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Molecular assessment of phylogenetic relationships in selected species/genera in the naviculoid diatoms (Bacillariophyta). I. The genus *Placoneis*

by

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With 19 figures and 4 tables

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Abstract: As part of a larger study to reconstruct evolutionary relationships within the naviculoid diatoms, phylogenetic analyses of several freshwater naviculoid species were performed using three different genes (SSU rRNA gene, LSU rRNA gene and rbcL gene), and the morphology of the sequenced species was investigated. This study focused on species of *Placoneis*, a genus that was separated from *Navicula* based on its chloroplast morphology, a feature that places it within the Cymbellales. The phylogenetic analyses also clearly place *Placoneis* in this order, but the relationships between the different genera varied with different genes. *Navicula hambergii*, whose allocation to *Navicula sensu stricto* was known to be wrong, is shown to belong to the genus *Placoneis* and is transferred to that genus. Its transfer is supported by both the phylogenetic analyses and the morphological investigation.

Introduction

The genus *Navicula* was described by Bory de Saint-Vincent in 1822 based on *Navicula tripunctata* (O.F.Müller) Bory. Within the diatoms, this genus was probably the largest and most diverse because "*Navicula* had traditionally been a dump for all bilaterally symmetrical raphid diatoms lacking particularly distinctive features" (Round et al. 1990, p. 566). Nevertheless, with electron microscopy and the investigation of living cells, the true morphological diversity of the genus became apparent, and many taxonomic revisions have been and are being conducted. As a result, many former *Navicula* spp. have been transferred to new genera, e.g., *Haslea* Simonsen

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(1974), *Proschkinia* Karayeva (1978), *Parlibellus* E.J.Cox (1988), *Luticola* D.G.Mann (Round et al. 1990), *Hippodonta* Lange-Bertalot et al. (1996), *Eolimna* Lange-Bertalot & W.Schiller in W.Schiller & Lange-Bertalot (1997), *Mayamaea* Lange-Bertalot (1997), or old genera, e.g., *Sellaphora* Kützing, *Placoneis* Mereschkowsky, and *Dickieia* Berkeley have been resurrected (Mann 1989, Cox 1987, 2003, Mann 1994). Today, most diatomists agree that *Navicula* (*sensu stricto*) should be used only for species that belong to *Navicula* section *Lineolatae sensu* Cleve (1895) and Hustedt (1930). *Navicula sensu stricto* encompasses approximately 200 species, which predominantly (about 150 species) inhabit freshwater environments (Witkowski et al. 1998). However, the validity of the new or resurrected genera has not yet been assessed using molecular techniques.

It has long been evident that there is useful information about evolutionary history in gene sequences. The wide application of this method began with the appearance of the polymerase chain reaction (PCR) in mid-1980 (Saiki et al. 1988). Coupled with the direct dideoxynucleotide sequencing of amplified products, the technique became a powerful tool in life sciences. Sequences of several genes are now being used to assess phylogenetic relationships in the diatoms [18S, 16S, *tuf*A, *rbc*L in Medlin et al. (1996, 2000); Cox 1 in Ehara et al. (2000); and *rpo*A (Fox & Sorhannus 2003).

The SSU rRNA gene is the most widely used gene for inferring phylogenetic relationships (Van der Auwera & De Wachter, 1998, Ludwig & Klenk 2001). Thousands of partial and complete sequences (approx. 1800 bp in eukaryotes) from prokaryotes, single-celled and multicellular eukaryotes can be found in internet-available databases, such as GenBank (http://www.ncbi.nlm. nih.gov/). In diatoms, the gene has been used to study their position within the heterokont algae (e.g., Daugbjerg & Andersen 1997), to reconstruct the evolution of the major classes (e.g., Medlin & Kaczmarska 2004) or to assess the monophyly of diatom orders or genera (e.g., Beszteri et al. 2001) and the presence of cryptic species (Sarno et al. 2005).

The LSU rRNA gene comprises more highly variable areas than the SSU rRNA gene (Van der Auwera & De Wachter 1998). This likely carries a stronger phylogenetic signal for discriminating closely related species as compared to the slower evolving SSU rRNA gene, but it may cause problems for reconstructing deep phylogenies because of saturation effects, i.e., the signal might be indistinct. Furthermore, highly variable sequences are difficult to align. Because of the large size of LSU rDNA (over 3300 bp) complete sequences of this gene are rare and typically sequences used for phylogenetic analyses are derived from parts of the gene, most notably approximately 600 bp from the 5' end of 28S rDNA (D1/D2 region), one of the most highly variable regions in the gene.

Not all eukaryotic DNA is stored within the cell nucleus. Organelles, such as mitochondria or chloroplasts, contain their own DNA and such genomes usually consist of a single DNA molecule with each gene normally represented only once. The chloroplast genome contains predominantly protein-coding genes, which are used for phylogenetic analyses. The enzyme ribulose-1,5-bisphosphate carboxylase (RUBISCO) is responsible for carbon fixation. The *rbc*L gene encoding the large subunit of RUBISCO is located in a single-copy region of the chloroplast genome. It is typically 1428-1434 bp in length and insertions or deletions are extremely rare (Soltis & Soltis 1998).

The relative rates of evolution of the SSU rRNA and *rbc*L genes vary among different groups. The *rbc*L gene generally evolves about three times faster than SSU rDNA in angiosperms but is slower in the Orchidaceae (Soltis & Soltis 1998). Within the phaeophytes, a slightly faster mutation rate of the *rbc*L gene has been observed (Draisma & Prud'homme van Reine, electronic source). Compared to SSU rDNA, the *rbc*L gene appears more suited in diatoms to studies of evolution at order to generic levels of taxonomic hierarchy (Mann et al. 2001).

In order to estimate evolutionary relationships within the Naviculaceae (*sensu* Krammer & Lange-Bertalot 1986) and to identify taxonomic problems, phylogenetic analyses of several freshwater naviculoid species were performed from cultures established from collecting sites in north Germany. Three different genes (SSU, LSU, and *rbc*L) were sequenced for each culture and phylogenies were reconstructed for each gene and a phylogenetic analysis based on a combined data set of all three genes was conducted. The morphology of the sequenced species was also investigated. We present here the first part of this study: an assessment of the genus *Placoneis*.

Materials and methods

Cultures

The cultures used in this study were established within the scope of the ALGATERRA project (http://www.algaterra.net/). Between November 2001 and September 2003, 220 samples were taken from 83 sites, representing several terrestrial, freshwater and brackish habitats in northern Germany (Fig. 1).

Cultures were initiated from these samples using a DY-IV medium (Andersen et al. 1997) mixed 2:1 with filter-sterilized (pore size: 0,1 µm) water from the sampling sites. After one to four days, clonal cultures were isolated from these initial cultures. For isolates from alkaline, acid or brackish habitats, the media was adjusted by addition of sodium hydroxide, hydrochloric acid or IMR-media (Eppley et al. 1967). Most of these isolates still contained small flagellates and in order to remove these flagellates from the cultures a small number of diatom cells was transferred to fresh medium several times and then grown on agar plates (prepared from liquid media) for one to three weeks. A small number of diatom cells were transferred from these plates to liquid medium. If necessary the entire procedure was repeated several times. All isolates were grown under a 14/10 light/dark cycle with photon flux densities between 30 and 120 µM photons m^{-2} s^{-1} at 15°C. A list of all cultured species is presented in Table 1.

