Phylogeny of the Bacillariaceae with emphasis on the genus *Pseudo-nitzschia* (Bacillariophyceae) based on partial LSU rDNA

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In order to elucidate the phylogeny and evolutionary history of the Bacillariaceae we conducted a phylogenetic analysis of 42 species (sequences were determined from more than two strains of many of the *Pseudo-nitzschia* species) based on the first 872 base pairs of nuclear-encoded large subunit (LSU) rDNA, which include some of the most variable domains. Four araphid genera were used as the outgroup in maximum likelihood, parsimony and distance analyses. The phylogenetic inferences revealed the Bacillariaceae as monophyletic (bootstrap support ⩾ 90%). A clade comprising *Pseudo-nitzschia*, *Fragilariopsis* and *Nitzschia americana* (clade A) was supported by high bootstrap values (⩾ 94%) and agreed with the morphological features revealed by electron microscopy. Data for 29 taxa indicate a subdivision of clade A, one clade comprising *Pseudo-nitzschia* species, a second clade consisting of *Pseudo-nitzschia* species and *Nitzschia americana*, and a third clade comprising *Fragilariopsis* species. *Pseudo-nitzschia* as presently defined is paraphyletic and emendation of the genus is probably needed. The analyses suggested that *Nitzschia* is not monophyletic, as expected from the great morphological diversity within the genus. A cluster characterized by possession of detailed ornamentation on the frustule is indicated. Eighteen taxa (16 within the Bacillariaceae) were tested for production of domoic acid, a neurotoxic amino acid. Only *P. australis*, *P. multiseries* and *P. seriata* produced domoic acid, and these clustered together in all analyses. Since *Nitzschia navis-varingica* also produces domoic acid, but is distantly related to the cluster comprising the *Pseudo-nitzschia* domoic acid producers, it is most parsimonious to suggest that the ability of species in the Bacillariaceae to produce domoic acid has evolved at least twice.

Key words: Bacillariaceae, diatoms, domoic acid, *Fragilariopsis*, LSU rDNA, morphology, *Nitzschia*, phylogeny, *Pseudo-nitzschia*, taxonomy

Introduction

Pseudo-nitzschia H. Peragallo 1899 is one of the most common diatom genera among marine phytoplankton. It occurs in polar, temperate, subtropical and tropical areas worldwide (Hasle, 1964, 1965*a*, *b*, 1972*a*; Kaczmarska *et al.*, 1986; Fryxell *et al.*, 1991). The genera *Fragilariopsis* Hustedt in Schmidt *emend.* Hasle 1993 and *Nitzschia* Hassall 1845 closely resemble *Pseudo-nitzschia* and the relationships among these genera have been debated for many years (e.g. Peragallo, 1921; Hustedt, 1952, 1958*a*, *b*; Kolbe, 1954; Paasche, 1961; Geissler & Gerloff, 1963; Hasle, 1964, 1965*b*, 1972*b*, 1993, 1994, 1995; Round *et al.*, 1990). Concurrently the diagnoses and taxonomic affiliations of both *Pseudo-nitzschia* and *Fragilariopsis* have changed.

Pseudo-nitzschia was described in H. & M. Peragallo (1897–1908) by H. Peragallo. It appears from this book that the plate with the name *Pseudo-nitzschia* was first published in 1899. *Fragilariopsis* was erected in 1913 by Hustedt. Hustedt (1958*b*) reduced *Pseudo-nitzschia* to a section within *Nitzschia*, noting that the raphe of *Pseudo-nitzschia* is only slightly reduced compared with *Nitzschia*. In contrast, the raphe of *Fragilariopsis* was considered functionally fully reduced and hence its generic status was preserved. In 1972, Hasle (1972*b*) reduced *Fragilariopsis* to a section within *Nitzschia*, due to the morphological similarities between *Pseudo-nitzschia* and *Fragilariopsis*. Round *et al.* (1990) recommended retaining *Fragilariopsis* as a separate genus, based upon morphological characters as well as the already great diversity within *Nitzschia*. Hasle (1993, 1994) re-erected *Pseudo-nitzschia* as a separate genus after comparing morphological characters of *Pseudo-nitzschia* and *Nitzschia* subgenus *Nitzschia* (Mann, 1986). However, the close relationship between *Pseudo-nitzschia* and *Fragilariopsis* was still emphasized by Hasle (1993, 1994).

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Due to the taxonomic uncertainties, a phylogenetic study of the Bacillariaceae is therefore of potential interest as a means of clarifying the phylogenetic positions and the taxonomy of the genera.

Several *Pseudo-nitzschia* species have been shown to produce the neurotoxin domoic acid, and blooms of *Pseudo-nitzschia* may result in accumulation of the toxin in the marine foodweb, thereby affecting both marine organisms and, potentially, humans (Bates *et al.*, 1989; Work *et al.*, 1993; Lefebvre *et al.*, 1999; Scholin *et al.*, 2000). Hence, *Pseudo-nitzschia* has received much attention since 1987, when a large bloom affected more than a hundred humans in Canada, resulting in the first awareness of toxin-producing diatoms (Bates *et al.*, 1989). Several different *Pseudo-nitzschia* species have subsequently been reported to produce domoic acid (Martin *et al.*, 1990; Garrison *et al.*, 1992; Lundholm *et al.*, 1994; Rhodes *et al.*, 1996, 1998; Trainer *et al.*, 1998; Sarno & Dahlman, 2000). The recent demonstration that *Nitzschia navis-varingica* Lundholm et Moestrup is a major producer of domoic acid (Kotaki *et al.*, 2000) and an earlier paper mentioning production of domoic acid in *Amphora coffeaeformis* (Agardh) Kützing (Maranda *et al.*, 1990) indicates that the question of domoic acid production should be extended to other diatom genera. Sala *et al.* (1998), however, questioned the identity of the organism identified as *Amphora coffeaeformis*. In this context, a phylogenetic study of the Bacillariaceae may therefore be expected to give information on the phyletic nature of toxin production (i.e. mono- versus polyphyly) and reveal additional species that might produce domoic acid.

Before assessing the monophyly of the Bacillariaceae, and of *Pseudo-nitzschia* in particular, it is necessary to establish at which level monophyly can be concluded, from the presently available molecular and morphological data. A monophyletic origin of the diatoms within the stramenopiles/ heterokonts has been firmly established (e.g. Medlin *et al.*, 1993; Leipe *et al.*, 1994; van de Peer *et al.*, 1996; Andersen *et al.*, 1998; Guillou *et al.*, 1999; Daugbjerg & Guillou, 2001). Within the diatoms, a monophyletic origin of the pennate diatoms, and within this group a monophyletic origin of the raphid diatoms, has also been shown, using either small subunit (SSU) rDNA or partial large subunit (LSU) rDNA (Medlin *et al.*, 1993, 1996, 2000; Philippe *et al.*, 1994). Based on morphological evidence a monophyletic origin of the Bacillariaceae has long been unquestioned (Van Heurck, 1885; Peragallo, 1897–1908; Hustedt, 1930; Simonsen, 1979; Round *et al.*, 1990). The analyses of Medlin *et al.* (2000), based on SSU rDNA, support this view, indicating that the Bacillariaceae is monophyletic. In our phylogenetic study of *Pseudo-nitzschia* we therefore decided to sample as many representatives

of the genera within Bacillariaceae as possible, as well as a few representatives of other raphid genera.

The nuclear-encoded LSU rDNA comprises more variable areas than SSU rDNA and, as comparative analyses seem to indicate a stronger phylogenetic signal in LSU than in SSU (Van Der Auwera & De Wachter, 1998; see Soltis & Soltis, 1998), we decided to use partial sequences of the LSU rDNA gene. Prior to this study, the only LSU rDNA sequences available for raphid diatoms in the most variable regions (the first approx. 800 base pairs) were those obtained by Miller & Scholin (1994). In this study we present a phylogenetic analysis of the Bacillariaceae with emphasis on *Pseudo-nitzschia* based on the first *c.* 840 base pairs of LSU rDNA, including the highly variable regions B13_1–B16, the entire C and D5–D5_1 (Ben Ali *et al.* 1999). This corresponds to D1–D3 (Lenaers *et al.*, 1989).

Materials and methods

Cultures and field material

All cultures (Table 1) were clonal, non-axenic and were isolated or acquired from Eun Cho (KoreaA, rensubfrau), Lars Holtegaard (STH14, STH19, Hobart5), Karin Jensen (*Navicula* strain), Yuichi Kotaki (VSP974-1, 99SK2-4 99NG1-16 and VHL987), Kristian Priisholm (no7), Jette Skov (VPB-B3), Yang Zhenbo (Zhenbo7B), The Culture Collection of Algae at the University of Texas at Austin (UTEX strains), Provasoli-Guillard National Center for Marine Phytoplankton (CCMP strains) or Universität zu Köln, Botanisches Institut (M strains). Cultures were grown at 24 °C in a L:D 12:12 h regime or at 15 °C or 4 °C in a L:D 16:8 h regime, mostly in 32 psu L1 medium (Guillard & Hargraves, 1993) and all at a photon fluence rate of 20–60 μmol m^{-2} s^{-1}. The heterotrophic *Nitzschia alba* was grown in L medium to which an organic stock solution was added (Guillard, 1960). The freshwater species were grown in WEES (McFadden & Melkonian, 1986). The identity of all cultures, including those received from culture collections, was carefully examined. The identities of some cultures were changed (Table 1).

