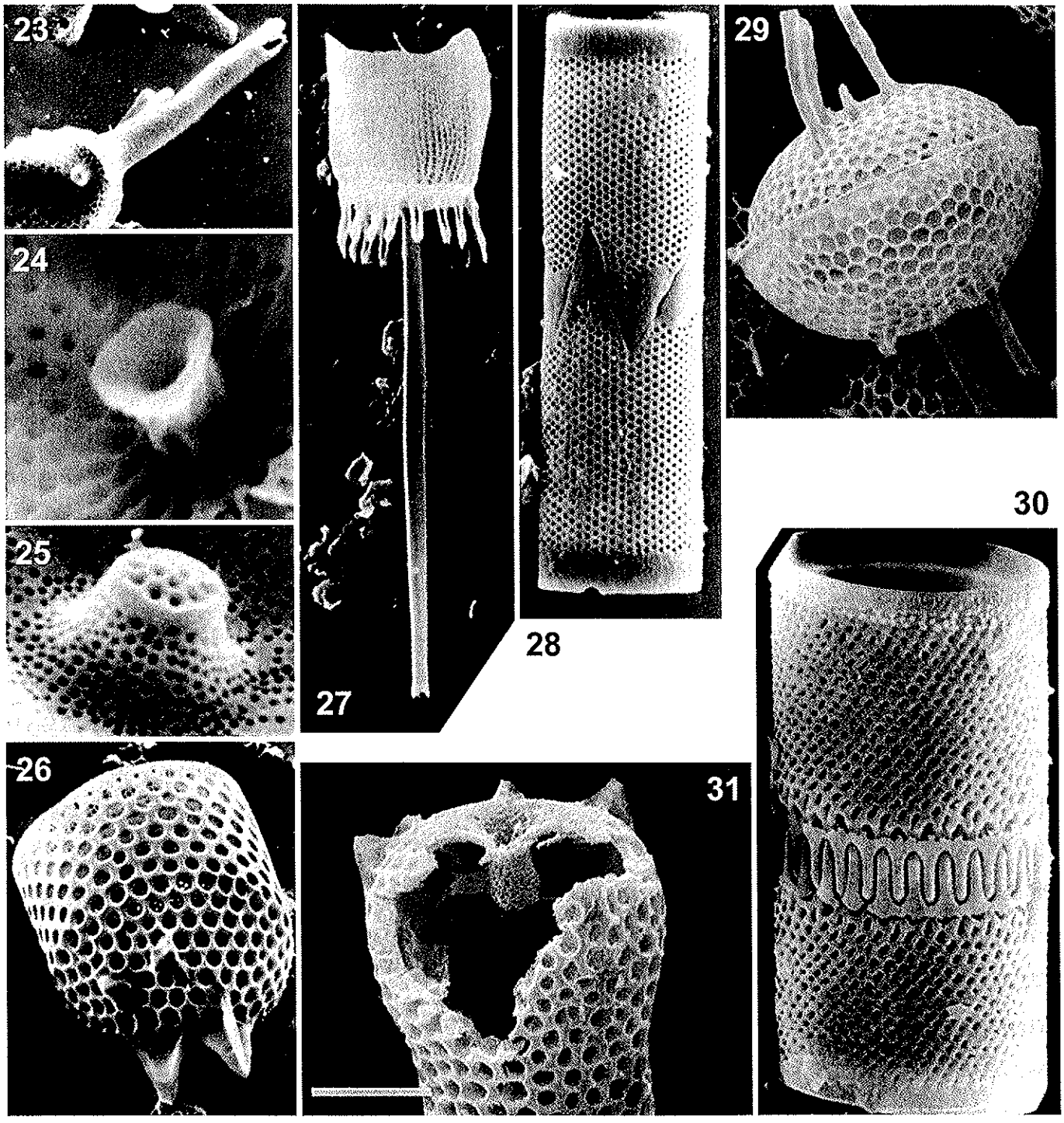
SPERMATOZOID ULTRASTRUCTURE: Although the ultrastructure of only a few spermatozoid cells has been studied, there appear to be some differences between those that fall into clade 1 compared with those from clade 2. They certainly bear further investigation in other genera from these clades to ascertain whether or not they are consistent, although these type of data may be very difficult to obtain. The spermatozoids of both clades have two transitional plates in the helix, a feature that they share with the sister group of the diatoms, the Bolidophyceae. As the spermatozoids mature, either they extrude their plastids as the final spermatozoids develop (merogenous) or they retain them (hologenous) (Figs 13, 14; see references and illustrations in Round *et al.* 1990). In general, the hologenous forms predominate among the genera of clade 2a, whereas the merogenous forms are primarily found in clade 1; known exceptions are listed in Jensen *et al.* (2003). Both types are present in *Odontella, Ditylum* Bailey and *Chaetoceros.* Nonflagellated male gametes in pennates retain their plastids as well and may be regarded as hologenous.