DNA Methods

DNA ISOLATION: Culture material was concentrated by filtration and quick-frozen in liquid nitrogen. Nucleic acids were extracted using the Invisorb Spin Plant Mini Kit (Invitek GmbH, Berlin, Germany). The given protocol was only modified by a duplication of the two washing steps.

PCR: For each culture, the small subunit rRNA coding gene (SSU rDNA), the D1-D2 region of the large-subunit rRNA gene (LSU rDNA) and the middle part of the ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit gene (*rbc*L) were amplified using the polymerase chain reaction (PCR; Saiki et al. 1988, Medlin et al. 1988). In the *rbc*L gene sequence of *Rhizosolenia setigera* (GenBank accession number: AF015568) the sequence of the primers F3 and R3 can be found at the position 292-314 and 1028-1051, respectively. The primers and conditions used for PCR are shown in the Tables 2 and 3. The PCR-products were purified by MinElute™ PCR Purification Kit (QIAGEN, Germany) according to the manufacturer's protocol. PCR products with multiple bands were purified by excising from a 1% agarose gel.

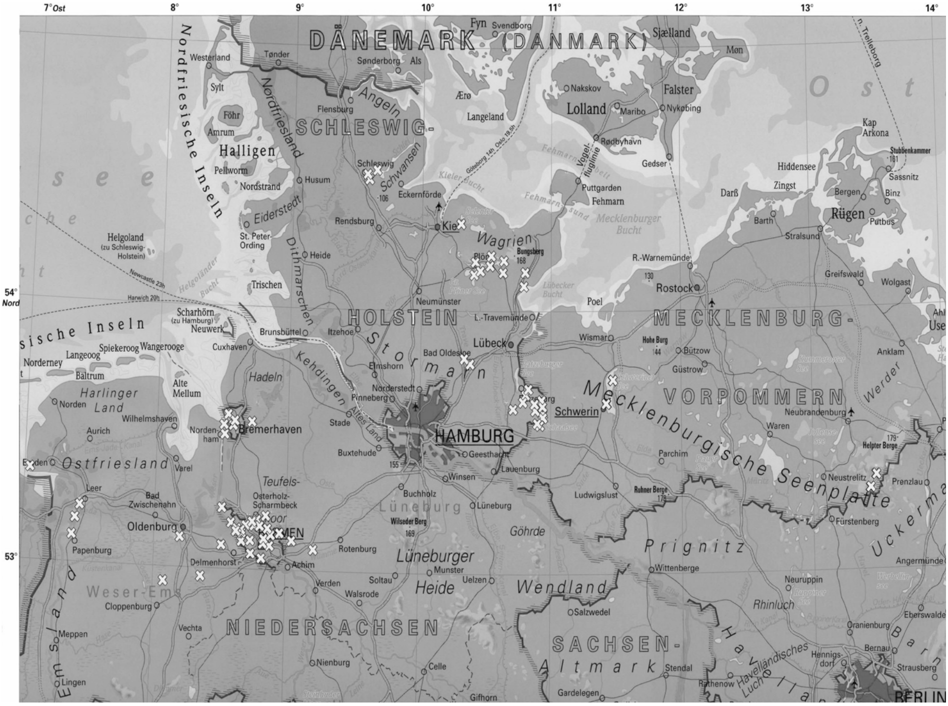


Fig. 1. Sampling sites (map from Stiefel Verlag GmbH, Lenting).

SEQUENCING: PCR products were sequenced directly on both strands using Big Dye Terminator v3.1 sequencing chemistry (Applied Biosystems, CA, USA). For the LSU rRNA gene and the *rbc*L-gene the sequencing reactions were made using the same primers already used in the PCR. Because of the length of the SSU rRNA gene, additional internal primers (Elwood et al. 1985) were used. Sequencing products were purified by DyeEx™ Spin Kit (QIAGEN, Germany) and electrophoresed on an ABI 3100 Avant sequencer (Applied Biosystems, CA, USA).

Sequence Analysis

Sequences exported from corrected electropherograms were assembled using SeqMan (Lasergene package, DnaStar, Madison, WI, USA). Accession numbers for the three genes are presented in Table 1. For the protein-coding *rbc*L-gene, the protein-sequence was also checked. Three species had internal stop codons in the primary sequence and these species are marked as pseudogenes in their GenBank entry. The alignment of the SSU rDNA sequences was done with ARB using the secondary structure. The sequences of the D1-D2 region and the *rbc*L Gene were aligned using ClustalX (Thompson et al. 1997) and checked manually using ProSeq v 2.9 beta (Filatov 2002). The rRNA genes show hypervariable regions for which it is difficult to obtain an unambiguous alignment. These highly variable sites (e.g., V4) were excluded from the alignment. The final data set contained 3226 bp of which 896 were informative for parsimony analyses.

To obtain three combinable alignments with the same set of species an alignment was computed for each gene using only the sequences of the cultures established for this study (Table 1). A second alignment was made for each gene using additional sequences obtained from GenBank (Table 4). For the individual genes, the analysis was performed on the combined datasets of GenBank and ALGATERRA sequences. For the analysis of the combined genes, only the sequences generated in

Table 1: List of diatom cultures established and sequenced within ALGATERRA.

Preparation	**Species**	**Author**	**Strain**	**Collection site**	**SSU**	**LSU**	***rbc*L**
1425	*Adlafia brockmannii*	(Hustedt)	AT_111Gel10	53°11,39N; 08°47,05E Hamme: river, plankton	AM502020	AM710576	AM710487
1438	*Achnanthidium minutissimum*	(Kützing) Czarnecki	AT_196Gel02	54°10,97N; 10°37,92E Ukelei See: lake, plankton	AM502032	AM710588	AM710499
1427	*Amphora* cf. *fogediana*	Krammer	AT_212.06	54°19,86N; 10°17,72E Dobersdorfer See: lake, benthos	AM502022	AM710578	AM710489
1264	*Amphora libyca*	Ehrenberg	AT_117.10	53°09,51N; 08°42,57E Lesum: river, plankton	AM501959	AM710513	AM710425
1263	*Amphora normannii*	Rabenhorst	AT_105Gel5	53°09,90N; 08°45,10E Wümme: river, benthos	AM501958	AM710512	AM710424
1265	*Amphora pediculus*	(Kützing) Grunow	AT_117.11	53°09,51N; 08°42,57E Lesum: river, plankton	AM501960	AM710514	AM710426
1554	*Amphora sp.*	Ehrenberg ex Kützing	AT_221.04	53°06,41N; 08°11,23E Hunte: river, plankton	AM501957	AM710600	AM710511
1256	*Asterionella formosa*	Hassall	AT_67-2b	53°13,79N; 08°41,06E Geeste: river, plankton	AM712617	AM778963	AM778961
1550	*Caloneis amphisbaena*	(Bory) Cleve	AT_177.07	53°04,08N; 08°29,04E Hasbruch: ditch, benthos	AM501954	AM710596	AM710507
23	*Caloneis budensis*	(Grunow) Krammer	AT_220.06	53°06,41N; 08°11,23E Hunte: riverside, soil	AM502003	AM710559	AM710470
1446	*Caloneis lauta*	J.R.Carter & Bailey-Watts	AT_160Gel04	52°57,65N; 08°20,67E Poggenpohls Moor: soil, moss	AM502039	AM710595	AM710506
1415	*Cocconeis pediculus*	Ehrenberg	AT_212.07	54°19,86N; 10°17,72E Dobersdorfer See: lake, benthos	AM502010	AM710569	AM710477
1418	*Cocconeis placentula*	Ehrenberg	AT_212Gel11	54°19,86N; 10°17,72E Dobersdorfer See: lake, benthos	AM502013	AM710566	AM710480
1318	*Craticula cuspidata*	(Kützing) D.G.Mann	AT_200.05	54°11,69N; 10°36,24E Krumm See: lake, benthos	AM501998	AM710554	AM710465