Removal of organic material

For studies of valve morphology, a subsample was taken soon after the culture was established or acquired and fixed in 2% glutaraldehyde. The organic material was removed by oxidation by adding 2 ml 30% H_2SO_4 and 10 ml saturated aqueous solution of $KMnO_4$ to a 10 ml sample. The sample was cleared after 24 h by addition of 10 ml saturated aqueous solution of oxalic acid and subsequently rinsed several times with distilled water (Christensen, 1988). For studies of girdle band structure, two different methods were used: (1) that of Hasle (1970) or (2) 10 ml of 60% HNO_3 was added to a concentrated subsample of about 1 ml. Grains of $NaNO_2$ were added until fizzing had nearly stopped. The sample was then left for 24 h and subsequently rinsed three times with distilled water (modified after Hargraves & Guillard, 1974;

Table 1. Cultures sequenced in this study

Taxon	Strain designation	Origin	Accession no.
Amphora coffeaeformis	Amphora	Nivå Bay, Denmark	AF417682
Asterionellopsis glacialis	CCMP1717	Kastisna Bay, Gulf of Alaska	AF417687
Bacillaria paxillifer	Tenerife7	Tenerife, Canary Islands	AF417678
Cylindrotheca closterium	K-520	Kattegat, Denmark	AF417666
Cylindrotheca fusiformis	UTEX2083	Sandy Hook, NJ, USA	AF417665
Entomoneis sp.	99SK2-4	Ishigaki Island, Japan	AF417683
Fragilaria capucina (**F.* sp.)	M1767	Fühlinger See, Cologne, Germany	AF417684
Fragilariopsis curta	1-A	Ross Sea 74°0′2″ S, 140°0′76″ W, ice	AF417659
Fragilariopsis cylindrus	2-E-F	Ross Sea, 74°0′2″ S, 140°0′76″ W, ice	AF417657
Fragilariopsis kerguelensis	4-20	Ross Sea, 65°2570′ S, 149°58′1″ W	AF417658
Fragilariopsis rhombica	5-17	Ross Sea, 66°58′63″ S, 149°58′53″ W, ice	AF417656
Fragilariopsis vanheurkii	3-18	Ross Sea, 74°59′ S, 145°019′ W	AF417660
Hantzschia amphioxys	UTEX657	CT, USA	AF417677
Navicula cf. *erifuga* (**N.* sp.)	Navicula	Langelinie, Denmark	AF417679
Nitzschia cf. *agnita*	STH14	Store Havelse, Denmark	AF417664
Nitzschia alba (**N.* sp.)	M1354	Near Roscoff, Bretagne, France	AF417670
Nitzschia communis (**N.* sp.)	M1762	Botanical Garden, Cologne, Germany	AF417661
Nitzschia frustulum	UTEX2042	La Jolla, CA, USA	AF417671
Nitzschia fusiformis	STH19	Store Havelse, Denmark	AF417668
Nitzschia laevis	M1285	Tropical basin in Aquazoo, Düsseldorf, Germany	AF417673
Nitzschia lecointei	5-21	Ross Sea, 73°59′36″ S, 150°0′36″ W	AF417667
Nitzschia navis-varingica	VSP974-1	Do Son, Vietnam	AF417675
Nitzschia navis-varingica	VHL987	Ha Long Bay, Vietnam	AF417674
Nitzschia pellucida	99NG1-16	Ishigaki Island, Japan	AF417672
Nitzschia cf. *promare* (**N.* cf. *arctica*)	CCMP1116	Baffin Bay, 76°25′ N, 82°55′ W	AF417676
Nitzschia cf. *pusilla* (**N. laevis*)	CCMP560	Martha's Vineyard, MA, U.S.A.	AF417663
Nitzschia cf. *pusilla* (**N. laevis*)	UTEX2047	Woods Hole, MA, U.S.A.	AF417662
Nitzschia cf. *vitrea* (**N. curvilineata*)	UTEX2033	New Haven, CT, U.S.A.	AF417669
Pauliella taeniata (◆ *Achnanthes taeniata*)	CCMP1115	Baffin Bay, 76°25′ N, 82°55′ W	AF417680
Phaeodactylum tricornutum	CCMP1327	Great South Bay, Long Island, NY, USA	AF417681
Pseudo-nitzschia australis	ØM1	Aveiro, Portugal	AF417651
Pseudo-nitzschia delicatissima	1001 2b	Kattegat, Denmark	AF417645
Pseudo-nitzschia fraudulenta	Limens1	Limens (42°14′36″ N and 88°49′50″ W) Spain	AF417647
Pseudo-nitzschia inflatula	No7	Phuket, Thailand	AF417639
Pseudo-nitzschia micropora, sp. ined.	VPB-B3	Van Phong Bay, Vietnam	AF417649
Pseudo-nitzschia multiseries	OFPm984	Ofunato Bay, Japan	AF417655
Pseudo-nitzschia multistriata	KoreaA	Chinhae Bay, Korea	AF417654
Pseudo-nitzschia pseudodelicatissima	P-11	Gafanha, Portugal	AF417640
Pseudo-nitzschia cf. *pseudodelicatissima*	Hobart5	Hobart, Tasmania, Australia	AF417641
Pseudo-nitzschia pungens	KBH2	Khan Hoa Bay, Vietnam	AF417650
Pseudo-nitzschia pungens	P-24	Costa Nova, Portugal	AF417648
Pseudo-nitzschia seriata	Lynæs 8	Lynæs, Isefjord, Denmark	AF417653
Pseudo-nitzschia seriata	Nissum3	Nissum Bredning, Denmark	AF417652
Pseudo-nitzschia subfraudulenta	rensubfrau	Chinhae Bay, Korea	AF417646
Pseudo-nitzschia cf. *subpacifica*	P28	Costa Nova, Portugal	AF417643
Pseudo-nitzschia cf. *subpacifica*	RdA8	Ria de Arosa, Spain	AF417642
Pseudo-nitzschia cf. *subpacifica*	Zhenbo7B	Port Shelter, Hong Kong	AF417644
Synedropsis hyperboreoides	5-15	Ross Sea, 66°58′63″ S, 149°58′53″ W, ice	AF417685
Thalassionema frauenfeldii	CCMP1798	Channel between Guana and Tortula, Caribbean Sea	AF417686

*Indicates specification or re-identification compared with the name provided.
◆Indicates that the generic affiliation has changed (Round & Basson, 1997).

R. R. L. Guillard, Bigelow Laboratory for Ocean Sciences, USA, personal communication). Permanent slides for light microscopy were prepared by mounting the cleaned material in Naphrax (Northern Supplies Limited, Ipswich, UK).

Light microscopy

Live cultures and Naphrax slides were studied using a Zeiss Axiophot, a BH-2 Olympus microscope or an Olympus Provis AX70. Measurements of cell dimensions and observations of cell shape and chain formation were mainly based on light microscopy.

Electron microscopy of whole mounts

For transmission electron microscopy (TEM), drops of cleaned material were placed on formvar-coated copper grids, dried, and studied in a JEOL-100SX electron microscope. The cells were checked for width and length, for density of interstriae, fibulae and poroids on valves and bands, for pattern of perforations in poroid hymens

and for detailed structure of the frustule (Table 2). For most taxa a minimum of 20 cells was measured.

DNA extraction, amplification and sequencing

Cells were concentrated by centrifugation and frozen. Extraction followed the CTAB method (Doyle & Doyle, 1987) with the following modifications. Cells were incubated in 500 μl preheated 2×CTAB (2% hexadecyltrimethylammonium bromide) buffer for 1–2 h at 60 °C. Genomic DNA was extracted using 500 μl 24:1 chloroform:isoamyl alcohol and precipitated and cleaned using ethanol and 3 M sodium acetate. Double-stranded DNA was amplified in a 50 μl reaction containing 5 μl 10 × *Taq* buffer (0·67 M Tris/HCl pH 8·5, 0·02 M MgCl$_2$, 0·166 M (NH$_4$)$_2$SO$_4$, 0·1 M 2-mercaptoethanol), 20 μl 0·5 μM dNTP mix, 5 μl 10 μM of each primer, 5 μl 100 mM TMA (tetramethylammonium chloride) and 1 U *Taq* polymerase (Amersham, UK). For some taxa the TMA was replaced by 2 mM TMA oxalate in order to increase specificity and yield of the polymerase chain reaction (PCR) reaction (Kovárová & Dráber, 2000). The PCR primers used were: D1R-F (ACC CGC TGA ATT TAA GCA TA; Scholin *et al.*, 1994) and D3B-R (TCG GAG GGA ACC AGC TAC TA; Nunn *et al.*, 1996). The amplification conditions were: one initial denaturation of 94 °C for 2 min, followed by 30 cycles each consisting of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s; and finally 72 °C for 2 min. The PCR products were visualized on EtBr-stained 2% Nusieve gels. The PCR products were purified using QIAquick PCR Purification Kit (Qiagen, Germany) as recommended by the manufacturer. Twenty to forty nanograms of PCR product were used in each 20 μl sequencing reaction. Nucleotide sequences were determined using Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, California) as recommended by the manufacturer. Sequencing was done using an ABI Prism 377 DNA sequencer (Perkin Elmer). Sequencing primers were the two PCR amplification primers and D2C-R (CCT TGG TCC GTG TTT CAA GA; Scholin *et al.*, 1994).

Alignment and phylogenetic analyses

Sequences were initially aligned using Clustal W (Thompson *et al.*, 1994) and afterwards edited manually in BIOEDIT (Hall, 1999). Information on secondary structure of LSU rDNA of *Saccharomyces cerevisiae* Meyen ex E. C. Hansen and *Ochromonas danica* Pringsheim (Ben Ali *et al.*, 1999; Wuyts *et al.*, 2001) was included in the alignment, but removed before final analyses. The alignment also included sequences published by Miller & Scholin (1994): *N. americana* Hasle (U41390; named *P. americana* in Miller & Scholin (1994) following Hasle (1993)). Transfer to *Pseudo-nitzschia* was, however, based on a mix-up of *N. americana* and a similar species producing stepped colonies, hence the species was referred to *Nitzschia* again by Hasle & Syvertsen (1997). A separate study will describe *N. americana* and two new morphologically closely related *Pseudo-nitzschia* species (Lundholm *et al.*, in press). The species sequenced by Miller & Scholin (1994) is identical to the originally described *N. americana*. *P. australis* Frenguelli (U40850 and U41393), *P. delicatissima* (Cleve) Heiden in Heiden et Kolbe (U41391), *P. multiseries* (Hasle) Hasle (U41389)

and *P. pungens* (Grunow *ex* Cleve) Hasle (U41262, U41392) were included as well as a sequence of *Cylindrotheca closterium* (Ehrenberg) Reimann *et* Lewin (AF289049). Of 918 aligned nucleotide positions (including gaps), 872 were considered unambiguous and analysed using parsimony, maximum likelihood and distance methods. Three positions (81, 346 and 853) showed differences between the sequences of Miller & Scholin (1994) and all sequences performed during this study, including a sequence of *Cylindrotheca closterium* (AF289049). The differences appeared in highly conserved regions and the three positions were omitted from the analyses. The alignment used for analyses corresponds to positions 31–993 in *Prorocentrum micans* (Lenaers *et al.*, 1989) and to positions 190–1090 in *Saccharomyces cerevisiae* (Ben Ali *et al.*, 1999) except deleted internal positions. The final data set contained 56 taxa and was rooted using four araphid diatoms, as the use of several outgroup taxa has been indicated to improve analyses (Swofford *et al.*, 1996).

All analyses were performed using PAUP* (version 4.0b.8) (Swofford, 1998). Parsimony analyses were done using heuristic searches with random addition of sequences (1000 replicates) and a branch-swapping algorithm (tree-bisection reconnection, TBR). Gaps were treated as missing data and characters treated as multistate and unordered. A parsimony analysis in which gaps were coded according to Simmons & Ochoterena (2000) was performed but did not provide further resolution of the tree topology.