Thin sections of the spermatozoids were recently produced from the genera *Coscinodiscus* and *Chaetoceros* (Jensen *et al.* 2003). *Coscinodiscus* has an elongated nucleus in its mature spermatozoid as well as in its spermatocyte (Fig. 15) but some members of clade 2 also have elongated nuclei in the spematozoid. The microtubular root system from the single basal body extends over the entire nucleus (Jensen *et al.* 2003). The mitochondria are also elongated and there are no Golgi bodies in the mature sperm, although earlier stages, e.g. spermatocytes, can have a G–ER–M unit (Schmid 1988, fig. 2; Fig. 15). Spermatozoids of species in clade 2 belonging to the genera *Chaetoceros* (Jensen *et al.* 2003), *Lithodesmium* (Manton *et al.* 1969a, b, 1970) and *Thalassiosira* (Idei *et al.* 1994, unpublished observations) have been thin-sectioned and are shown to have a rounded nucleus; those illustrated here (Fig. 16) are for *Lithodesmium.* So far, only *Melosira* (from clade 1) has been reported to have a rounded nucleus (Jensen *et al.* 2003) and may or may not have an elongated cell shape. In diatoms from clade 2a, the microtubular root from the basal body extends only over the upper surface of the nucleus. Mitochondria are small and rounded and Golgi bodies, when present, are around or near the nucleus (Fig. 17; Manton *et al.* 1969a) but they are not associated with a mitochondrion.

PYRENOID ULTRASTRUCTURE: Recently, Schmid (2001) has detailed pyrenoid structure in many genera and species of diatoms. We present here a summary of this work and relate the structure of the pyrenoids to the clades recovered in the molecular trees shown in Figs 1, 2. In general, clade 1 centric species have a single membrane-bound embedded pyrenoid that is transversed by one or more sets of lamellae not connected to thylakoids (Fig. 18). The radial Thalassiosirales and the bi- or tripolar centrics of clade 2a also have a single membrane-bound embedded pyrenoid per plastid. These two groups also appear to have slightly different pyrenoid structures. In the radial centrics of the Thalassiosirales the pyrenoids are often free of traversing membranes. In those species that do show lamellae invading the pyrenoids, the lamellae are usually positioned along the periphery of the pyrenoid. One notable exception to this pattern is in *Stephanodiscus,* which has many membranes traversing the pyrenoid (Fig. 19). The bi- or tripolar species of clade 2a also have a single membrane-bound embedded pyrenoid, often crossed by a single thylakoid like that of clade 1. The most significant difference between these centrics and those of clade 1 is a tendency in this group to form plastids with many pyrenoids. The pennate diatoms of clade 2b possess either embedded or, rarely, protruding pyrenoids (Figs 20–22). There is a wide range of variation in the morphology of the pyrenoids even among species of the same pennate genus (see examples in Schmid 2001). Embedded pyrenoids are membrane bound with tubular invaginations across the pyrenoids that are continuous with the thylakoids (Figs 21, 22). All plastids with multiple pyrenoids are of the embedded type. Protruding pyrenoids, so far as is known, occur only singly in plastids. Plastids of this sort may also contain cytoplasmic tubular intrusions throughout the matrix of the pyrenoid (see Schmid 2001, fig. 45). Pyrenoids in clade 1 appear to be simpler and less variable than those in clade 2, especially in the pennates (Schmid 2001).