Preparation	Species	Author	Strain	Collection site	SSU	LSU	*rbc*L
1320	*Craticula cuspidata*	(Kützing) D.G.Mann	AT_219.03	53°06,41N; 08°11,23E Hunte: river, benthos	AM502000	AM710556	AM710467
283	*Craticula halophilioides*	(Hustedt) Lange-Bertalot	AT_5Nav02	53°09,65N; 08°43,40E Maschinenfleet: canal, plankton	AM501977	AM710544	AM710443
1308	*Craticula halophilioides*	(Hustedt) Lange-Bertalot	AT_36klein	53°12,72N; 08°26,85E Weser: river, benthos	AM501989	AM710532	AM710455
1284	*Craticula molestiformis*	(Hustedt) Lange-Bertalot	AT_70Gel14a	53°13,79N; 08°41,06E Geeste: riverside, moss	AM501978	AM710533	AM710444
1493	*Cyclotella choctawatcheeana*	Prasad	L1840	Geeste: river, plankton	AM712618	AM778964	AM778962
1414	*Cymbella affinis*	Kützing	AT_204Gel02	54°09,09N; 10°27,45E Großer Madebroken See: lake, plankton	AM502009	AM710565	AM710476
1423	*Cymbella affinis*	Kützing	AT_213.04	54°19,86N; 10°17,72E Dobersdorfer See: lake, periphyton	AM502018	AM710574	AM710485
1421	*Cymbella aspera*	(Ehrenberg) Cleve	AT_210Gel07	54°09,98N; 10°25,19E Trammer See: lake, periphyton	AM502016	AM710572	AM710483
1431	*Cymbella helmckei*	Krammer	AT_194Gel07	54°08,53N; 10°39,70E Großer Eutiner See: lake, benthos	AM502026	AM710582	AM710493
1317	*Cymbella naviculiformis*	(Auerswald) Cleve	AT_117.04	53°04,08N; 08°29,04E Hasbruch: ditch, benthos	AM501997	AM710553	AM71046
1324	*Cymbella naviculiformis*	(Auerswald) Cleve	AT_221.02	53°06,41N; 08°11,23E Hunte: river, plankton	AM502004	AM710560	AM710471
1422	*Cymbella proxima*	Reimer	AT_210Gel13	54°09,98N; 10°25,19E Trammer See: lake, periphyton	AM502017	AM710573	AM710484
1441	*Encyonema caespitosum*	Kützing	AT_214Gel03	54°19,86N; 10°17,72E Dobersdorfer See: lake, benthos	AM502035	AM710591	AM710502
1266	*Encyonema minutum*	(Hilse) D.G.Mann	AT_137.13	53°41,96N; 11°29,15E Schweriner See: lake, plankton	AM501961	AM710515	n/a

Preparation	Species	Author	Strain	Collection site	SSU	LSU	*rbc*L
1267	*Eolimna minima*	(Grunow) Lange-Bertalot	AT_70Gel18	53°13,79N; 08°41,06E Geeste: riverside, moss	AM501962	AM710516	AM710427
1268	*Eunotia formica*	Ehrenberg	AT_111Gel9	53°11,39N; 08°47,05E Hamme: river, plankton	AM502040	AM710517	AM710428
1321	*Eunotia implicata*	Nörpel, Lange-Bertalot & Alles	AT_219.07	53°06,41N; 08°11,23E Hunte: river, benthos	AM502001	AM710557	AM710468
1269	*Eunotia* sp.	Ehrenberg	AT_73Gel2	53°38,11N; 10°44,56E Pinnsee: lake, periphyton	AM501963	AM710518	AM710429
1254	*Fragilaria crotonensis*	Kitton	AT_185Gel3	53°07,20N; 09°03,52E Wümme: river, plankton	AM712616	AM713192	AM713181
1410	*Fragilaria* sp.	Lyngbye	AT_124.05b	53°33,00N; 10°55,16E Schaalsee: lake, benthos	AM502006	AM710562	AM710473
1445	*Frustulia vulgaris*	(Thwaites) De Toni	AT_108Gel03	53°10,89N; 08°45,70E Hamme: river, benthos	AM502038	n/a	n/a
1424	*Gomphonema acuminatum*	Ehrenberg	AT_219Gel10	53°06,41N; 08°11,23E Hunte: river, benthos	AM502019	AM710575	AM710486
1439	*Gomphonema affine*	Kützing	AT_196Gel03	54°10,97N; 10°37,92E Ukelei See: lake, plankton	AM502033	AM710558	AM710500
1322	*Gomphonema affine*	Kützing	AT_219Gel06	53°06,41N; 08°11,23E Hunte: river, benthos	AM502002	AM710589	AM710469
1409	*Gomphonema* cf. *angustatum*	(Kützing) Rabenhorst	AT_109Gel8b	53°10,89N; 08°45,70E Hamme: river, plankton	AM502005	AM710561	AM710472
1315	*Gomphonema* cf. *parvulum*	(Kützing) Kützing	AT_161.15	52°57,65N; 08°20,67E Poggenpohls Moor: puddle, soil	AM501995	AM710551	AM710462
1270	*Gomphonema micropus*	Kützing	AT_117.09	53°09,51N; 08°42,57E Lesum: river, plankton	AM501964	AM710519	AM710430
1271	*Gomphonema micropus*	Kützing	AT_117Gel21	53°09,51N; 08°42,57E Lesum: river, plankton	AM501965	AM710520	AM710431
1313	*Gomphonema productum*	(Grunow) Lange-Bertalot & Reichardt	AT_160Gel27	52°57,65N; 08°20,67E Poggenpohls Moor: soil, moss	AM501993	AM710549	AM710460
1552	*Gomphonema truncatum*	Ehrenberg	AT_195Gel09	54°08,53N; 10°39,70E Gr. Eutiner See: lake, periphyton	AM501956	AM710598	AM71050