Maximum likelihood analyses were performed using heuristic searches with 10 random addition replicates and the TBR branch-swapping algorithm. The optimal model was found using Modeltest version 3.04b (Posada & Crandall, 1998). The optimal model using a 0·01 level of significance appeared to be the Tamura–Nei substitution model (a b a a e a) (Tamura & Nei, 1993) with equal base frequencies and included parameters for rate heterogeneity between sites: the proportion of sites assumed to be invariable was estimated and on the remaining sites a gamma distribution with four rate categories was applied (Swofford *et al.*, 1996). The exact parameters were estimated from consecutive heuristic searches and reoptimizing parameters until the values of the parameters converged (D. Swofford, Smithsonian Institution, MD, personal communication). The optimal parameters were a substitution matrix: (a b a a e a) = (1, 2·574, 1, 1, 4·106, 1), the proportion of invariable sites was 0·558 and the shape of the gamma distribution, $\alpha = 0·687$ indicating a large variation in evolutionary rate between sites.

Distance analyses were performed with minimum evolution as the optimality criterion and a neighbor-joining tree as the starting tree (Saitou & Nei, 1987), using either the same model as in maximum likelihood (Hillis *et al.*, 1996), or a LogDet transformation of data (Lockhart *et al.*, 1994). The latter model allows unequal rates in different lineages and different nucleotide frequencies between taxa. Based on comparison of log-likelihood scores of the two trees, the LogDet tree was chosen. Bootstrap analyses were used to determine the robustness of nodes (Felsenstein, 1985): 1000 replicates in parsimony and LogDet, and 33 in ML, which is computationally more intensive.

Congruences of trees generated by maximum likelihood, parsimony and distance analyses were tested using Kishino–Hasegawa tests (Kishino & Hasegawa, 1989).

Table 2. Selected morphological features of species belonging to the Bacillariaceae (all observations original)

Species	Raphe raised above valve	Poroids in wall of raphe canal	Central nodule	No. of striae compared with fibulae	No. of rows of poroids	Pattern of perforations in poroid hymen	Copulae	Secondary structure on frustule	Growth form/ Colony type
Nitzschia cf. *agnita*	?	+	−	>	1	Scattered	1–2 rows	−	Solitary
N. pellucida	+	+	+	>	1	Scattered-hexagonal	1–3 rows	+valve and bands	Solitary
N. laevis	?	+	+	>	1	Hexagonal	1–2 rows	−	Ribbon-shaped
N. cf. *promare*	+	+	+	>	1	Scattered	1 row	+valve and bands	Ribbon-shaped
N. navis-varingica	+	+	+	>	1	Scattered	1–3 rows	+valve and bands	Solitary
Bacillaria paxillifer	+	+	−	>	1	Hexagonal	1–2 rows	+valve and bands	Special
Hantzschia amphioxys	+	+	+	>	1	Scattered	Special	−	Solitary
N. cf. *vitrea*	+	+	−	>	1	Hexagonal	1–2 rows	−	Solitary
N. lecointei	?	+	+	>	1	Hexagonal	1–2 rows	−	Solitary
N. alba	?	+	+	>	1	Circular	1–2 rows	−	Solitary
N. cf. *pusilla*	+	+	−	>	1	Scattered	1–2 rows	−	Ribbon-shaped
N. communis	+	+	−	>	1	Circular-scattered	1 row	−	Ribbon-shaped
N. fusiformis	?	+	+	>	1	Circular	1–2 rows	−	Solitary
N. frustulum	?	+	+	>	1	Hexagonal	1 row	−	Ribbon-shaped
Pseudo-nitzschia cf. *subpacifica*	−	−	+	>	2	Hexagonal	Striae	−	Stepped
P. inflatula	−	−	+	>	1	Hexagonal	?	−	Stepped
P. cf. *cuspidata*	−	−	+	>	1	Hexagonal	1 row (may be divided)	−	Stepped
P. pseudodelicatissima	−	−	+	>	1	Hexagonal	Striae	−	Stepped
P. delicatissima	−	−	+	>	2	Hexagonal	1 row	−	Stepped
P. micropora sp. ined.	−	−	−	>	2	Hexagonal	Striae	−	Stepped
P. fraudulenta	−	−	+	=	2–3	Hexagonal-circular	Striae	−	Stepped
P. subfraudulenta	−	−	+	>	2–3	Hexagonal-circular	Striae	−	Stepped
P. cf. *subpacifica*	−	−	+	>	2	Hexagonal	Striae	−	Stepped
P. pungens	−	−	−	=	2	Hexagonal	1 row	−	Stepped
P. multiseries	−	−	−	=	3–4	Hexagonal	Striae	−	Stepped
P. australis	−	−	−	=	2	Hexagonal	Striae	−	Stepped
P. seriata	−	−	−	=	4 (2+2)	Hexagonal	Striae	−	Stepped
P. multistriata	−	−	−	>	2	Hexagonal	Striae	−	Stepped
N. americana	−	−	−	>	2 (3)	Hexagonal	Striae	−	Solitary
Fragilariopsis rhombica	−	−	−	≅	1–2	Circular	1 row	−	Ribbon-shaped
F. kerguelensis	−	−	−	=	2	Circular	No poroids	−	Ribbon-shaped
F. curta	−	−	−	≅	1–2	Scattered?	1 row	−	Ribbon-shaped
F. cylindrus	−	−	−	=	2	Hexagonal	1–2 rows	−	Ribbon-shaped
F. vanheurkii	−	−	+	>	2	Hexagonal	1 row	−	Ribbon-shaped

The trees were compared with the best tree, which was found to be the maximum likelihood tree, at a significance level of < 0.05. User-defined trees were generated based on the maximum likelihood tree using MacClade (version 3.08) (Maddison & Maddison, 1992) and compared with the best tree. In addition constrained maximum likelihood analyses were performed using the same model as above with five random addition replicates.

Toxin analyses

One hundred and fifty millilitre cultures (Tenerife 7, UTEX2083, 1-A, 2-E-F, 4-20, 5-17, 3-18, STH14, UTEX2042, STH19, UTEX2047, CCMP1115, ØM1, OFPm984, Lynæs 8, Nissum 3, KoreaA, P-11, P-24, RdA8, Zhenbo7B, 5-15) were grown in 250 ml flasks at 24 °C in a L:D 12:12 h regime in 32 psu L1 medium at a photon fluence rate of 100 μmol m^{-2} s^{-1}. For two taxa (CCMP1115, 5-15) grown at 4 °C, this fluence rate was too high. They were therefore grown at a photon fluence rate of 50 μmol m^{-2} s^{-1}. The samples were freeze-dried, resuspended in re-distilled water and tested using FMOC-HPLC by Y. Kotaki in Japan (see Kotaki *et al.*, 2000) or UV-DAD HPLC at the University of Copenhagen. For UV-DAD HPLC the samples were sonicated for 1 min and filtered through a (Whatman) PTFE syringe filter (pore size 0.2 μm). The analyses were conducted on a Waters model equipped with a 600 controller, 717 plus autosampler and 996 PDA, Waters spherisorb 5 μm ODS, 4.6 × 250 reversed phase analytical column. Elution was isocratic with 85:15 acetonitrile + 0.1% TFA: water + 0.1% TFA with a 50–100 μl injection volume. The standard of domoic acid was DACS-1C (Certified Reference Materials Program (CRMP), NRC, Institute for Marine Biosciences, Halifax, Nova Scotia). Runtime was 15 min, absorption at 242 nm (Quilliam *et al.*, 1989).

Terminology follows Anonymous (1975), von Stosch (1975), Ross *et al.* (1979) and Mann (1981).

Results

Morphology of the Bacillariaceae

Detailed examination of the taxa included in this study revealed that the morphology of most taxa corresponded to published species descriptions. Some varied however: for example, the isolates of *P.* cf. *subpacifica* (Hasle) Hasle had fewer poroids (7–9 per μm) and a smaller width (3–5 μm) compared with the 9 or 10 poroids and a width of 5–7 μm in the description of *P. subpacifica* by Hasle (1965a). They otherwise resembled *P. subpacifica*. The isolate designated Hobart 5 and tentatively identified as *P.* cf. *pseudodelicatissima* agreed with the species description except for the presence of a lanceolate rather than a linear valve. This feature is one of the important characters distinguishing this and the closely related but lanceolate species *P. cuspidata*. *Pseudo-nitzschia cuspidata* is also broader (3 μm) than *P. pseudodelicatissima* (1.3–2.5 μm) (Hasle 1965a). As Hobart 5 is narrow and the distinction

between linear and lanceolate can be difficult, identification was settled as *P.* cf. *pseudodelicatissima*. The isolates Hobart 5 and P-11, both identified as *P. pseudodelicatissima*, had different poroid patterns. In P-11 the poroid was subdivided into two sections (see Hasle 1965a, fig. 8), while in Hobart 5 it was divided into several small parts (see Skov *et al.*, 1999, fig. 10G). Both poroid patterns have, however, been described for both *P. pseudodelicatissima* and *P. cuspidata*. Except for having a smaller width, the heterotrophic isolate M1354 agreed exactly with the original species description (Lewin & Lewin, 1967).

Detailed examination of valves and girdle bands of the taxa of the Bacillariaceae revealed some morphological features which could be relevant in the context of a phylogenetic analysis (Table 2). *Cylindrotheca* species were not included, as their morphology is different from the other taxa within the Bacillariaceae. All species within *Pseudo-nitzschia* and *Fragilariopsis*, including *N. americana*, had at least two features in common: (1) they lacked poroids in the wall of the raphe canal, whereas the wall of the raphe canal of all other taxa was perforated by poroids (Figs 1, 2, 5) and (2) the raphe was not raised above the plane of the valve. Most – if not all – of the other species of Bacillariaceae examined had the raphe raised on a keel (see Lundholm & Moestrup, 2000, fig. 5E).

