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Pseudo-nitzschia pungens (Bacillariophyceae): A cosmopolitan diatom species?

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Abstract

Genetic, reproductive and morphological variation were studied in 193 global strains of the marine diatom species *Pseudo-nitzschia pungens* (Grunow *ex* Cleve) Hasle to assess potential intraspecific variation and biogeographic distribution patterns. Genetic differentiation between allo- and sympatric strains was investigated using the ITS1–5.8S–ITS2 rDNA region. Three ITS clades were found. Clones of opposite mating type were sexually compatible within clades I or II, and viable F1 hybrid offspring were produced in crosses between them. The molecular differences between these clades were correlated with slight but consistent morphological differences. At present, nothing can be said about morphology and mating behavior for clade III clones because only ITS data were available. The three ITS clades showed different geographic distributions. Clade II was restricted to the NE Pacific, whereas clones belonging to clade III originated from geographically widely separated areas (Vietnam, China and Mexico). ITS clade I was recovered in all locations studied: the North Sea (Belgium, The Netherlands, France), the eastern and western N Atlantic (Spain, Canada), the NW and S Pacific (Japan, New Zealand) and the NE Pacific (Washington State). Clade I thus appears to be globally distributed in temperate coastal areas and provides the first strong evidence to date for the global distribution of a biologically, genetically and morphologically defined diatom species.

Keywords: Breeding test; Cosmopolitanism; Diatom; Genetic diversity; ITS rDNA; *Pseudo-nitzschia*; Secondary structure; Sexual reproduction

1. Introduction

During the last two decades, the pennate diatom genus *Pseudo-nitzschia* H. Peragallo has been the focus of much attention. Representatives of this genus are a typical component of marine phytoplankton worldwide and, based on morphology, most appear to have a cosmopolitan distribution (Hasle, 2002). The genus contains at least 12 species that are able to produce domoic acid, a neurotoxin responsible for amnesic shellfish poisoning (Bates, 2000; Bates and Trainer, 2006), and 'harmful algal blooms' (HABs) involving these organisms have caused a series of environmental

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and public health problems (e.g. Bates and Trainer, 2006). As a consequence, the genetic and morphological diversity of *Pseudo-nitzschia* has received much attention because, without confident delineation of species boundaries, efforts to understand, monitor and predict events occurring in natural populations (especially during HABs) are severely compromised.

Recent taxonomic research on diatoms suggests that traditional species boundaries, based largely on variation in the morphology of the cell's siliceous exoskeleton (the frustule), have been drawn too widely and that real species diversity has probably been greatly underestimated (Mann, 1999). As for other groups of organisms (Dettman et al., 2003; Agapow et al., 2004), it is expected that better insight into diatom species taxonomy and evolution can be obtained through comparison of biological, phylogenetic and morphological species circumscriptions (using breeding, molecular and morphological data). *Pseudo-nitzschia* is one of the few diatom genera that has been subject to this approach. This has led to the revision of existing species and the description of several new species (Lundholm et al., 2003, 2006; Orsini et al., 2004; Hasle and Lundholm, 2005; Amato et al., 2007). To date, *Pseudo-nitzschia pungens* (Grunow *ex* Cleve) Hasle has received little attention, despite being one of the most commonly reported, potentially toxic representatives of the genus worldwide (Hasle, 2002). Clones of *P. pungens* isolated from various geographic areas exhibited different abilities to produce domoic acid. Toxic clones have only been reported from New Zealand (Rhodes et al., 1996), Washington State (Trainer et al., 1998) and Monterey Bay, California (Bates et al., 1998). No toxic clones have so far been reported from the North Sea (Vrieling et al., 1996), where *P. pungens* is the most common representative of the genus.

In a recent study, we obtained 24 clones of *P. pungens* from the North Sea and the Westerscheldt estuary and experimentally examined the principal cell and life cycle features, including auxosporulation and the mating system (Chepurnov et al., 2005). North Sea *P. pungens* is heterothallic and must therefore outbreed, which is consistent with the high genetic diversity observed in North Sea populations by Evans et al. (2005). In the present study, we aimed at assessing genetic, morphological and reproductive variation in global populations of *P. pungens*. We extended the sampling from the North Sea and adjoining areas and also included *P. pungens* clones from five geographically more distant areas in the Atlantic and Pacific Oceans. We identified genetic variation by using sequences of the ITS1–5.8S–ITS2 rDNA region, documented morphological differences, and involved all available clones in crossing experiments to reveal mating barriers.

2. Materials and methods

2.1. Sampling and culturing

A first set of *P. pungens* clones, established in 2002–2003, contained 24 isolates from different sites in the southern North Sea (the Netherlands, Belgium and northern France). These strains are listed by Chepurnov et al. (2005) and were used for intensive study of the life cycle and mating system. Fourteen of these strains were also used in the present study.