Preparation	Species	Author	Strain	Collection site	SSU	LSU	*rbc*L
1272	*Hippodonta capitata*	(Ehrenberg) Lange-Bertalot, Metzeltin & Witkowski	AT_124.24	53°33,00N; 10°55,16E Schaalsee: lake, benthos	AM501966	AM710521	AM710432
1273	*Luticola goeppertiana*	(Bleisch) D.G.Mann	AT_104Gel12a	53°09,90N; 08°45,10E Wümme: river, plankton	AM501967	AM710522	AM710433
1274	*Mayamaea atomus* var. *atomus*	(Kützing) Lange-Bertalot	AT_115Gel7	53°11,79N; 08°48,11E Hamme: river, benthos	AM501968	AM710523	AM710434
1553	*Mayamaea atomus* var. *atomus*	(Kützing) Lange-Bertalot	AT-199Gel01	54°11,69N; 10°36,24E Krumm See: lake, plankton	n/a	AM710599	AM710510
1275	*Mayamaea atomus* var. *permitis*	(Hustedt) Lange-Bertalot	AT_101Gel4	53°40,20N; 10°50,21E Schwarze Kuhle: lake, periphyton	AM501969	AM710524	AM710435
1417	*Navicula capitatoradiata*	H.Germain	AT_212Gel07	54°19,86N; 10°17,72E Dobersdorfer See: lake, benthos	AM502012	AM710568	AM710479
1310	*Navicula cari*	Ehrenberg	AT_82.04c	53°36,36N; 10°54,02E Küchensee: lake, periphyton	AM501991	AM710546	AM710457
1279	*Navicula cryptocephala*	Kützing	AT_114Gel8c	53°13,63N; 08°53,22E Hamme: river, periphyton	AM501973	AM710528	AM710439
1316	*Navicula cryptocephala*	Kützing	AT_176Gel5	53°04,08N; 08°29,04E Hasbruch: ditch, plankton	AM501996	AM710552	AM710463
1416	*Navicula cryptotenella*	Lange-Bertalot	AT_212Gel01	54°19,86N; 10°17,72E Dobersdorfer See: lake, benthos	AM502011	AM710567	AM710478
1420	*Navicula cryptotenella*	Lange-Bertalot	AT_210Gel05	54°09,98N; 10°25,19E Trammer See: lake, periphyton	AM502015	AM710571	AM710482
1435	*Navicula cryptotenella*	Lange-Bertalot	AT_202Gel03	54°09,86N; 10°32,81E Dieksee: lake, benthos	AM502029	AM710585	AM710496
1280	*Navicula gregaria*	Donkin	AT_117Gel5	53°09,51N; 08°42,57E Lesum: river, plankton	AM501974	AM710529	AM710440
1278	*Navicula radiosa*	Kützing	AT_114Gel6	53°13,63N; 08°53,22E Hamme: river, periphyton	AM501972	AM710583	AM710438

Preparation	Species	Author	Strain	Collection site	SSU	LSU	*rbc*L
1433	*Navicula radiosa*	Kützing	AT_200.04	54°11,69N; 10°36,24E Krumm See: lake, benthos	AM502027	AM710590	AM710494
1440	*Navicula radiosa*	Kützing	AT_205.02b	54°09,09N; 10°27,45E Gr. Madebroken See: lake, benthos	AM502034	AM710527	AM710501
1282	*Navicula reinhardtii*	Grunow	AT_124.15	53°33,00N; 10°55,16E Schaalsee: lake, benthos	AM501976	AM710531	AM710442
1411	*Navicula* sp.1	Bory	AT_145.08	54°06,55N; 10°48,68E Neustädter Binnenwasser: brackish water, plankton	AM502007	AM710555	AM710474
1319	*Navicula* sp.2	Bory	AT_201Gel01	54°11,69N; 10°36,24E Krumm See: lake, benthos	AM501999	AM710563	AM710466
1434	*Navicula tripunctata*	(O.F.Müller) Bory	AT_202.01	54°09,86N; 10°32,81E Dieksee: lake, benthos	AM502028	AM710584	AM710495
1276	*Navicula veneta*	Kützing	AT_108Gel1	53°10,89N; 08°45,70E Hamme: river, benthos	AM501970	AM710525	AM710436
1277	*Navicula veneta*	Kützing	AT_110Gel19	53°11,39N; 08°47,05E Hamme: river, benthos	AM501971	AM710526	AM710437
1281	*Navicula veneta*	Kützing	AT_117Gel20b	53°09,51N; 08°42,57E Lesum: river, plankton	AM501975	AM710530	AM710441
1551	*Neidium affine*	(Ehrenberg) Pfitzer	AT_177.12	53°04,08N; 08°29,04E Hasbruch: ditch, benthos	AM501955	AM710597	AM710508
1426	*Pinnularia acrosphaeria*	Rabenhorst	AT_161.08	52°57,65N; 08°20,67E Poggenpohls Moor: puddle, soil	AM502021	AM710577	AM710488
1286	*Pinnularia anglica*	Krammer	AT_100Gel1	53°40,20N; 10°50,21E	AM501980	AM710535	AM710446

Table 2: Primers used for PCR

Gene	Primer	Sequence (5' ® 3')	Author
SSU rRNA	1F	AAC CTG GTT GAT CCT GCC AGT	Medlin et al. (1988), without polylinker
	1528R	TGA TCC TTC TGC AGG TTC ACC TAC	Medlin et al. (1988), without polylinker
LSU rRNA	DIRF	ACC CGC TGA ATT TAA GCA TA	Scholin et al. (1994)
	D2CR	CCT TGG TCC GTG TTT CAA GA	Scholin et al. (1994)
*rbc*L	F3	GCT TAC CGT GTA GAT CCA GTT CC	Beszteri, unpubl.
	R3	CCT TCT AAT TTA CCA ACA ACT G	Beszteri, unpubl.

Table 3: PCR programs

Cycle step	SSU and LSU rRNA		*rbc*L	
	Temperature	Time	Temperature	Time
Initial denaturation	94°C	7 min	94°C	10 min
	Cycle		Cycle	
Denaturation	94°C	2 min	94°C	1 min
Annealing	54°C	4 min	56°C	1 min
Elongation	72°C	2 min	72°C	2 min
Cycle repetitions	35		31	
Final elongation	72°C	7 min	72°C	10 min

the ALGATERRA project were used so that only species with sequence data for all three genes were used.

Phylogenetic analyses were performed using PAUP* 4.0b10 (Swofford 1998). In all analyses, the data set was rooted using one centric (*Cyclotella choctawatcheeana*) and two araphid diatoms (*Fragilaria crotonensis* and *Asterionella formosa*), as the use of several outgroup taxa improves the analyses (Swofford et al. 1996). For maximum likelihood (ML) and distance based tree calculations, likelihood scores of different nucleotide substitution models were compared on a neighbor joining tree using Modeltest 3.0 (Posada & Crandall 1998). Based on the Akaike Information Criterion (AIC) the best fit model (GTR +I +G) was identified for all genes. This was used for phylogenetic analyses using ML and neighbor joining (NJ) tree inference with ML distances. Maximum parsimony (MP) and ML trees were obtained in heuristic searches, with 10 random taxon additions. To assess confidence in clades recovered, bootstrapping of MP and NJ analyses was made with 1000 replicates (Felsenstein 2004). If necessary, a time limit of 15 minutes was set for each replicate. For the combined dataset, 100 replicates of the partition homogeneity test, as implemented in PAUP, were performed.