FOSSIL SUPPORT FOR MOLECULAR DATA: The earliest, exceptionally well-preserved diatoms are known from Lower Cretaceous Antarctic deposits (Gersonde & Harwood 1990) and contain mainly radial centric forms, although a few bipolar forms have also been reported. If we assume that some of the diatoms present in these deposits are ancestors of modern taxa, we can relate the frustule architecture of these fossil diatoms to designs seen in modern taxa representing the two molecular clades. We can also use the morphological features present in the fossil taxa to characterize the molecular tree and to compare them to similar contemporary species (Figs 23–31; Medlin *et al.* 1995). Taxonomically important modern valve features, such as labiate or strutted processes, are absent from the Lower Cretaceous diatoms, but Lower Cretaceous diatoms possess different, previously unknown types of tubes or processes. On the basis of those novel structures and on the type of linking apparatus, Gersonde & Harwood (1990) classified Lower Cretaceous diatoms into four groups. The two clades recovered in our rRNA tree can be correlated with three of the four Cretaceous groups if we use criteria based on the presence or absence of a central invagination and details of the elaborate linking structures between cells (Medlin *et al.* 1995, 2000). Nikolaev & Harwood (1999, 2001) have also attempted to link the genera in the 135 Ma deposit with modern taxa. They proposed the same links as those we have proposed here and earlier (Medlin *et al.* 1995, 2000).

Taxa of Gersonde & Harwood's (1990) groups 1 and 4 are characterized by the absence of a central structure or tube and the presence of robust, peripheral linking implements for chain formation (Figs 27–30). These may represent features characteristic of the ancestral stock of clade 1 in our molecular tree (Medlin *et al.* 1995, 2000). Linking structures in *Stephanopyxis* (Haga 1997, fig. 6) are similar to those of group 1 taxa (Gersonde & Harwood 1990). The structures holding the external tubes of the labiate processes in *Stephanopyxis* are also formed from part of the areolar wall (compare Fig. 27 with Fig. 30). Similarly, colony-holding spines are formed from the main part of the wall in both group 4 taxa of Gersonde & Harwood (1990) and in modern *Aulacoseira* (com-



Figs 23–31. Fossil taxa paired with modern taxa illustrating the features unifying the two groups. Figs 23–30 reproduced from Medlin *et al.* (2000), courtesy of Polish Academy of Sciences; Fig. 31 reproduced from Nikolaev & Harwood (1997), courtesy of Biopress.

Figs 23–25, 31. Fossil taxa (*Rhynchopyxis, Gladiopsis, Praethalassiosiropsis* and *Archaegladiopsis,* respectively) purported to be ancestors of modern taxa of clade 2, showing the central structure in the centre of the valve.

Fig. 26. *Ditylum:* a modern taxon of clade 2, with central structure.

Figs 27, 28. Fossil taxa (*Amphlypyrgus* and *Archepyrgus*) with strong peripheral linking processes (here spines) and no central structure in centre of valve; purported to be ancestors of modern taxa of clade 1.

Figs 29–30. *Stephanopyxis* and *Aulacoseira*: modern taxa of clade 1, with strong peripheral linking structures and no central structure in centre of valve.

pare Figs 28, 29). The fossil genus *Archepyrgus* Gersonde & Harwood and the modern *Aulacoseira* both even possess the ringleist on the valve margin. Nikolaev & Harwood (1999) also drew attention to possible phylogenetic links between these fossil and modern genera. In general, modern taxa from clade 1 do possess one of two types of peripherally located tubes, e.g. the labiate processes, but lack any structure or tube in the valve centre. It has recently come to our attention (P.A. Sims, unpublished observations) that a diatom resembling *Stephanopyxis* has been found in the Gersonde and Harwood material in both light and scanning electron microscope preparations. Two specimens have been found, both with labiate processes. Thus, we suggest that the ability to make a labiate process was present in an ancestral lineage prior to its divergence into clades 1 and 2 but, because these two lineages have very different labiate processes structurally (macro and micro), it would be best to assume that they are very different structures until evidence suggests otherwise. Because clade 2 diverges into clades 2a and 2b, it is likely that their labiate processes share a common ancestor, because pennates first appear in the fossil record at the same time as clade 2 centrics with labiate processes. Macrolabiate processes are not found in any of the clade 2a bipolar centrics and Schmid (1987) has documented that only the macrolabiate processes are involved in anchoring of the nucleus. This suggests that the macro- and microlabiate processes in clade 1 are not homologous. Thus, not all labiate processes would be homologous structures [see the variety of labiate processes in Medlin *et al.* (2000, fig. 3)] and it would be erroneous to code them as different states of the same structure in any cladistic study.