In 2004, planktonic samples were collected monthly on the Belgian North Sea coast (five stations, 91 strains isolated), and adjoining coastal lagoon at Oostende (Spuikom, 12 strains isolated) and the Westerscheldt estuary at Terneuzen and Vlissingen (two stations, 36 strains isolated) (Fig. 1A) using a plankton net with mesh size 20 μm. Within a few hours, an aliquot of the material was transferred to polystyrene 50-mm Petri dishes and examined under an Axiovert 135 inverted microscope (Zeiss, Jena, Germany). Monoclonal cultures were established by isolating single chains by micropipette and were grown in f/2 medium (Guillard, 1975) based on filtered and sterilized seawater (~30–32 psu) from the North Sea (18 °C, 12-h light:12-h dark period and 25–50 μmol photons m^{-2} s^{-1} from cool-white fluorescent lights).

Other *P. pungens* clones (Fig. 1B and Table 1) originated from the North-East Atlantic (Spain, 4 strains), North-West Atlantic (Canada, 16 strains), North-East Pacific (Washington State, USA, 12 strains), North-West Pacific (Japan, 5 strains) and the South Pacific (New Zealand, 3 strains). Detailed information about the precise sampling locations and dates, suppliers, initial strain designations and EMBL accession numbers, is available in supplementary Table S1.

2.2. DNA extraction, amplification and sequencing

DNA of *P. pungens* cells was extracted using a bead-beating method with phenol extraction and ethanol precipitation (Zwart et al., 1998). The internal transcribed spacer region (ITS1–5.8S–ITS2) of rDNA was amplified by PCR, using the primers 1800F (Friedl, 1996) and ITS4 (White et al., 1990). PCR mixtures contained 1–5 μl of template DNA, primers at a

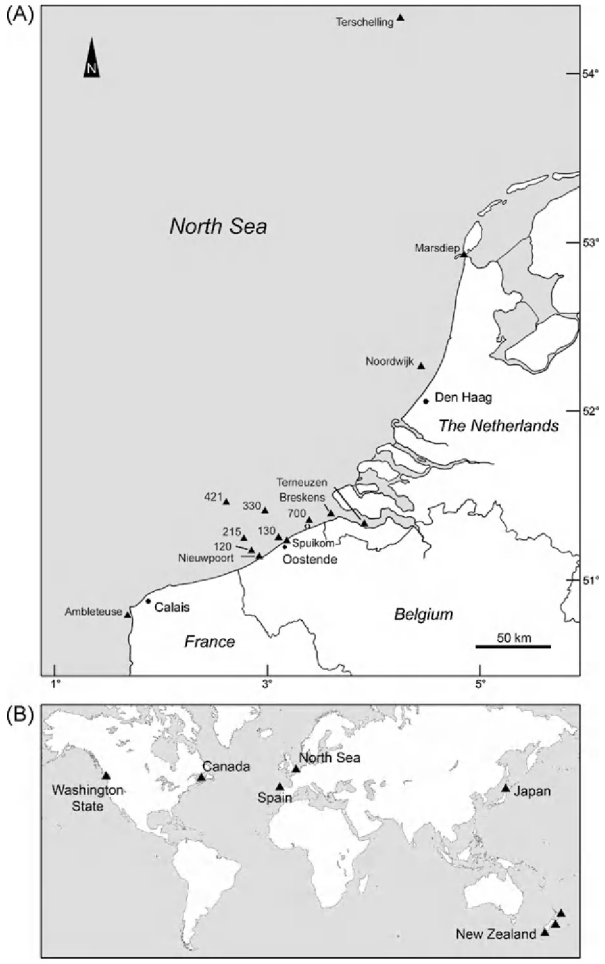

Fig. 1. Locations of sampling stations. (A) The North Sea and Westerscheldt estuary; (B) World-wide. (▲) Sampling locations. The numbers indicate the sampling station according to the VLIZ monitoring campaign (http://www.vliz.be/).

concentration of 0.5 μM, deoxynucleoside triphosphates at 200 μM each, bovine serum albumin (BSA) at 0.4 μg μl^{-1}, 5 μl of 10 X PCR buffer [Tris–HCl, $(NH_4)_2SO_4$, KCl, 15 mM $MgCl_2$, pH 8.7 at 20 °C; "Buffer I", Applied Biosystems, Foster City, USA] and 2.5 units of Taq polymerase (AmpliTaq, Perkin-Elmer, Wellesley, USA); mixtures were adjusted to a final volume of 50 μl with sterile water (Sigma, St. Louis, USA). The cycling parameters were: initial denaturation at 94 °C for 7 min; 30 cycles of 1 min at 94 °C, 1 min at 48 °C and 2 min at 72 °C; and finally 72 °C for 10 min. Amplified products were purified using the Qiaquick PCR purification kit (Qiagen, Hilden, Germany) and directly sequenced with the aid of a Big Dye™ Terminator Cycle Sequencing Ready reaction Kit (Applied Biosystems, Foster City, USA), using the same forward and reverse primers as applied for PCR amplifications. Sequencing was done with a capillary sequencer (ABI3100, Applied Biosystems, Foster City, USA). Sequences run in both directions were overlapping. The sequence data were checked and edited using BioNumerics (Applied Maths, Belgium) software. Strains that gave no or a very weak PCR product required a new PCR, with nested primer combinations. First a PCR was run with primers and cycling conditions as described by Lundholm et al. (2003). A nested PCR was performed on these PCR products with the same primers and cycling conditions mentioned above. To study sequence variation within a single strain, purified PCR products were cloned using the pGEM-T Kit (Promega, Madison, USA), as recommended by the manufacturer. Vector primers T7 and SP6 were used as sequencing primers. For six strains (see Table 1; supplementary Table S1), five to seven cloned sequences were analyzed.