Several other features, such as presence of a central nodule, number of striae, number of fibulae, number of striae compared with fibulae, number of rows of poroids, the pattern of perforations within the poroids, the poroids on copulae, secondary structures on the frustule and the growth form, varied considerably without any obvious pattern (Table 2). However, all *Pseudo-nitzschia* species grew in stepped colonies, a feature not observed outside *Pseudo-nitzschia* apart from the special variable colony shape of *Bacillaria paxillifer* (O. F. Müller) Hendey. All taxa within *Fragilariopsis* formed ribbon-shaped colonies, a feature shared with several *Nitzschia* species (Table 2). The pattern of perforations of the poroids was observed to vary notably between and even within genera, e.g. *Fragilariopsis* (Table 2, Figs 3, 4). No variation was found within species. Secondary structural features were observed in a small number of taxa, especially silica warts on the girdle bands and on the valve mantle (Fig. 6). The girdle bands also differed considerably. Most species of *Fragilariopsis* examined possessed a longitudinal row of small poroids near the advalvar margin of the bands. *Fragilariopsis kerguelensis* (O'Meara) Hustedt was subjected to a very thorough examination but seemed to lack poroids in the girdle bands. In *Pseudo-nitzschia* most girdle bands contained striae with a varying number of transverse rows of
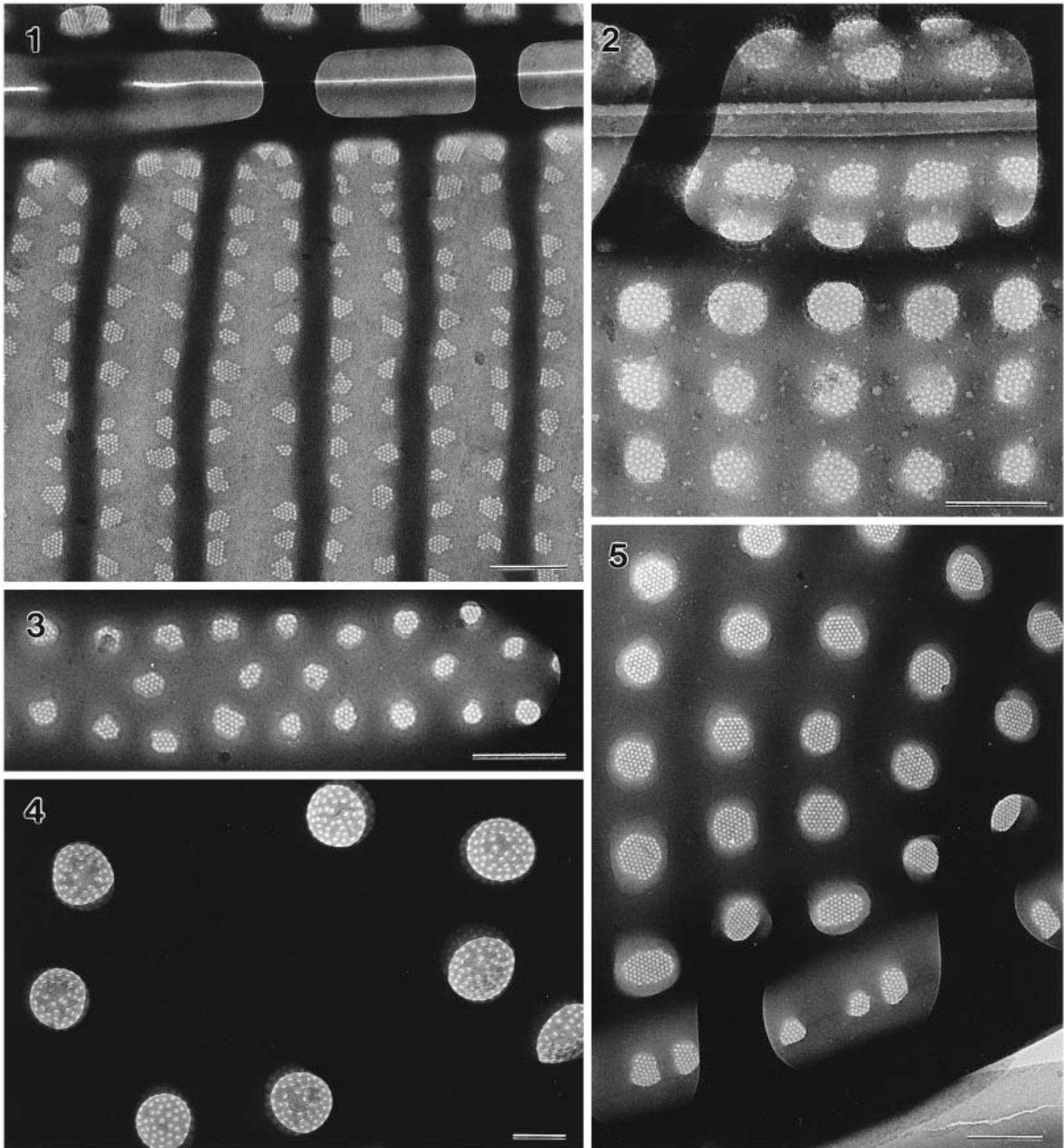
Figs 1–5. Transmission electron micrographs. Fig. 1. *Pseudo-nitzschia delicatissima*: wall of raphe canal without poroids, hexagonal pattern in poroids. Fig. 2. *Nitzschia* cf. *agnita*: poroids in wall of raphe canal, scattered pattern in poroids. Fig. 3. *Fragilariopsis cylindrus*: hexagonal pattern in poroids. Fig. 4. *Fragilariopsis kerguelensis*: circular pattern in poroids. Fig. 5. *Nitzschia laevis*: poroids in wall of raphe canal, hexagonal pattern in poroids. Scale bars represent 0·2 μm.
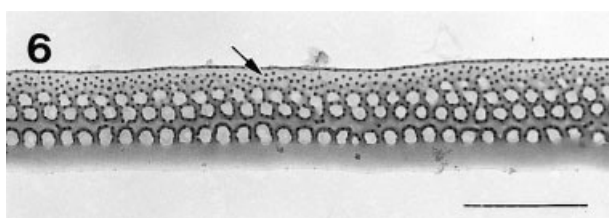
Fig. 6. Transmission electron micrograph. *Nitzschia pellucida*. Girdle band with silica warts seen as dark dots. Arrow shows silica warts. Scale bar represents 2 μm.

poroids, but some species differed in having one longitudinal row of large poroids. Species of *Hantzschia* Grunow, *Bacillaria* and *Nitzschia* all possessed one or more longitudinal rows of poroids on the copulae.