Gersonde & Harwood's (1990) group 2 taxa possess a central tube-like structure and their peripheral chain-linking mechanisms are reduced (Fig. 24). We have hypothesized that this group forms the ancestral stock for clade 2a of our rRNA tree (Medlin *et al.* 1995). Morphological changes in the valve centre of these Lower Cretaceous diatoms can be hypothesized to have occurred from a valve with an invagination (e.g. the uvular process of *Archaegladiopsis* Nikolaev & Harwood, Fig. 31; see also Nikolaev & Harwood 1994, 1997) 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, Fig. 25; Gersonde & Harwood (1990)]. The next step would be for the perforate plate to be lost, then the annular process or multistrutted processes of *Gladiopsis speciosa* (Schulz) Gersonde & Harwood (Fig. 24) (Sims 1994) and *Thalassiosiropsis* Hasle (Hasle & Syvertsen 1985) can be formed as the pores in the wall of the central tube coalesce. If the perforations in the wall of the central tube are further reduced, then the sipho-shaped process in *G. ellipsoidea* (Schulz) Gersonde & Harwood would be formed (Glezer *et al.* 1988, figs 6, 7). Finally, a valve with an open central tube without perforations in the tube wall (e.g. the rhyncho-shaped process of *Rhynchopyxis* Gersonde & Harwood, Fig. 23; Gersonde & Harwood 1990; Nikolaev & Harwood 1994, 1997) can be produced. As originally defined, *Gladiopsis* Forti & Schulz contained taxa with at least three or more types of central valve structures: the uvular process, the annular or multistrutted process and the sipho-shaped process (Nikolaev & Harwood 1994, 1997). Nikolaev & Harwood (1997) have proposed one new genus, *Archaegladiopsis* for those taxa formerly placed in *Gladiopsis* with an uvular process. The line of development from the perforate process of *Praethalassiosiropsis* into the multistrutted (annular) process of *Thalassiosiropsis* (D. Harwood, unpublished observations) appears to stop at the Palaeocene (Sims 1994), with no connections in the fossil record between the central process of *Thalassiosiropsis* and the true strutted processes of *Thalassiosira,* although Hasle & Syvertsen (1985) point out the similarity in the processes of the two genera and propose that the multistrutted process is a primitive form of the modern strutted process. True *Thalassiosira* spp. (i.e. with true strutted processes) do not appear until the Miocene (Hasle 1985), so a search for more direct ancestors spanning the gap between the last *Thalassiosiropsis* spp. and modern Thalassiosirales should perhaps be directed towards taxa with a central structure or tube more closely resembling that of *Rhynchopyxis*. The simple pore structure in the valves of *Ditylum* could be the precursors of the satellite pores. This scenario would imply that the satellite pores of true Thalassiosirales may be a later addition to the central tube or strutted process in this lineage.

Such evolutionary sequences are supported by the phylogenetic relationships inferred from the rRNA tree. If we remove *Ditylum* (Fig. 26) from the base of the Thalassiosirales in our rRNA tree, we obtain a significantly less robust tree (Medlin *et al.* 1995). It also implies that the basic valve designs present in the fossil material can be related to both the morphology and the position in our phylogenetic tree of extant species. In an analysis of partial LSU (28S) rRNA sequences, Sörhannus *et al.* (1995) recovered the Thalassiosirales as outgroup to *Lithodesmium* and the pennates. Clearly, a close and significant relationship (Bayesian analysis, 100%) association between the Thalassiosirales and the Lithodesmiales is recovered with the molecular analyses. Significantly, modern taxa in clades 2a and b have a central structure in the valve, i.e. a portula (labiate or strutted), annulus or sternum.