Microscopy

For identification and morphological investigations of the cultures, light and scanning microscopy were used. Living cells as well as cleaned frustules were examined and photographed by bright field microscopy using a ZEISS Axioplan microscope with a AxioCam MRc digital camera. To remove all organic material, the cells were oxidized with $KMnO_4$ for 12-16 hours. Then HCl was added and the mixture boiled until it turned light yellow. The liquid was discarded and the frustules were

Table 4: List of species of diatoms obtained from GenBank and their accession numbers of the used gene sequences

Species		Authority	SSU rRNA	LSU rRNA	*rbc*L
Achnanthes	*bongranii*	(M.Peragallo) A.Mann	AJ535150		
Achnanthes	*brevipes*	C.Agardh	AY485476		
Achnanthes	*minutissima*	Kützing	AJ866992		
Achnanthes	sp. 1	Bory	AY485496		
Achnanthes	sp. 2	Bory	AJ535151		
Achnanthidium	cf. *longipes*	C.Agardh	AY485500		
Amphora	cf. *capitellata*	Frenguelli	AJ535158		
Amphora	cf. *proteus*	W.Gregory	AJ535147		
Amphora	*coffeaeformis*	(C.Agardh) Kütz.	AY485498	AF417682	
Amphora	*montana*	Krasske	AJ243061		
Amphora	sp.	Ehrenberg ex Kützing	AB183590		
Anomoeoneis	*sphaerophora*	(Kützing) Pfitzer	AJ535153		
Bacillaria	*paxillifer*	(Müller) Hendey	M87325	AF417678	
Campylodiscus	*ralfsii*	C.Agardh	AJ535162		
Cocconeis	cf. *molesta*	Kützing	AJ535148		
Cyclotella	*choctawatcheeana*	A.K.S. Prasad		AJ878463	
Cylindrotheca	*closterium*	(Ehrenberg) Reimer & Lewin	M87326		
Cymatopleura	*elliptica*	(Brébisson) W.Smith	AJ867030		
Cymbella	*cymbiformis*	W.Smith	AJ535156		
Diadesmis	*gallica*	W.Smith	AJ867023		
Dickieia	*ulvacea*	Berkeley	AY485462		
Encyonema	cf. *sinicum*	Krammer			AY571754
Encyonema	*triangulatum*	(Ehrenberg) Kützing	AJ535157		
Entomoneis	*alata*	(Ehrenberg) Ehrenberg	AY485497		
Entomoneis	*paludosa*	(W.Smith) Reimer	AY485468		
Entomoneis	cf. *alata*	(Ehrenberg) Ehrenberg	AJ535160		
Entomoneis	sp.	Ehrenberg		AF417683	
Eolimna	*minima*	(Grunow) Lange-Bertalot	AJ243063		
Eolimna	*subminuscula*	(Mangin) Moser	AJ243064		
Eunotia	*minor*	(Kützing) Grunow			AY571744
Eunotia	*bilunaris*	(Ehrenberg) Mills	AJ866995		
Eunotia	cf. *pectinalis* f. *minor*	(Kützing) Rabenhorst	AJ535146		
Eunotia	*formica* var. *sumatrana*	Hustedt	AB085830		
Eunotia	*monodon* var. *asiatica*	Skvortsov	AB085831		
Eunotia	*pectinalis*	(Kützing) Rabenhorst	AB085832		
Eunotia	sp.	Ehrenberg	AJ535145		
Fragilaria	*crotonensis*	Kitton	AF525662		
Asterionella	*formosa*	Hassall	AF525657		
Fragilariopsis	*cylindrus*	Hasle	AY672802	AF417657	
Gomphonema	*capitatum*	Ehrenberg			AY571751
Gomphonema	*parvulum*	(Kützing) Kützing	AJ243062		
Gomphonema	*pseudaugur*	Lange-Bertalot	AB085833		
Gyrosigma	*limosum*	Sterrenburg & Underwood	AY485516		
Haslea	*crucigera*	(W.Smith) Simonsen	AY485482		
Haslea	*nipkowii*	(Meister) Poulin & G.Massé	AY485488		

Species		Authority	SSU rRNA	LSU rRNA	*rbc*L
Haslea	*ostrearia*	(Gaillon) Simonsen	AY485523		
Haslea	*pseudostrearia*	G.Massé, Rincé & E.J.Cox	AY485524		
Lyrella	*atlantica*	(A.Schmidt) D.G.Mann	AJ544659		AY571747
Lyrella	*hennedyi*	(W.Smith) Stickle & D.G.Mann			AY571755
Lyrella	sp.	N.I. Karajeva			AY571756
Lyrella	sp. 2	N.I. Karajeva	AJ535149		
Navicula	*atomus* var. *permitis*	(Hustedt) Lange-Bertalot	AJ867024		
Navicula	cf. *duerrenbergiana*	Hustedt			AY571749
Navicula	cf. *erifuga*	Lange-Bertalot		AF417679	
Navicula	*cryptocephala* var. *veneta*	(Kützing) Rabenhorst	AJ297724		
Navicula	*diserta*	Hustedt	AJ535159		
Navicula	*lanceolata*	(C.Agardh) Kützing	AY485484		
Navicula	*pelliculosa*	(Brébisson ex Kützing) Hilse	AY485454		
Navicula	*phyllepta*	Kützing	AY485456		
Navicula	*ramosissima*	(C.Agardh) Cleve	AY485512		
Navicula	*salinicola*	Hustedt			AY604699
Navicula	*saprophila*	Lange-Bertalot & Bonik	AJ867025		
Navicula	*sclesviscensis*		AY485483		
Navicula	sp.	Bory	AY485513		
Navicula	sp. 2	Bory	AY485502		
Navicula	sp. 3	Bory	AY485460		
Nitzschia	*amphibia*	Grunow	AJ867277		
Nitzschia	*communis*	Rabenhorst	AJ867278	AF417661	
Nitzschia	cf. *frustulum*	(Kützing) Grunow	AJ535164	AF417671	
Nitzschia	*sigma*	(Kützing) W.Smith	AJ867279		
Nitzschia	*vitrea*	G.Norman	AJ867280		
Pauliella	*taeniata*	(Grunow) Round & Basson	AY485528	AF417680	
Peridinium	*balticum* endosymbiont		Y10566		
Peridinium	*foliaceum* endosymbiont		Y10567		
Petroneis	*humerosa*	(Brébisson ex W.Smith) Stickle & D.G.Mann			AY571757
Phaeodactylum	*tricornutum*	Bohlin	AY485459	AF417681	
Pinnularia	cf. *interrupta*	W.Smith	AJ544658		
Pinnularia	*rupestris*	Hantzsch	AJ867027		
Pinnularia	sp.	Ehrenberg	AJ535154		
Placoneis	cf. *paraelginensis*	Lange-Bertalot			AY571753
Placoneis	*constans*	(Hustedt) E.J.Cox			AY571752
Pleurosigma	*intermedium*	W.Smith	AY485489		
Pleurosigma	*planktonicum*	H.- J.Schrader	AY485514		
Pleurosigma	sp.	W.Smith	AY485515		
Pleurosigma	sp. 2	W.Smith	AF525664		
Pseudogomphonema	cf. *kamtschaticum*	(Grunow) Medlin			AY571748
Pseudogomphonema	sp. 1	Medlin	AJ535152		