Phylogenetic inference

Most of the variation observed in LSU rDNA sequences was in the highly variable regions B13-1
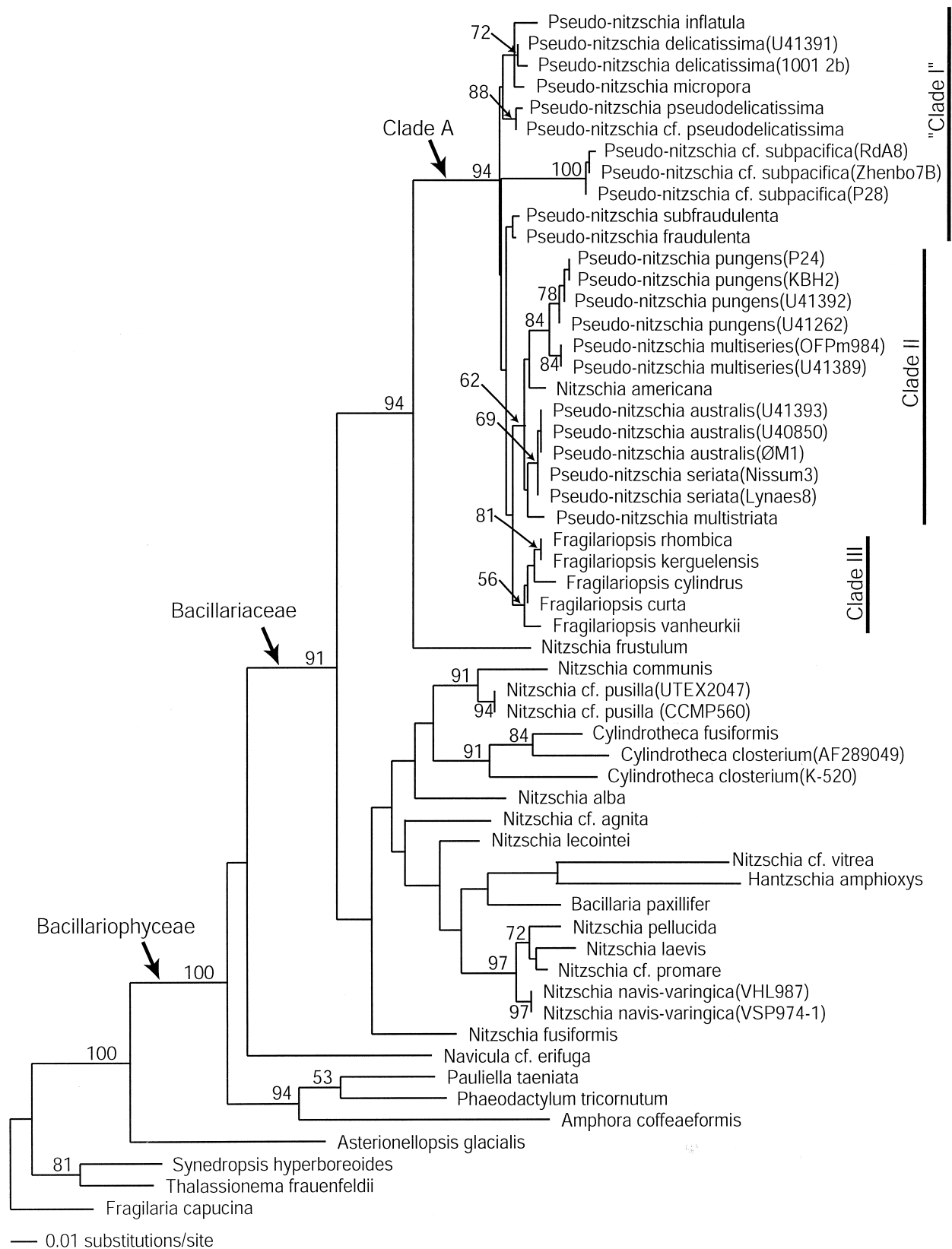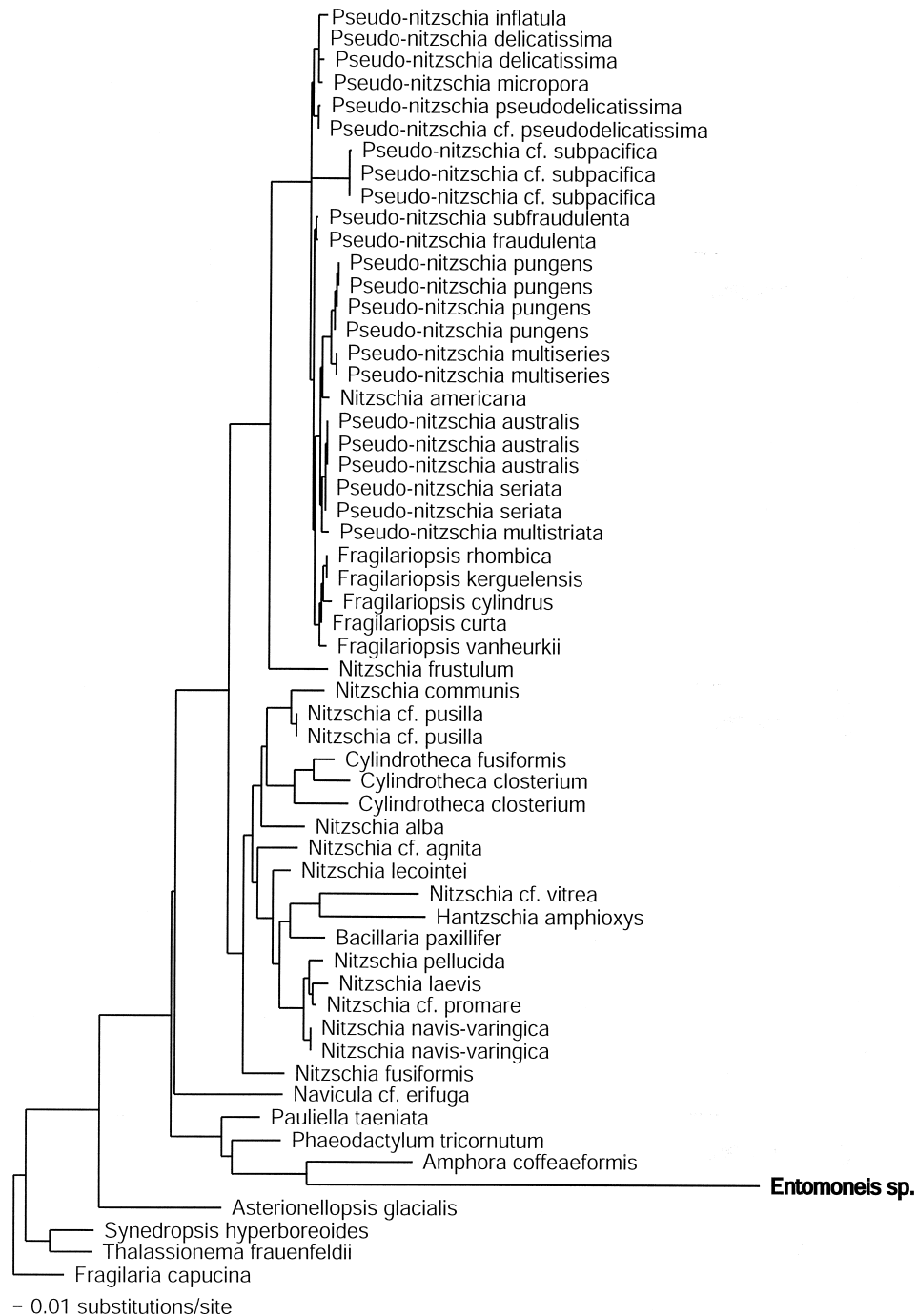
Fig. 7. A maximum likelihood tree based on one of the most variable regions of LSU rDNA (872 positions included) illustrating the phylogeny of the Bacillariaceae. The tree was rooted using four araphid taxa. The $-$Ln likelihood of the tree is 6302·198. The analysis is based on the Tamura–Nei substitution model with invariable sites and on the remaining sites a gamma distribution with four rate categories. Major systematic clades have been marked by arrows. Within clade A three clades are labelled I, II and III. Clade I is shown as 'Clade I' as it does not resolve as a monophyletic group in the maximum likelihood analysis. Bootstrap values based on 33 replicates are shown on the branches. Only values above 50% are given. *Pseudo-nitzschia micropora* has not yet been formally described (Priisholm *et al.*, 2002).
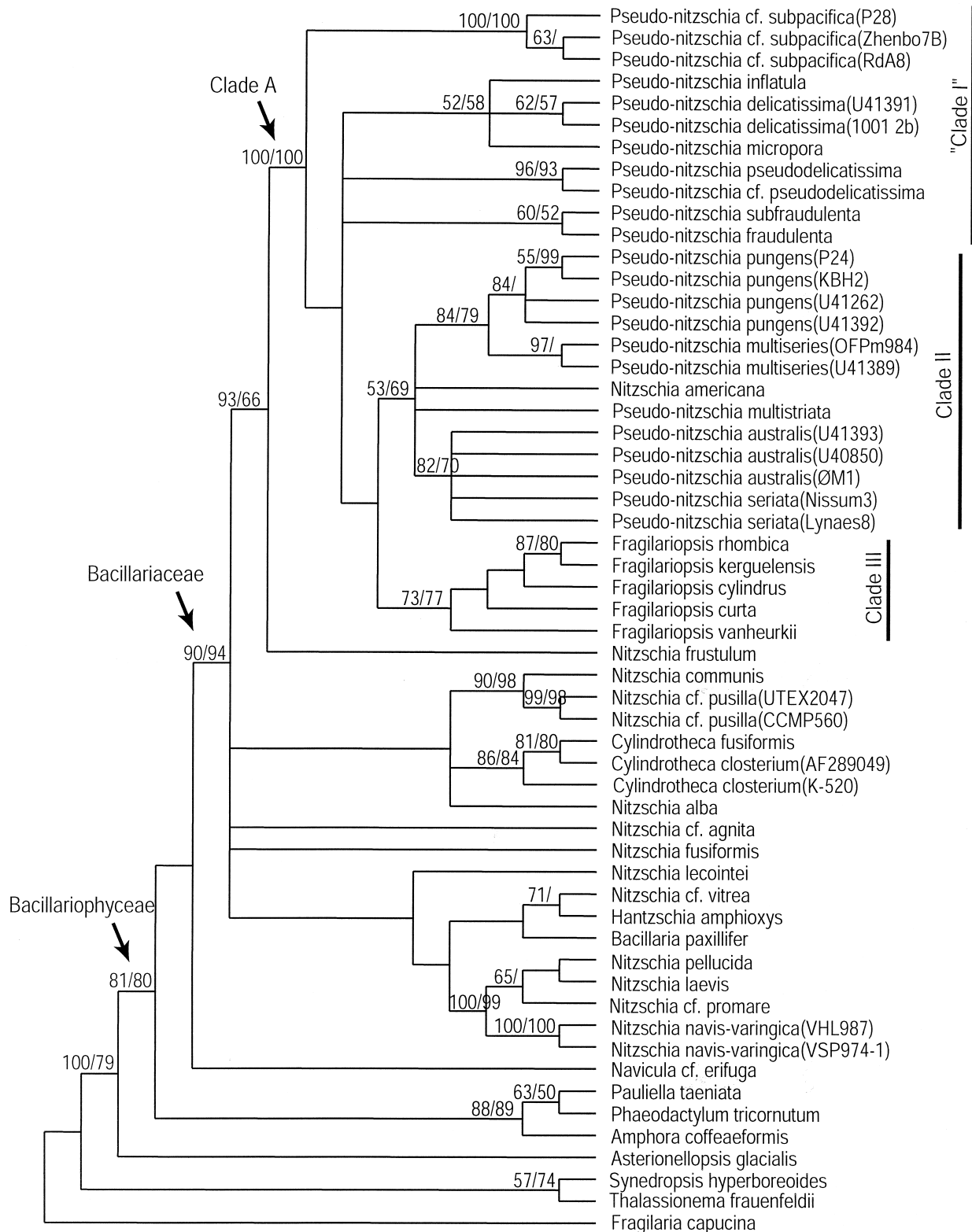
Fig. 8. A maximum parsimony tree based on the most variable region of LSU rDNA (872 positions included) illustrating the phylogeny of the Bacillariaceae. The tree has been rooted using four araphid taxa. The tree is a strict consensus of 270 equally parsimonious trees. Length of tree = 1109 steps. Two hundred and eight of 872 characters were parsimony informative. Major systematic clades have been marked by arrows. Within clade A three clades are labelled I, II and III. Clade I is shown as 'Clade I' as it does not resolve as a monophyletic group in the maximum parsimony analysis. Bootstrap values from parsimony (1000 replicates) are shown before the slash, bootstrap values from the distance analysis (1000 replicates) after the slash. Only values above 50 % are shown. *Pseudo-nitzschia micropora* has not yet been formally described (Priisholm *et al.*, 2002).

Fig. 9. A maximum likelihood tree based on the most variable region of LSU rDNA (872 positions included) illustrating the phylogeny of the Bacillariaceae. The tree has been rooted using four araphid taxa. This analysis shows a long branch to *Entomoneis* sp. The tree has a −Ln likelihood of 6726·098. The analysis is based on the Tamura–Nei substitution model with invariable sites and on the remaining sites a gamma distribution with four rate categories. *Pseudo-nitzschia micropora* has not yet been formally described (Priisholm *et al.*, 2002).

to B16, C and D5 to D5_1 (corresponding to base pairs 102–247, 422–580 and 689–705). As a result of choosing the araphid taxa as an outgroup, the maximum likelihood, parsimony and distance trees showed the raphid diatoms (Bacillariophyceae *sensu* Round *et al.*, 1990) as a monophyletic group (Figs 7, 8). The monophyletic origin was well supported by bootstrap values in all analyses. Initially *Entomoneis* sp. Ehrenberg was included in the analyses, but the branch leading to *Entomoneis* sp. was very long (Fig. 9) and hence it was excluded in the final

analyses to avoid the problem of long-branch attraction (Felsenstein, 1978). A blast search ensured that the *Entomoneis* sp. sequence was related to other pennate diatoms. Exclusion of *Entomoneis* sp. did not change tree topology. The overall topology of the tree did not change either by changing the outgroup to comprise fewer araphid taxa, by including the four raphid taxa outside Bacillariaceae in the outgroup or by excluding taxa with relatively longer branches (*Amphora coffeae-formis*, *Nitzschia* cf. *vitrea* Norman and/or *Hantz-*

Table 3. Tests of congruence using the Kishino–Hasegawa test: the best tree (the maximum likelihood tree; Fig. 7) compared with the tree topologies calculated using MP (maximum parsimony), LogDet (Log Det transformation), with topologies of different constrained trees and with user-defined tree topologies based on the best tree (ML)