Finally, from our rRNA analysis and from cytological data, it can be shown that *Coscinodiscus* is not related to *Thalassiosira*. Historically, many *Thalassiosira* species were identified as *Coscinodiscus,* and many taxonomists still confuse the two genera. Any scenario that reconstructs a recent evolutionary relationship between the orders to which these taxa belong would be in serious conflict with several lines of evidence. Not only do they belong to two entirely different clades of diatoms as shown by our rRNA tree, but also they have completely different valve structures and tube processes secreting chitan (Fryxell & Hasle 1972; Herth 1979), different responses to microtubule poisons (Schmid *et al.* 1981; Schmid 1984b) and different arrangements of Golgi bodies and different types of spermatozoid formation. In addition, the placement of the Thalassiosirales near any member of clade 1, e.g. *Stephanopyxis* (Nikolaev & Harwood 2001), is also in direct conflict with the molecular data.

CONCLUSIONS

It is now clear that the diatoms belong to the pigmented heterokont algae, and with their sister group, the Bolidophyceae, diverge simultaneously within a lineage giving rise to all other pigmented heterokont algae. Molecular clock calculations based on linearized trees from both nuclear and plastid genes

and first-appearance dates of the diatoms indicate that it is unlikely that the diatoms existed before the Permian–Triassic boundary, approximately 250 Ma (Medlin *et al.* 1997). The phylogeny of the diatoms recovered with these genes does not agree with their current classification system, which is based on the features of the siliceous cell wall, gross cytoplasmic details and type of gametes produced.

The molecular data can be used to show relationships at higher taxonomic levels within the diatoms and are best correlated with other, previously unappreciated cytoplasmic features. Although not all the cytoplasmic features have been elucidated for all the taxa sequenced (and perhaps they never will be), there is sufficient agreement with a majority of taxa sequenced to use the morphological data sets to support the molecular evidence. On the other hand, with the support of molecular data, unsuspected relationships between other morphologies may also be discovered. Moreover, molecular evidence from 18S and RUBISCO has also identified the true sister group of the diatoms as the biflagellated Bolidophyceae. Because the Bolidophyceae are picoplankton with a simple cellular organization (Guillou *et al.* 1999), it was not possible to determine the arrangement of the Golgi body or the single mitochondrion. These cells are so small that all the organelles are closely aligned, even though the Golgi was consistently found beneath the basal body. However, G–ER–M units are known from the oomycetes and the red algae, whereas an association of the Golgi around the nucleus is also known in the Labyrinthuloides. Thus, it would appear that both features are present in ancestors of the diatoms and the potential host cells of their plastids. It can be argued that the two traits then segregated themselves in the two separate lineages as they evolved. Ancient polymorphisms can be estimated in the same way a molecular clock is calibrated, by cladistic events. The time elapsed since divergence can be measured by the accumulation rate for neutral mutations. Once lineages diverge, shared ancient polymorphisms should degrade at a rate related to the mutation rate. Thus, after sufficient time since separation of lineages, there should be almost no shared polymorphisms based on a neutral accumulation of new alleles and drift of allele frequencies. However, there are cases in which polymorphisms have been maintained after very long periods of independent evolution. The implication would be that the polymorphism maintains a function that influences fitness, i.e. selection is maintaining the polymorphism. We believe that this is why there are a few exceptions to the Golgi arrangements in clades 1 and 2a.

No traces of scales or silica were found in the Bolidomonads (Guillou *et al.* 1999). However, organic scales in cell walls are known in the sister groups of the diatom and bolidophyte lineage, i.e. in the chrysophyte algae and in the Thraustochytrids and Labyrinthuloides (Margulis *et al.* 1989), which are earlier divergences in the heterokont algae (Medlin *et al.* 2000) and could have been present in even more ancestral heterokonts. In addition, the possibility that these cells might be the vegetative state of the Parmales, which bear a carapace of closely fitting silica scales, has been speculated (see Mann & Marchant 1989). The taxonomic position of the Parmales has not been ascertained by molecular data. Significantly, there is only a short internode between the divergence of the Bolidophyceae and diatoms and the subsequent diversification of the diatoms themselves (Guillou *et al.* 1999). Therefore, we postulate that the earliest fossil diatoms are (relatives of) progenitors of extant clades and that the origin of the extant diversity of the diatoms must predate their first appearance in the fossil record. The short internode leaves too little time for the existence of a more ancient diversification and subsequent extinction, such as can be seen in the haptophyte 18S tree in which there is an extremely long internode before the modern diversification of taxa (Edvardsen *et al.* 2000). Because of this long internode, these workers hypothesized that early divergences in the haptophyte lineage are likely to have become extinct. This node is several times longer than that separating the Bolidophytes from the diatoms.