Species		Authority	SSU rRNA	LSU rRNA	*rbc*L
Pseudo-gomphonema	sp. 2	Medlin	AF525663		
Rossia	sp.	M.Voigt	AJ535144		
Sellaphora	*bacillum*	(Ehrenberg) Mann			AY571745
Sellaphora	*laevissima*	(Kützing) Mann	AJ544655		
Sellaphora	*pupula*	(Kützing) Mereschkowsky	AJ544649		AY571746
Sellaphora	*pupula* var. *captitata*	(Skvortsov & K.I.Meyer) Poulin	AJ535155		
Seminavis	cf. *robusta*	Danielidis & D.G.Mann			AY571750
Stauroneis	*constricta*	(W.Smith) Cleve	AY485521		
Surirella	*angusta*	Kützing	AJ867028		
Surirella	*brebissoni*	Krammer & Lange-Bertalot	AJ867029		
Surirella	*fastuosa* var. *cuneata*	(A.Schmidt) H.Peragallo & M.Peragallo	AJ535161		
Tryblionella	*apiculata*	(W.Gregory) D.G.Mann	M87334		
uncultured *Eunotia*-like diatom			AY821975		
Undatella	sp.	Paddock & P.A.Sims	AJ535163		

washed 4 times with distilled water. The cleaned frustules were stored in distilled water. Permanent slides were made in Naphrax. For electron microscopy, coverslips were attached to aluminium specimen stubs with double-sided adhesive tape. Cleaned frustules were pipetted onto stubs, which were platinum-coated with a sputter coater (Emscope SC 500). Electron micrographs of cleaned frustules were taken at 10kV accelerating voltage on a Quanta FEG 200F, a PHILIPS XL30 ESEM or an I.S.I. DS-130.

Results

The phylogenetic trees generated in this study clearly show that *Placoneis,* consisting in our analysis of *Placoneis elginensis,* and an unidentified species, is distinct from *Navicula sensu stricto* and that *N. hambergii* belongs to *Placoneis* because it diverged at the base of or within the genus in most trees (Figs 2-5). The monophyly of *N. hambergii* and *Placoneis* was well supported, but its relationship/monophyly to other genera in the order Cymbellales varied with the gene used. In the SSU tree with the ML analysis, *Placoneis* is monophyletic and sister to clade containing *Cymbella, Gomphonema,* and *Encyonema,* but this relationship is unsupported (Fig. 2). In the LSU tree using ML analyses, *Placoneis* is sister to a clade with *Cymbella* and *Gomphonema,* and *Encyonema* is sister to both of these (Fig. 3). In the *Rbc*L tree, *Cymbella* is not monophyletic and one species falls at the base of the *Placoneis* clade (Fig. 4). *Gomphonema* and *Encyonema* are separate lineages basal to the *Placoneis/Cymbella* clade. In the combined analysis of all three genes, *Placoneis* is a well-supported monophyletic clade sister to *Cymbella*. Again *Encyonema* and *Gomphonema* are basal to this lineage. All four analyses place *Placoneis* in the Cymbellales (Fig. 5).

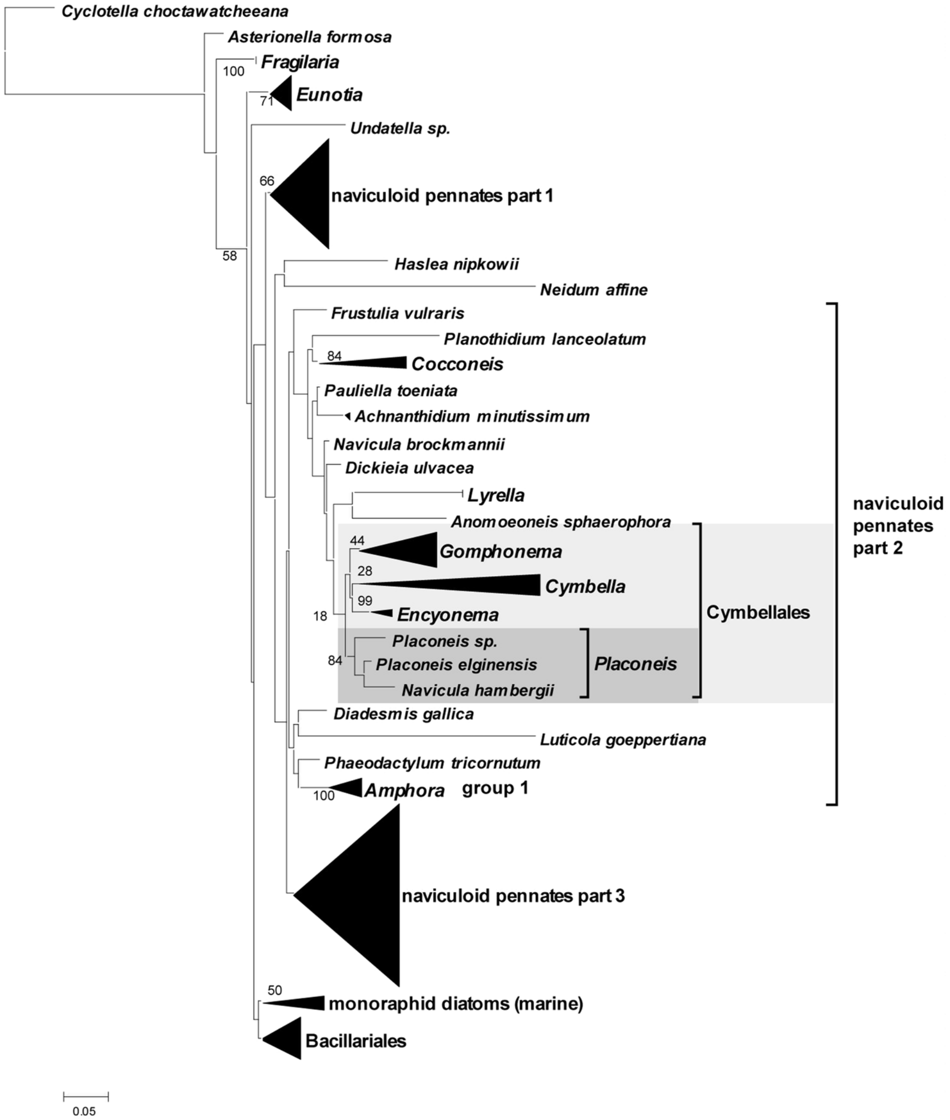


Fig. 2. Phylogeny inferred with the ML analysis using SSU rDNA sequences from GenBank and the AlgaTerra cultures. Bootstrap values obtained from 1000 replications based on NJ analyses (GTR +I +G model) have been plotted at the nodes. Condensed regions will be shown in detail in separate papers.