Tree topologies/constrained trees	KH test of constrained ML searches			KH test of user-defined tree topologies including MP and LogDet		
	Diff. in −lnL[a]	SD	P	Diff. in −lnL[b]	SD	p
Best (ML)	(6302·1982)[c]			(6302·1982)[c]		
MP				34·8667	12·4708	0·0053*
LogDet				34·7563	14·712	0·0184*
Psn. and *N. americana* monophyletic	1·2972	5·8317	0·8240	8·2236	5·9215	0·1653
N. americana outside clade A	5·0117	7·7825	0·5198	27·5792	10·3216	0·0077*
Psn. monophyletic	15·8590	11·1709	0·1561	29·39198	10·4222	0·0049*
Clade I monophyletic	1·2511	5·8354	0·8303	7·4761	5·7826	0·1964
Nitz. monophyletic	57·4501	20·6173	0·0054*	229·2832	30·7107	< 0·0001*
Ornamented spp. monophyletic	30·4805	15·6276	0·0514	53·0663	15·1009	0·0005*
Ornamented *Nitz.* monophyletic				5·8279	4·4347	0·1891

Nitz., *Nitzschia*; ornamented spp., all species found to be ornamented in this study; *Psn.*, *Pseudo-nitzschia*.
[a]Difference in −log-likelihood between the best tree and the constrained tree.
[b]Difference in −log-likelihood between the best tree and the user-defined tree.
[c]−log likelihood of the best tree (ML).
*Indicates trees which are significantly different from the best tree.

schia amphioxys (Ehrenberg) Grunow in Cleve *et* Grunow), indicating a stable tree topology. Following the results of the Kishino–Hasegawa test, the tree based on maximum likelihood was significantly better than both the parsimony and the LogDet tree (Table 3). The topological differences between the maximum likelihood and the maximum parsimony tree were focused in two places. The first was the branching of a clade consisting of *N. pellucida*, *N. laevis* and *N.* cf. *promare*. The second was the position of *P.* cf. *subpacifica* within clade A (see below). The differences between the distance and the maximum likelihood trees were mainly due to a different branching pattern in some of the terminal branches with little or no bootstrap support, and to the position of *Navicula*. None of the well-supported branches differed among the three types of analyses conducted.

In all analyses a group comprising *Pauliella taeniata* (Grunow) Round *et* Basson, *Amphora coffeaeformis* and *Phaeodactylum tricornutum* Bohlin formed a highly supported group, which excluded *Navicula* cf. *erifuga* Lange-Bertalot as the only other raphid taxon outside the Bacillariaceae. In the maximum likelihood analysis (Fig. 7) the Bacillariaceae formed a monophyletic group, which was recovered in the trees based on maximum parsimony (Fig. 8) and distance analysis (not shown) and supported by high bootstrap values (91%, 90% and 95%, respectively). A clade labelled 'clade A', including all *Pseudo-nitzschia* species, and all *Fragilariopsis* species plus *Nitzschia americana*, appeared in all trees and was significantly supported by bootstrap values (≥ 94%). A user-defined tree forcing *N. americana* outside clade A

resulted in a tree topology significantly different from the best tree, whereas a constrained maximum likelihood analysis forcing *N. americana* outside clade A resulted in a not significantly different topology (Table 3). On the contrary, a monophyletic origin of *Pseudo-nitzschia* including *N. americana* was not rejected by the Kishino–Hasegawa test (Table 3).

The topology of the branches within Bacillariaceae but outside clade A varied slightly between the different analyses; however, the three *Cylindrotheca* species always clustered together (≥ 84% bootstrap support; Figs 7, 8). A well-supported group (bootstrap values ≥ 97%) comprising the two isolates of *Nitzschia navis-varingica*, *N. pellucida* Grunow in Cleve *et* Grunow, *N. laevis* Hustedt non *N. levis* Frenguelli and *N.* cf. *promare* Medlin always clustered together. Another well-supported group consisted of *Nitzschia communis* Rabenhorst and the two strains of *N.* cf. *pusilla*. *Nitzschia frustulum* (Kützing) Grunow in Cleve *et* Grunow had a relatively isolated position in all three analyses, appearing as a sister-group to clade A and supported by high bootstrap values (91% in maximum likelihood, 93% in maximum parsimony and 66% in distance analyses). *Nitzschia* was polyphyletic in all three analyses. The Kishino–Hasegawa test showed significant differences between both a user-defined tree forcing *Nitzschia* to be monophyletic and the similarly constrained tree, and the maximum likelihood tree (Table 3). The other branches outside clade A were either not resolved or not supported by bootstrap values.

Within clade A two clades were resolved in all analyses: a clade labelled 'clade II' (Figs 7, 8)

comprising *P. pungens*, *P. multiseries*, *P. australis*, *P. seriata* (Cleve) H. Peragallo in H. *et* M. Peragallo, *P. multistriata* (Takano) Takano and *N. americana* was moderately supported by bootstrap values in all analyses (62 % in maximum likelihood, 53 % in maximum parsimony and 69 % in distance analyses). The five *Fragilariopsis* species included in the study comprised clade III, which received moderate support (56 % in maximum likelihood, 73 % in maximum parsimony and 77 % in distance analyses). These two clades clustered together and in all three analyses had a common monophyletic origin, although not supported by bootstrap values.

The branches between the remaining taxa were very short and revealed an uncertain branching pattern. This group of taxa consisted of *P. inflatula* (Hasle) Hasle, *P. delicatissima*, *P. micropora* Priisholm *et* Moestrup, *sp. ined.*, *P. pseudodelicatissima*, *P. fraudulenta* (Cleve) Hasle, *P. subfraudulenta* (Hasle) Hasle and *P.* cf. *subpacifica*. In the distance analysis the taxa grouped in one clade, while in the other analyses they clustered close to each other or were not resolved. The Kishino–Hasegawa test of topologies forcing these taxa to form a monophyletic clade (clade I) was not significantly different from the best tree (Table 3). Isolates supposed to belong to the same morphological taxa always clustered close together except in the LogDet analysis where isolates of *P. pungens* and *P. multiseries* intermingled. The same was seen in the distance analysis based on the maximum likelihood model. In all three analyses *P. delicatissima* and *P. micropora* clustered close together. *Pseudo-nitzschia pungens* and *P. multiseries* were sister-groups in all analyses, supported by high bootstrap values. In clade III, *F. rhombica* (O'Meara) Hustedt and *F. kerguelensis* always clustered together, supported by high bootstrap values ($\geqslant 80$ %).

Production of domoic acid

The toxin analyses showed that *P. australis* (ØM1, from Portugal), *P. multiseries* (OFPm984, from Japan) and *P. seriata* (Lynæs8 and Nissum3, both from Denmark) produce domoic acid. *Bacillaria paxilifer*, *Cylindrotheca fusiformis* Reiman *et* Lewin, *Fragilariopsis curta* (van Heurck) Hustedt, *F. cylindrus* (Grunow) Krieger, *F. kerguelensis*, *F. rhombica*, *F. vanheurkii* (M. Peragallo) Hasle, *Nitzschia* cf. *agnita* Hustedt, *N. frustulum*, *N. fusiformis* Grunow, *N.* cf. *pusilla* (Kützing) Grunow *emend.* Lange-Bertalot (UTEX 2047), *Pauliella taeniata*, *Pseudo-nitzschia multistriata*, *P. pungens* (KBH2 and P-24), *P.* cf. *subpacifica* (RdA8, Zhenbo7B) and *Synedropsis hyperboreoides* Hasle, Syvertsen *et* Medlin did not produce domoic acid in detectable amounts.

Discussion

Using three different methods for studies of phylogenetic inference, the present work has demonstrated that the Bacillariaceae is a monophyletic group (bootstrap values $\geqslant 90$ %). This was also indicated in a study based on SSU rDNA by Medlin *et al.* (2000). It indicates, as stated by Medlin *et al.* (2000), that fibulae have evolved more than once, namely in *Entomoneis* and in the other genera of Surirellaceae, in addition to the Bacillariaceae.

Clade A forms another major group supported by high bootstrap values. The branch leading to clade A is relatively long, indicating either a long period of isolated evolution or a very rapid sequence evolution. Clade A, which is significantly supported in the bootstrap analyses, comprises *Fragilariopsis* and *Pseudo-nitzschia* species and *Nitzschia americana*. This is not surprising since the taxa included are morphologically very similar. *Nitzschia americana* does not form stepped colonies and hence has been included in *Nitzschia* (Hasle & Syvertsen, 1997). It differs from *Pseudo-nitzschia* species in being solitary and having more obtuse rounded ends like *Fragilariopsis* species. Otherwise it shares all features of the frustule with the other species of clade A.

Morphological features of clade A

Clade A can be defined by a complex of at least five characters, especially features of the raphe. These characters, many of which have been used to separate *Pseudo-nitzschia* (and *Fragilariopsis*) from *Nitzschia sensu stricto* (Mann, 1986; Hasle, 1993, 1994) are: (1) The raphe is not raised above the level of the valve face, whereas *Nitzschia* subgenus *Nitzschia*, and most other *Nitzschia* species, have the raphe raised on a keel (Mann, 1984). (2) Most *Nitzschia* species have poroids perforating the wall of the raphe canal, whereas poroids in the outer wall of the raphe canal are absent in the species in clade A. In this study all taxa belonging to the Bacillariaceae except those of clade A had poroids in the raphe canal. A preliminary survey of published electron micrographs of *Nitzschia* species revealed that out of 65 species, four had no poroids in the wall of the raphe canal (see below). (3) In *Nitzschia* subgenus *Nitzschia* a conopea lines each side of the raphe, whereas this structure is absent in the taxa of clade A. (4) Species within clade A have either several rows of poroids in the striae, or one row of poroids in which the poroids are subdivided into two or more parts. In contrast, *Nitzschia* species mainly have uniseriate striae (Table 2 and Round *et al.*, 1990). (5) The outer surfaces of the frustules in clade A are uniformly flat. The interstriae do not protrude outwards, as in many *Nitzschia* species. However, members of *Nitzschia* subgenus *Nitzschia*