Based on features that the diatoms share with either the bolidomonads or earlier divergences in the heterokont tree, we hypothesize the ancestral diatom cell to have been a diploid biflagellated scaly unicell with the ability to produce either a G–ER–M unit or a perinuclear arrangement for the Golgi, because these features are present in earlier divergences in heterokonts and are probably ancestral polymorphisms.

The evolution of the ability to metabolize silica appears to have occurred since the divergence of pigmented heterokonts from their nonpigmented ancestors. No traces of silica scales have been found in Bolidophyceae or in any earlier heterotrophic divergences in the heterokonts, although silica metabolism occurs in the Chrysophyceae, Parmophyceae, Dictyochophyceae and Xanthophyceae, groups closely related to the diatom–bolidomonad lineage (Fig. 1). Just exactly why the diatoms use silica has been frequently debated among diatomists (see discussion on the diatom list server http://www.indiana.edu/~diatom/silica.dis). Although defence is often invoked as the most likely reason for silica metabolism and wall evolution in the diatoms in the past (Hamm *et al.* 2003), Medlin (2002) has reviewed lines of evidence from mammalian cell lines that suggest that silica is necessary to prevent cell ageing, to induce normal formation of connective tissues, to reduce the effects of toxic metals and to inhibit fungal attack as well as to place cells in a prolonged resting state. In her hypothesized scenario of why the diatoms have evolved silica metabolism, a simple naked biflagellate cell evolved silica metabolism, which conferred advantages of prevented ageing to the cell and placed it in a prolonged resting state. Silica became involved in the metabolic processes of the cell, presumably by supplying bioactive surfaces for reactions to take place. As silica accumulated in the cells, it was sequestered in the ER and eventually found its way into a vacuole whose internal pH was acidic. Once inside the vacuole, the silica began to polymerize; the polymerized silica was then extruded from the cell because it was inaccessible, in its polymerized state, to the chemical reactions needed to prevent cell ageing. The interaction of silica with the condensation of amino acids into proteins can be demonstrated in vitro (see references in Perry & Keeling-Tucker 2000). Kröger *et al.* (1999) have shown that the proteins (silaffins) help to precipitate the silica in an accelerated nonrandom fashion. Thus, the cell evolved a continuous need to replenish its internal silica pool. In this respect, the diatoms have developed an absolute requirement for silica before the cell can divide (Darley & Volcani 1969). The species-specific cell wall structure of the diatoms has evolved as the cell utilized both membrane-mediated (micro-) morphogenetic and macromorphogenetic mechanisms to mould the wall features (Kröger &

Table 3. Comparison of morphological features across the three molecular clades.

Feature	Clade 1	Clade 2a	Clade 2b
Morphological symmetry	radial centrics	bipolar centrics plus the radial Thalassiosirales	pennates
Golgi	G–ER–M, one exception	perinuclear, one exception	perinuclear and its variations, no exceptions
Pyrenoid	one per plastid, lamellae cross pyrenoids not connected to thylakoids	one or multiple numbers per plastid usually not crossed by lamellae, if so lying along periphery of pyrenoids, occasionally like clade 1; not connected to thylakoids	one per plastid, very complicated structures, lamellae crossing pyrenoids are connected to thylakoids
Auxospores	isometric	nonisometric properizonia	perizonium
Sexual reproduction	oogamy, mostly merogenous, sperm with elongated nuclei	oogamy, mostly hologenous, sperm with rounded nuclei	isogamy
Processes, raphe	marginal	central, also marginal in Thalassiosirales	central

Sumper 1998) and to guide the naturally mediated silica polymerization induced by the acidic conditions of the silica deposition vesicle.