Although it was already known that *N. hambergii* did not belong to *Navicula sensu stricto* (e.g., Krammer and Lange-Bertalot 1986), the species had not been reassigned to another genus, although Metzeltin et al. (2004, p. 8) noted that "*Navicula hambergii* belongs very probably to *Placoneis*". All features defining

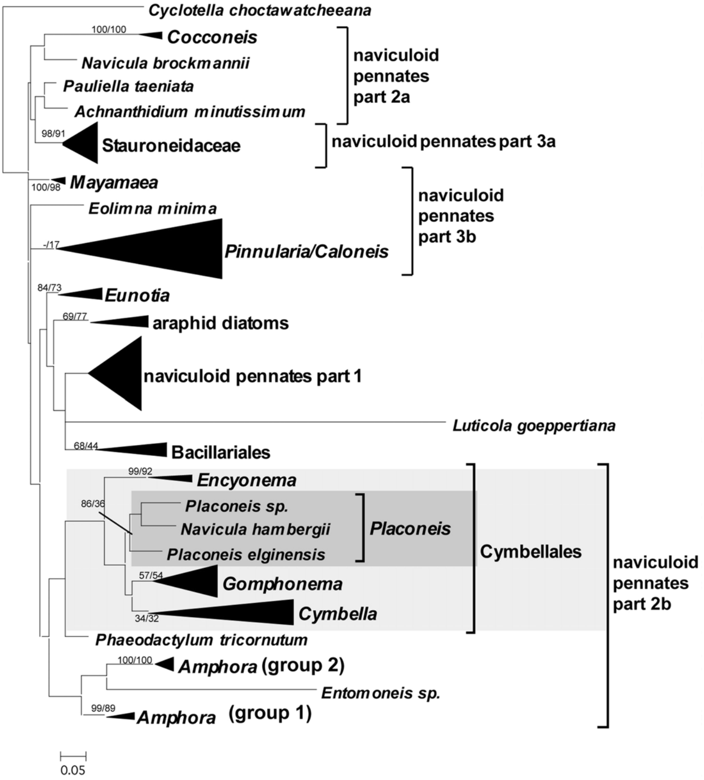


Fig. 3. Details of the ML tree analysis from LSU rDNA sequences from GenBank and AlgaTerra cultures. Bootstrap values obtained from 1000 replications based on NJ analyses using GTR +I +G model and on parsimony analyses have been plotted at the nodes. Collapsed clades will be discussed in future papers.

Placoneis were found in *N. hambergii* and supported its transfer to *Placoneis*. Morphological investigations of *Navicula hambergii* and *Placoneis elginensis* indicated that these two species were near relatives. The single chloroplast, with a central bridge from which lobes project into the four quadrants of the cell (Figs 6, 7, 13, 14), is typical for species belonging to *Placoneis*. The striae are radiate (Figs 8, 15). At the centre of the valve the striae are irregularly abbreviated (*P. elginensis*, Figs 8, 12) or alternately longer and shorter (*N. hambergii*, Figs 15, 19). With SEM it can be seen, that, externally, the striae consist of small round areolae (Figs 12, 19). Internally, the areolae are almost square and closed by vola-like occlusions (Figs 9, 16). Both species have a straight raphe with slightly expanded external central endings and at both poles the hook-like raphe fissures curve to the same side (Figs 8, 12, 15, 19). The internal central raphe endings of both species are hooked (Figs 11, 18) and the internally helictoglossae at the polar raphe endings are straight and knob-like (Figs 10, 17).

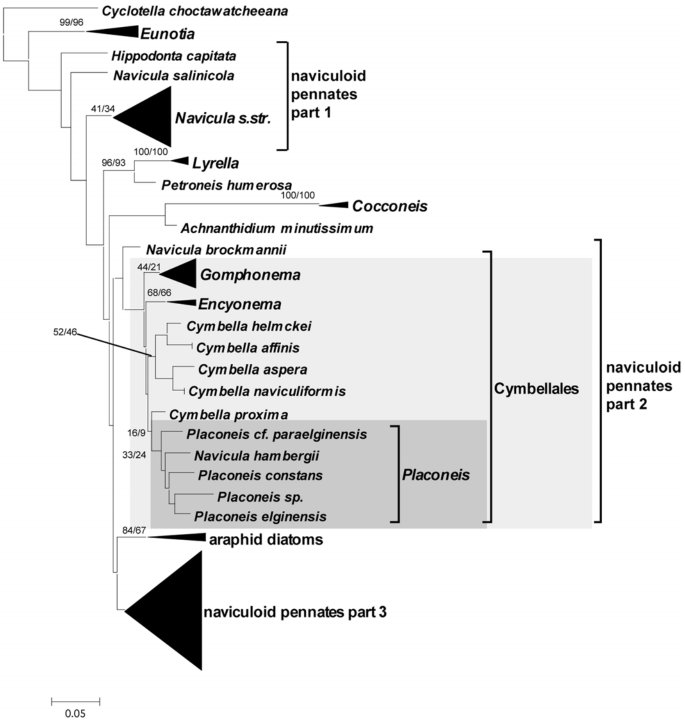


Fig. 4. Details of the ML tree analysis from *rbc*L sequences from GenBank and the AlgaTerra cultures. Bootstrap values obtained from 1000 replications based on NJ analyses using GTR +I +G model and on parsimony analyses have been plotted at the nodes. Collapsed clades will be discussed in future papers.

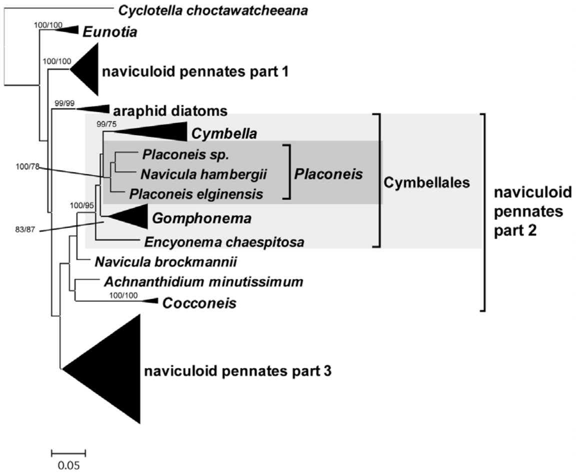
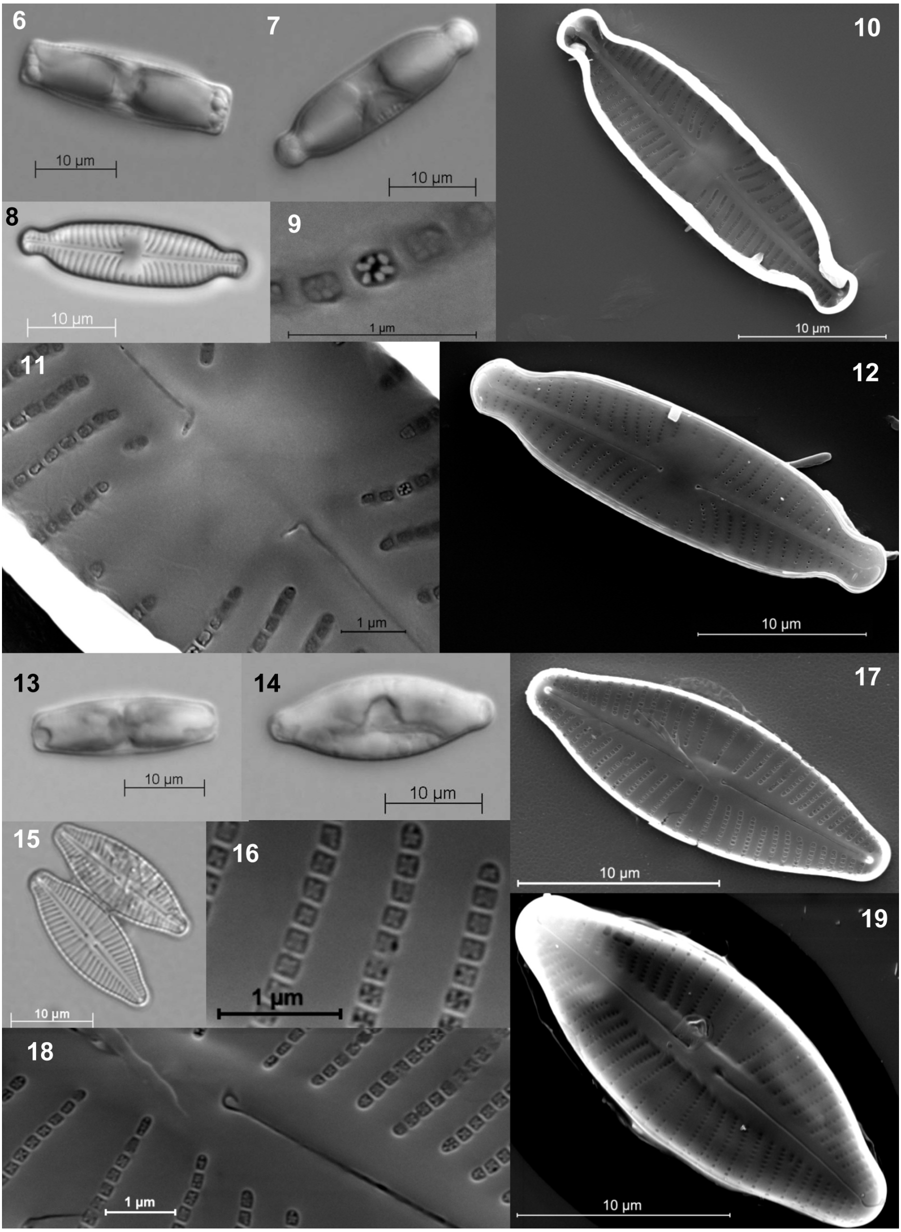