Table 4. Generic characters of *Fragilariopsis* and *Pseudo-nitzschia* as presently described (mainly based on Hasle, 1993, 1994)
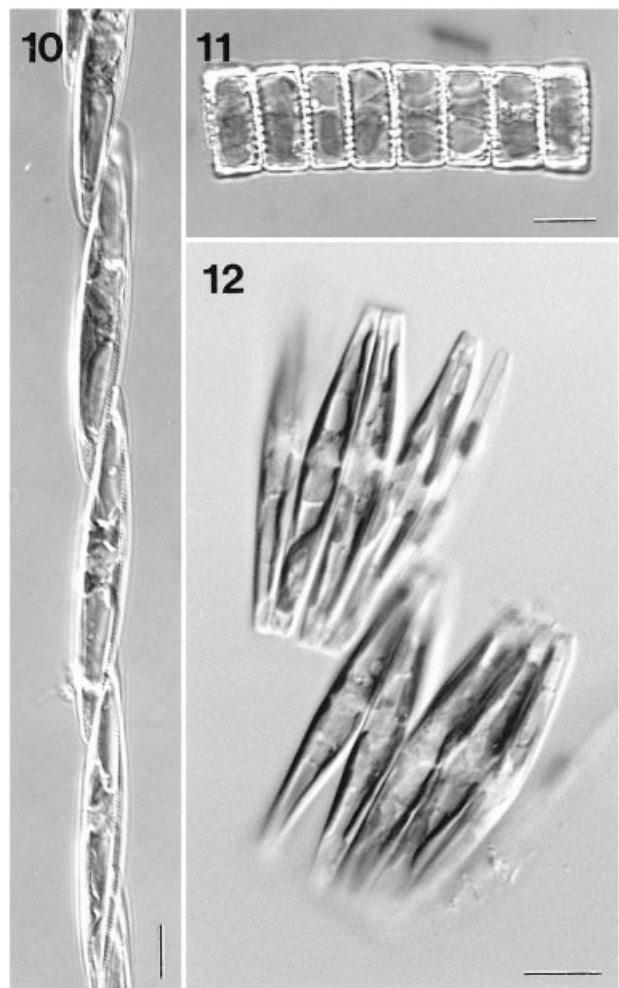
Feature	*Fragilariopsis*	*Pseudo-nitzschia*
Habitat	Marine, planktonic and benthic	Marine and planktonic
Colony type	Ribbon-shaped colonies	Stepped colonies
Valve shape	Broad and elliptical to linear-lanceolate	Linear to lanceolate
Chloroplasts	Two symmetical about the median transapical plane	Two symmetical about the median transapical plane
Raphe	Not raised above valve face, strongly eccentric	Not raised above valve face, strongly eccentric
Wall of raphe canal	No poroids	No poroids
Conopea	Absent	Absent
Larger central interspace	Present in a few species	Present in several species
Striae	Often two rows of poroids, rarely one or more than two	Often one or two rows of poroids, rarely more
Fibulae and striae	Often the same number	The same or more striae than fibulae
Girdle bands	Single row of poroids or striated	Single row of poroids or striated

also possess this feature despite being very different from the taxa in clade A otherwise (Mann, 1986).

Apart from these features, the cells of clade A are linear to lanceolate or fusiform in valve view. The raphe is extremely eccentric. The raphe lacks terminal fissures (the outer raphe fissure does not proceed beyond the helictoglossa) (Mann, 1978). The valve angle near the raphe is 90° and the mantle is relatively low (unpublished observations). The girdle usually consists of three bands perforated by striae or a longitudinal row of poroids.

Pseudo-nitzschia, Fragilariopsis *and* Nitzschia

The main difference between *Pseudo-nitzschia* and *Fragilariopsis*, as currently described, is their mode of colony formation (Table 4): *Pseudo-nitzschia* forms stepped colonies (cells overlapping tip-by-tip) (Fig. 10) while *Fragilariopsis* forms ribbon-shaped colonies (the cells line up valve by valve) (Fig. 11). One species, however, *P. granii*, has only been observed as single cells. Being morphologically very similar to other *Pseudo-nitzschia* species (Hasle, 1964; Hasle & Heimdal, 1998) it has been retained in *Pseudo-nitzschia* although colony type is included in the diagnosis of the genus. *Nitzschia americana*, which clusters with *Pseudo-nitzschia* species in our analyses, likewise does not form colonies. Colony formation is therefore not a very stable character in this cluster. This is supported by a Kishino–Hasegawa test showing that a user-defined tree topology forcing *N. americana* outside clade A was significantly different from the best tree. This was, however, not supported by the constrained ML analysis in which *N. americana* was excluded from clade A. Another indication that colony-formation as a taxonomic character is artificial is that *Pseudo-nitzschia* sometimes forms ribbon-shaped colonies in culture (Fig. 12). The raphe in these cells has apparently stopped functioning. Following cell division, the cells are still held together but they are unable to slide in relation to each other, resulting in

Figs 10–12. Light micrographs. Fig. 10. *Pseudo-nitzschia seriata*, stepped colony in girdle view. Fig. 11. *Fragilariopsis kerguelensis*, ribbon-shaped colony. Fig. 12. *Pseudo-nitzschia seriata*, ribbon-shaped colonies. Scale bars represent 10 μm.

the formation of ribbon-shaped colonies as in *Fragilariopsis*. As the discrimination between *Pseudo-nitzschia* and *Fragilariopsis* is mainly based on colony type, these findings pose a problem and like the molecular analyses indicate that the two genera are very closely related and perhaps congeneric.

We have examined several other morphological features to determine whether there is congruence between molecular and morphological data (Table 2). *Pseudo-nitzschia* species have a hexagonal perforation pattern in the poroid hymen and this character was examined in detail. In *Fragilariopsis* taxa we found two or three different types of patterns. This finding supports Mann (1981), who found that some genera possessed only one kind of poroid whereas in other genera the poroid pattern varied. Poroid structure seems to be a feature that shows a limited number of possible morphologies in raphid diatoms, and therefore may not always indicate homology (Mann, 1981). Different types of poroids have probably evolved several times.

The arrangement and size of the poroids on the girdle bands of *Fragilariopsis* at first glance seem to constitute a synapomorphic character for this genus, as the included taxa as well as *F. sublineata* (own observations) possess one longitudinal advalvar row of small poroids. However, *F. oceanica* (Cleve) Hasle, which unfortunately was not included in the present analysis, has striated girdle bands that resemble the striae on the girdle bands of some species of *Pseudo-nitzschia* (Hasle 1965*b*, pl. 2, fig. 9). If *F. oceanica* clusters with the other *Fragilariopsis* species, then the structure of the girdle bands seems to constitute a character which is useful for species identification but not for delimitation of the two genera. Girdle bands of some *Pseudo-nitzschia* species are also perforated by one row of poroids. These poroids are, however, often larger than in *Fragilariopsis* and not always advalvarly situated.

Possession of a central nodule is scattered in *Pseudo-nitzschia*, *Fragilariopsis* and *Nitzschia*. Like the pattern in the poroid hymen it is probably a structure that has evolved several times and therefore does not necessarily indicate homology.

Some species presently included in *Nitzschia* appear to possess the same characters as those of clade A, but have never been observed in stepped or ribbon-shaped colonies. These include *N. braarudii* Hasle, *N. marina* Grunow in Cleve *et* Grunow, *N. norvegica* Hasle and *N. sicula* (Castracane) Hustedt, which were examined by Hasle (1960, 1964) and Hasle & Syvertsen (1997). All four species have been shown to lack poroids in the outer wall of the raphe canal and to have a valve morphology closely related to the taxa in clade A. *Nitzschia sicula* was originally placed in *Pseudo-nitzschia* by H. Peragallo (1897–1908), as the genus then also comprised solitary species. Mann (1978) placed *Nitzschia sicula* in *Fragilariopsis* while Hustedt included it in *Pseudo-nitzschia*. This clearly demonstrates the close morphological relationship between the involved genera, as also mentioned

by Hasle & Syvertsen (1997). Hasle (1972*b*) emphasized the morphological similarity between *N. americana*, *N. barbieri* Peragallo, *N. peragalli* Hasle, *N. sicula* and *Fragilariopsis*, but retained the four former species in *Nitzschia* as they did not form colonies. In the present study *N. americana* clusters within *Pseudo-nitzschia*.

The data presented here illustrate the need for an emendation of the genus *Pseudo-nitzschia*, which should probably include some species presently referred to *Nitzschia* (similar to *N. americana*). *Nitzschia* comprises more than 900 species and is in serious need of taxonomic revision (Mann, 1986). This will constitute a major effort since only a few taxa have been studied thoroughly by transmission or scanning microscopy and no recent comprehensive monograph exists. In particular, *Nitzschia* species belonging to the sections *Lanceolatae* and *Nitzschiella* resemble taxa within clade A and should therefore be examined in more detail. However, many *Nitzschia* species have never been assigned to any of the sections.

Examination of *Fragilariopsis doliolus* (Wallich) Medlin *et* Sims would also be of interest. Morphologically *F. doliolus* seems to be slightly different from the taxa of clade A. It has interstriae raised externally above the level of the valve, whereas the taxa in clade A have very flat outer surfaces, the interstriae being prominent only on the inner surface. The position of the poroids at the base of the ribs rather than within the striae also separates *F. doliolus* from other species of *Fragilariopsis*. *Fragilariopsis doliolus* was transferred from *Pseudoeunotia* Grunow to *Fragilariopsis* by Medlin & Sims (1993), who argued that only the symmetry of the cell separated *Pseudoeunotia* from *Fragilariopsis*. Unfortunately no culture of *F. doliolus* was available.

As mentioned above, clade A is defined by a number of morphological characters that are in congruence with the molecular data. These morphological characters also indicate that the genera *Pseudo-nitzschia* and *Fragilariopsis* cannot be defined on features such as colony type and valve shape.

Subdivision of clade A

The division of clade A into three groups is not well supported in terms of bootstrap values. In all analyses *Pseudo-nitzschia* appears to be paraphyletic, as it splits into two groups and *Fragilariopsis* spp. form a monophyletic group within *Pseudo-nitzschia*. However, clustering of *Fragilariopsis* species outside a clade comprising *Pseudo-nitzschia* and *N. americana* could not be rejected on the basis of the Kishino–Hasegawa test.

The type species of *Pseudo-nitzschia* (*P. seriata*)

and *Fragilariopsis* (*F. kerguelensis*) appear in clades II and III, respectively. The monophyly of clade II (some *Pseudo-nitzschia* species and *N. americana*) and clade III (*Fragilariopsis*) are supported by low bootstrap values in all three analyses. A monophyletic origin of these two clades is also indicated but not supported by bootstrap values above 50 %. The lack of bootstrap support is probably due to the lack of phylogenetic information in LSU rDNA, which is visualized as very short branch lengths (Fig. 7). The same argument applies to the remaining *Pseudo-nitzschia* species, which are monophyletic only in the LogDet analysis and supported by a low bootstrap value. A monophyletic origin of clade I cannot be rejected, as a tree topology showing this scenario was not significantly different from the best tree. The resolution within clade A can therefore not be properly established at this moment. A monophyletic origin of *Fragilariopsis* and a paraphyletic origin of *Pseudo-nitzschia* seem apparent and the close relationship between the two genera is confirmed.