The molecular phylogeny recovered by the analysis of the rRNA genes has revealed three monophyletic clades when a database containing 8600 sequences (including a reduced data set of 281 capable of being bootstrapped) is used. This is reduced to a grade of clades from the first centric to the last divergence of the raphid diatoms when the database is reduced to something manageable for in-depth statistical analysis. This analysis finds BT support for two of the three clades, but combines clades 2a and 2b into one monophyletic clade. We are able to cluster these grades of clades into groups (which we have referred to as clades 1, 2a and 2b) because these clusters of clades share certain cytoplasmic features (Table 3); these include the arrangement and structure of the Golgi apparatus, the type of the auxospore, the type and arrangement of the pyrenoid, the ultrastructure of the spermatozoids in extant taxa, and the cell wall morphology in the earliest best-preserved fossil deposits of diatoms from the early Cretaceous. The younger clades in the tree are best correlated with siliceous features of the diatom cell wall and can best be recognized at the order level in the diatoms. Following a taxonomic hierarchy, the next full nomenclatural level above order is that of class, if the intervening levels are not used. We have used the intermediate level of subdivision to recognize the divergence of clade 1 and clade 2. We can then order our clusters of clades into two subdivisions and then into three different classes. We propose that there is now sufficient evidence, based, not only on molecular data from several genes coming from the nuclear, plastid and mitochondrial genome within the diatom cell, but also on cytoplasmic details, to revise the classification system of the diatoms. **We appreciate that not all of the taxa sequenced have been investigated for all these cytoplasmic features compared. Certain features, such as the structure of the spermatozoids, are sketchy at best and there are some exceptions in each cytoplasmic category compared. These exceptions probably reflect ancestral polymorphisms that have been retained in one or more of the lineage descendants because they have inferred some adaptative fitness to that lineage.** We do think that the classification system proposed here more naturally reflects the phylogeny of the groups, and of course the morphological definitions can be further refined as more evidence becomes available. The evidence we present far exceeds the handful of diatom species in which oogamy has been documented, and yet the assumption that all centric diatoms are oogamous seems to have been generally accepted. The clades in each grouping are united by several lines of cytoplasmic detail and the differences in siliceous cell wall between the clades within a cluster (or class, as we wish to call them) define the orders that the clade represents in the modern classification of the diatoms. Evolution is a continuous process and absolute distinctions are infrequent in biology. **Nevertheless, we feel that the revised classification proposed more naturally reflects the evolutionary relationships in the diatoms, despite the fact that not all evidence is available for all species in our molecular tree (and indeed, may never be).** The recognition of araphid and raphid diatoms as distinct classes, with only one class for all centrics, seriously underrepresents the great diversity of centric diatoms and misrepresents the molecular and several lines of cytological data.

Division Bacillariophyta

Subdivision Coscinodiscophytina Medlin & Kaczmarska

Valvae cellularum vegetativarum radiatim constitutae. Unusquisque Golgi-corpus cum mitochondrio et reticulo endoplasmatico consociatus (breve 'G–ER–M'), corporibus per protoplastum dispersis. Reproductio sexualis oogama, generatione spermatozoidorum plerumque merogama; paries auxosporae squamosus.

Vegetative cells with radial valves, Golgi bodies associated with a mitochondrion (G-ER–M) and dispersed through the protoplasm (type 1). Sexual reproduction oogamous, spermatozoids predominately merogamous, auxospores with scaly walls.

Class Coscinodiscophyceae Round & Crawford, emend. Medlin & Kaczmarska

Extant and fossil centric cells with mainly peripheral processes (i.e. labiate processes, colony-linking processes), rarely or secondarily centrally located; cells are usually radially ornamented from a central point. Extant cells usually with the Golgi bodies arranged in a G–ER–M unit; cells have a single membrane-bound embedded pyrenoid transversed by one or more sets of membranes that are not in contact or contiguous with the thylakoids. Sexual reproduction oogamous; scaly auxospores expanding isometrically with pliable walls containing siliceous scales. Spermatozoid formation predominantly merogenous; elongated sperm with elongated nuclei and elongated mitochondria; Golgi bodies arranged in G-ER-M unit, but may not be present in the mature sperm.