Fig. 5. Phylogeny inferred with the ML analysis using the combined dataset of SSU rDNA, LSU rDNA and *rbc*L sequences from the AlgaTerra cultures. Bootstrap values obtained from 1000 replications based on NJ analyses using JC model and on parsimony analyses have been plotted at the nodes. Condensed clades will be shown in future papers.



Figs 6-12. *Placoneis paraelginensis*. Fig. 6. Girdle view of living cell, LM. Fig. 7. Valve view of living cell, LM. Fig. 8. Cleaned valve, LM. Fig. 9. Detail areolae, SEM, showing valve interiors. Fig. 10. Internal valve view with attached girdle bands. Fig. 11. Detail of internal central raphe endings, SEM. Fig. 12 External valve view, SEM. Figs 13-19. *Navicula hambergii*. Fig. 13. Girdle view, LM. Fig. 14. Valve view, LM. Fig. 15. Cleaned valve. Fig. 16. Detail of areolae, SEM. Fig. 17. Internal valve view, SEM. Fig. 18. Detail of internal central raphe endings, SEM. Fig. 19. External valve view, SEM.

Discussion

Mereschkowsky described the genus *Placoneis* in 1903 and used *P. exigua* as the type species. With this genus he separated a group of species from *Navicula sensu lato*, which have a single, asymmetrical chloroplast. Cox (1987) re-erected the genus and chose *P. gastrum* as the type species, because "delineation and nomenclature of *P. exigua* are confused" (Cox 1987, p. 153). In the same paper and a second investigation, (Cox 2003) she added several morphological features from SEM investigations to the description of the genus. One of the most important features of the genus *Placoneis* is the single chloroplast with a central bridge and lateral lobes, which lies under the valves. This is the feature that allies them most easily with the Cymbellales, a feature noted as early as 1891 by Cleve. The cells are symmetrical and parallel or elliptical sided in their central region. The striae are radiate near the centre of the valve, becoming more parallel at the apices. They are composed of small round areolae, which are internally closed by volae. The usually straight raphe slits lie in a narrow axial area. Externally, the central raphe endings are straight and slightly expanded and the polar raphe endings curve to the same side. The internal central raphe endings are usually deflected to the same side and at the internal polar end small helictoglossae are present, another feature shared with the Cymbellales. Reproductive features shared by the two genera are discussed in Mann and Stickle (1995).

These characters are distinct from *Navicula sensu stricto* and the separation/resurrection of *Placoneis* initially substantiated from morphological data is now supported from molecular data. In two of the molecular analyses, including the final combined analysis of all three genes, *Placoneis* was sister to *Cymbella*. Likewise, in the combined analysis and one other analysis, *Gomphonema* and *Encyonema* are basal to the *Placoneis/Cymbella* clade. Other new taxa assigned to the Cymbellales by Krammer (1982) and Krammer (1997) are not represented in this study because there were no cultures available for molecular analysis.

In a cladistic analysis of protoplast and frustular features of naviculoid diatoms, Cox and Williams (2006) obtained different phylogenetic positions for *Placoneis* depending on which characters were used in the analysis. Using all features, *Placoneis* appeared as an outgroup clade. The remaining Cymbellales are together in another more derived clade. This is because *Placoneis* differed very little from the features they considered as primitive in the naviculoid diatoms. *Placoneis* contained states other than the primitive state in nine out of the 35 characters that they coded. Of these nine characters, six of them are plastid characters and in the analysis of plastid data alone, *Placoneis* groups with the other Cymbellales, as it does in the nuclear molecular data, although *Placoneis* is not resolved as a monophyletic genus with their cladistic analysis. When the frustule data are partitioned from the other data, then *Placoneis* fell as a monophyletic genus as part of an unresolved polytomy of naviculoid genera. The frustular features of *Placoneis* that separate it primarily from the Cymbellales are the structure of the areolae coverings and the symmetry of the cell. In *Placoneis*, the areolae are closed by a distinct cribrum, which has been termed a rota and the cells are seldom dorsiventral (Cox & Williams 2006). Cox (2004) reassessed the structure and terminology for pore occlusions in the raphid diatom. She gave a new name for the pore occlusion in *Placoneis*, the tectulum. This covering is placed over the internal opening to the areolae (Cox, 2004, fig. 21) and the external opening is unobstructed. In contrast, other members of the Cymbellales have the external opening of the areolae constricted or expanded in some manner from the virgae of the striae. There is no cribrum closing the internal opening of the areolae. The variety of constriction/expansions from the exernal opening of the areolae are illustrated in Cox (2004, figs 24-27). This is likely one of the features separating *Placoneis* from the remaining Cymbellales; from our molecular tree, it could be interpreted that the Cymbellales have lost the cribrum, which has been retained by its basal member, *Placoneis*. Instead the Cymbellales have modified the external opening of the areolae by extensions from the striae across the areolar opening.

Based on the results of the molecular and morphological analyses of *Navicula hambergii* a new combination must be made:

Placoneis hambergii (Hustedt) Bruder comb. nov. (Fig. 6).

BASIONYM: *Navicula hambergii* Hustedt (1924, Die Bacillariaceen-Vegetation des Sarekgebirges. - In: Hamberg, A. (ed.): Naturwissenschaftliche Untersuchungen des Sarekgebirges in Schwedisch-Lappland, Botanik 3 (6): p. 562, pl. 17: fig. 2).

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