The apparent paraphyly of *Pseudo-nitzschia* can be solved in three ways:

(1) Clade A could be erected as a single genus comprising both *Pseudo-nitzschia* and *Fragilariopsis* species, as well as the *Nitzschia* species possessing the raphe, valve and frustule type of *Fragilariopsis* and *Pseudo-nitzschia* species. *Pseudo-nitzschia* would have priority as a name for this larger genus. The paucity of synapomorphic characters for *Pseudo-nitzschia* and *Fragilariopsis*, respectively, supports the erection of clade A as a genus. As mentioned above, colony type may change during culturing and solitary taxa also exist within the clade. Most other characters are shared by species of the two genera. Erecting clade A as a single genus would solve the problem arising when a species is morphologically similar to either *Pseudo-nitzschia* or *Fragilariopsis* but has not been observed to form colonies. It would also solve the problems arising in palaeontology where colony type is often impossible to determine (Hasle, 1972*b*).

(2) *Pseudo-nitzschia* and *Fragilariopsis* could be retained today, accepting paraphyletic genera. The ease with which colony shape can be distinguished by light microscopy and the well-established use of the two generic names support this argument. It does not solve the problems concerning solitary species morphologically similar to the taxa of clade A, or that paraphyletic genera should be avoided within systematics.

(3) The three groups could be erected as three separate genera. However, no reliable morphological characters appear to separate the three clades. Poroid pattern, number of poroid rows, valve shape, number of striae versus fibulae, presence/absence of a central nodule and structure of copulae all vary within the groups (Table 2). Additionally, the delimitation of clade I is not supported in the parsimony and maximum likelihood analyses.

We are reluctant to suggest a formal change of the nomenclature as the molecular support is still too sparse. The topology of the basal branches in diatom phylogeny has mainly been addressed by sequence comparison of the nuclear-encoded small subunit of rDNA (SSU rDNA). As the nuclear-encoded large subunit of ribosomal DNA (LSU rDNA) contains highly variable regions scattered within the conserved regions we considered this gene a useful marker to elucidate the phylogenetic relationships within Bacillariaceae, especially concerning *Pseudo-nitzschia*. However, within the sequence regions determined, the variation within clade A was not very large (between 0 and 4·8 % base pair divergence) and an even more variable DNA sequence should be chosen to acquire a better resolution for establishing the relationship among the taxa comprising clade A. An analysis of ITS1 and ITS2 or a highly variable gene is expected to provide better resolution.

Some relationships within clade A are nevertheless resolved. The well-supported sister-group relationship between *P. pungens* and *P. multiseries* is not surprising (1–2 % base pair divergence). The two species are morphologically very similar, differing only in the structure of the stria membrane and the copulae. Because of the many similarities Hasle (1965*a*) originally described *P. multiseries* as a form of *P. pungens*. The present analyses support the separation of *P. multiseries* as a distinct species based on both morphological and molecular data (Hasle, 1995; Manhart *et al.*, 1995).

In all analyses *P. australis* and *P. seriata* cluster together. Although they can be separated morphologically, they are very similar and Rivera (1985) even suggested them to be conspecific. Unfortunately, the present study does not contribute towards solving this question, as the resolution is poor (based on 0·12–0·13 % base pair divergence).

As expected, the species *P. fraudulenta* and *P. subfraudulenta* cluster together in all analyses (0·25 % base pair divergence). They are morphologically very similar, differing only with regard to the number of striae versus fibulae and shape of the valve: *Pseudo-nitzschia fraudulenta* has the same number of fibulae and striae and a lanceolate shape of the valve, whereas *P. subfraudulenta* has a more linear shape in valve view and has more interstriae than fibulae (Hasle, 1965). Hence morphology supports keeping these species separate.

The sister-group relationship between *F. rhombica* and *F. kerguelensis* is well supported in all three analyses. Both taxa are elliptic-lanceolate and possess a circular perforation pattern in the poroid hymen. This could be an example of the taxonomic

level at which the perforation pattern of the poroid hymen can be used as a taxonomic character.

Species of the Bacillariaceae outside clade A

Phylogenetic analyses of taxa within Bacillariaceae but outside clade A were limited due to lack of cultures. Species of *Cylindrotheca* differ morphologically from the other taxa of the Bacillariaceae (Lewin & Lewin, 1964) and are consequently not included in Table 2. Our study indicates that *Cylindrotheca* forms a separate genus, as the three taxa included cluster as a highly supported group within the heterogeneous *Nitzschia*. In all analyses the one isolate of *C. closterium* clusters together with *C. fusiformis* Reimann *et* Lewin and not with a second isolate of the same species. As *Cylindrotheca* species can be difficult to identify, misidentification of one of the strains may be the cause. The identity of the *Cylindrotheca closterium* from GenBank could not be confirmed, whereas the identity of the others has been thoroughly examined.

All other species of Bacillariaceae outside clade A have poroids in the outer wall of the raphe canal (Table 2). They all have two chloroplasts arranged symmetrically about the transapical plane, a more or less eccentric raphe, one row of poroids in the striae and a higher number of striae than fibulae (Table 2). These features apply to most *Nitzschia* species (Mann, 1984; Round *et al.*, 1990). The analyses support the view of *Nitzschia* as not being a monophyletic group as predicted by morphological studies (Mann, 1986, 1993; Hasle & Syvertsen, 1997). *Nitzschia* is a very large genus comprising several different morphological groups (Mann, 1986; Round, 1996*a*), some of which have been separated at the generic level: e.g. *Fragilariopsis*, *Pseudo-nitzschia*, *Psammodictyon* and *Tryblionella* (Round *et al.*, 1990). To examine the relationships among *Nitzschia* species would require increased taxon sampling. However, some conclusions can be drawn.

Nitzschia frustulum constitutes a sister-group to clade A in all three analyses. It differs in having poroids in the outer wall of the raphe canal and in having one row of poroids that are not subdivided. Some *Fragilariopsis* species, e.g. *F. separanda* Hustedt, also have one or two rows of undivided poroids (Hasle, 1965*b*). *Nitzschia frustulum* has a central nodule but no larger central interspace, whereas a central nodule in the taxa of clade A is, as far as we are aware, always combined with a larger central interspace. It is otherwise morphologically close to many of the species in clade A: it is linear to lanceolate in valve view, it has more interstriae than fibulae, the mantle is identical with the valve, the poroid hymen has a hexagonal pattern and the raphe is not lined by a conopea. Detailed ornamentation on the frustule is absent. It belongs to the *Lanceolatae* section in *Nitzschia*, of which several species are closely related and possibly together with *N. frustulum* form a sister-group to clade A.

The two strains of *N. navis-varingica*, potent producers of domoic acid, always clustered together with *N.* cf. *promare*, *N. pellucida* and *N. laevis*, supported by high bootstrap values. Except for *N. laevis*, these species possess detailed ornamentation on the frustule, especially on the mantle, the wall of the raphe canal and the copulae. The only other taxon showing detailed ornamentation of the frustule is *Bacillaria paxillifer*. On the copulae, the ornamentation comprises silica warts (all species), on the wall of the raphe canal silica ridges (*N. navis-varingica*, *N. pellucida* and *N.* cf. *promare*), whereas the ornamentation on the mantle consists of either silica ridges (*N. navis-varingica*, *N.* cf. *promare*) or silica warts (*N. pellucida*, *B. paxillifer*) (Lundholm & Moestrup, 2000 and unpublished observations). In the distance analysis *Bacillaria paxillifer* constitutes a sister-taxon, while in the parsimony and maximum likelihood analyses it is the most closely related taxon in the sister-group. A Kishino–Hasegawa test of a user-defined tree topology forcing all the ornamented species into a monophyletic cluster was significantly different from the best tree, while a maximum likelihood analysis with a similar constraint was not rejected (Table 3). Clustering of species with ornamentation supports the view of Round (1996*b*) that these minute features might prove useful as taxonomic characters. No other morphological character seems to reflect this clustering of species. Further analyses including more taxa can be expected to reveal the degree to which ornamentation of the frustule is useful in the systematics of *Nitzschia*.

The single *Hantzschia* species included in our analyses clustered within *Nitzschia*. This is not surprising as the two genera are morphologically very similar, differing with regard to the placement of the two raphes of the frustule (at the same side or diagonally opposed) and to the two frustule types formed during cell division (Mann, 1977, 1980). Cell division in *Nitzschia* species may result in frustules of the *Hantzschia* type, whereas *Hantzschia* species does not form *Nitzschia*-type frustules after cell division (Lauritis *et al.*, 1967; Mann, 1977, 1980).

Producers of domoic acid

The major producers of domoic acid within *Pseudo-nitzschia* (*P. multiseries*, *P. australis* and *P. seriata*) clustered together. Other species within this cluster, such as *N. americana*, have tested non-toxic (Villac

et al., 1993) or, like *P. pungens* and *P. multistriata*, are minor producers of domoic acid (Rhodes *et al.*, 1996; Sarno & Dahlmann, 2000). Minor producers such as *P. delicatissima*, *P. pseudodelicatissima* and *P. fraudulenta* are also found outside this cluster (Martin *et al.*, 1990; Rhodes *et al.*, 1996, 1998). *Nitzschia navis-varingica*, another major producer of domoic acid, is distantly related to other producers of domoic acid in *Pseudo-nitzschia*. The other *Nitzschia* species, the *Fragilariopsis* species and some *Pseudo-nitzschia* species tested did not produce domoic acid. Hence, the phylogeny based on partial LSU rDNA reveals that the ability to produce domoic acid has probably evolved more than once.

Concluding remarks

Based on partial LSU rDNA sequences the Bacillariaceae has been shown to form a monophyletic group. The close relationship found among species of *Fragilariopsis* and *Pseudo-nitzschia* and at least one species of *Nitzschia* supports the many discussions during the twentieth century concerning the relationship among the three genera. The analyses indicate that emendation of *Pseudo-nitzschia* using features other than colony type will probably reflect phylogeny better and solve many problems regarding the generic relationship of morphologically similar extant – and fossil – cells observed as single cells. However, sequence determination of a more variable DNA region is needed before any changes in nomenclature should be undertaken. Though few species were included in this study, *Cylindrotheca* seems to constitute a monophyletic group. *Nitzschia* has been shown not to be monophyletic, as *Cylindrotheca*, *Bacillaria* and *Hantzschia* as well as *Pseudo-nitzschia* and *Fragilariopsis* species cluster within *Nitzschia*.

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Note added in proof

Formal erection of *Pseudo-nitzschia micropora* is in press (Priisholm *et al.*, 2002).

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