Orders given in Round *et al.* (1990) that fall into this class are: Coscinodiscales, Corethrales, Rhizosoleniales, Melosirales, Orthoseirales, Aulacoseirales, Chrysanthemodiscales,

Stictocyclales, Asterolamprales, Arachnoidiscales, Stictodiscales, Ethmodiscales and Leptocylindrales.

Subdivision Bacillariophytina Medlin & Kaczmarska

Valvae cellularum vegetativarum plerumque bi-(multi-)polares, pennatae vel centricae, Golgi-corporibus circa nucleum locatis. Reproductio sexualis oogama (formis centricis) vel anisogama isogamave (formis pennatis), generatione spermatozoidorum plerumque hologama. Paries auxosporae plerumque taeniis et interdum squamis obsitus.

Vegetative cell valves mainly bi- or multipolar; centric or pennate, with Golgi bodies located around the nucleus (type 2). Sexual reproduction oogamous (centric forms) or anisogamous or isogamous (pennate forms), predominately hologamous. Most auxospore walls contain bands and may contain scales.

Class Mediophyceae (Jousé & Proshkina-Lavrenko) Medlin & Kaczmarska, stat. nov.

BASIONYM: Mediales Jousé & Proshkina-Lavrenko (Proshkina-Lavrenko 1949–1950, vol. 2, p. 210).

TYPE GENUS: *Chaetoceros.*

Extant and fossil centric cells with tube processes (i.e. labiate, strutted or rhyncho-shaped), these primarily located in the cell centre or within the annulus, rarely with additional peripherally located processes (except in the Thalassiosirales where there may be multiple rings of marginal strutted or occluded processes); cells usually bi- or multipolar with radial ornamentation. Extant cells usually have the Golgi bodies arranged perinuclearly. In the Thalassiosirales, cells usually have a single membrane-bound embedded pyrenoid that may be transversed by membranes (in which case these are folds of membranes lying along the periphery of the pyrenoid). In the bi- or tripolar centrics, there is a tendency to form plastids with multiple pyrenoids and if these are crossed by membranes then they are similar to those of clade 1. Sexual reproduction is oogamous; auxospores are properizonial (nonisometric), with primary walls containing silica scales and, added later, a system of bands (except in the Thalassiosirales, which retained the pattern of auxospore formation of clade 1) that force the auxospore to expand only in certain directions. Spermatozoid formation is predominately hologenous. Sperm are usually rounded and have rounded nuclei and mitochondria. Golgi bodies often surround the nucleus of the mature spermatozoid.

Orders given in Round *et al.* (1990) that fall into this class are: Chaetocerotales, Biddulphiales, Cymatosirales, Thalassiosirales, Triceratiales, Hemiaulales, Lithodesmiales, Toxariales and a suspected bipolar centric (= Ardissoneales).

Class Bacillariophyceae Haeckel, emend. Medlin & Kaczmarska

Extant and fossil cells with central sternum, with or without a raphe. Rapheless species usually have tube processes (i.e. labiate processes) most commonly located at the cell apices. Others carry a central or marginal raphe system. Cells are bipolar, usually with bilateral symmetry around an axial rib or raphe system. Extant cells usually have the Golgi bodies arranged perinuclearly; cells usually have a single membrane-bound embedded or rarely protruding pyrenoid transversed by membranes that are usually continuous with thylakoids. Pyrenoid structure is highly varied. Sexual reproduction is anisogamous or isogamous. Auxospores are perizonial (nonisometric) and have a perizonium that forces the auxospore to expand only in polar regions; early stages of the auxospore primary wall may contain scales. Spermatia retain chloroplasts.

Orders given in Round *et al.* (1990) that fall into this class are: Fragilariales, Tabellariales, Licmophorales, Raphoneidales, Thalassionematales, Rhabdonematales, Eunotiales, Lyrellales, Mastogloiales, Dictyoneidales, Cymbellales, Achnanthales, Naviculales, Thalassiophysales, Bacillariales, Rhopalodiales and Surirellales.

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