2.3. Sequence alignment and phylogenetic analysis

Sequences were aligned using BioNumerics (Applied Maths, Belgium) and corrected manually. The boundaries of the ITS regions and 5.8S gene were identified by comparison with published sequences (Manhart et al., 1995; Cangelosi et al., 1997; Lundholm et al., 2003). The alignment comprised ITS sequences of *P. pungens* that were obtained in the present study (direct and cloned sequences) and those available in GenBank [accession numbers AY257845 (Portugal), AY257846 (Mexico), AY544769 (China), DQ062665 (Vietnam), DQ166533 (Vietnam)]. Both to test the accuracy of our results and to check those sequences already available for *P. pungens* in GenBank, we obtained three *P. pungens* strains for which ITS sequences had been deposited in GenBank (AY257845, from Portugal; AY257846, from Mexico; and DQ062665, from Vietnam). The DNA was re-extracted and the ITS region sequenced. Our sequences and those from GenBank were identical for the Mexican and the Vietnamese strains but different for the Portuguese isolate (our Portuguese sequence was identical to the North Sea sequences, while the sequence found in GenBank was identical with the Mexican sequence). We therefore omitted the Portuguese sequence from the phylogenetic analysis.

Phylogenetic signal among parsimony-informative sites was assessed by comparing the measure of skewedness (g1-value; PAUP*) with the empirical threshold values in Hillis and Huelsenbeck (1992). All phylogenetic analyses were performed on the total ITS1–5.8S–ITS2 rDNA region with the aid of PAUP

Table 1
List of *P. pungens* monoclonal cultures involved in the present study

Clone	General region	Specific location	Sampling date
1, **2**	Westerscheldt, The Netherlands	Terneuzen	24/05/02
6	North Sea, The Netherlands	Terschelling	1/05/00
14	North Sea, The Netherlands	Marsdiep	25/04/02
16, **18**, **19**	North Sea, The Netherlands	Noordwijk	22/05/02
20, **24**, **28**	North Sea, Belgium	VLIZ station 130	30/06/03
40s, **44**	North Sea, Belgium	Ijzermonding Nieuwpoort	13/08/03
52, **57**	North Sea, France	Ambleteuse	13/09/03
V120(3)1, 2, 3, 4, **5**, 6, 7, 8	North Sea, Belgium	VLIZ station 120	24/03/04
V120(8)1, 2, 3, 4, 5, 6, 7, 8	North Sea, Belgium	VLIZ station 120	16/08/04
V215(3)1, **2**, 3, 4, **5**, 6, 7	North Sea, Belgium	VLIZ station 215	24/03/04
V215(7)1, 2, **3**, 4, 5, 6, **7**[a], 8, 9	North Sea, Belgium	VLIZ station 215	19/07/04
V215(10)1	North Sea, Belgium	VLIZ station 215	18/10/04
V215(12)1, 2, 3, 4, 5, 6, 7, 8, 9, 10	North Sea, Belgium	VLIZ station 215	08/12/04
V330(3)1, 2, 3, 4	North Sea, Belgium	VLIZ station 330	24/03/04
V700(4)1, 2, 3, 4, 5, 6, 7, 8	North Sea, Belgium	VLIZ station 700	19/04/04
V700(7)1, 2, 3, 4, **5**, 6, 7, 8, 9, 10, **11**, 12	North Sea, Belgium	VLIZ station 700	19/07/04
V421(3)1, 2, 3, **4**, 5, 6	North Sea, Belgium	VLIZ station 421	29/03/04
V421(8)1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11	North Sea, Belgium	VLIZ station 421	23/08/04
V421(10)1, 2, 3, 4, 5, 6, 7	North Sea, Belgium	VLIZ station 421	19/10/04
S(4)1, 2, 3, 4, 5, **6**[a], 7, 8, 9	Oostend Lagoon, Belgium	Spuikom Oostende	7/04/04
S(5)1, 2	Oostend Lagoon, Belgium	Spuikom Oostende	18/05/04
S(7)1	Oostend Lagoon, Belgium	Spuikom Oostende	13/07/04
W1(4)1, 2, 3, 4, 5, 6, 7, 8	Westerscheldt, The Netherlands	Breskens	21/04/04
W1(7)1, 2, 3, 4, 5, **6**[a], 7, 8, 9, 10	Westerscheldt, The Netherlands	Breskens	14/07/04
W4(4)1, 2, 3, 4, 5, 6, 7, 8	Westerscheldt, The Netherlands	Terneuzen	21/04/04
W4(7)1, **2**, 3, 4, 5, 6, 7, 8, 9, 10	Westerscheldt, The Netherlands	Terneuzen	14/07/04
Vigo-1, **Vigo-2**, **Vigo-3**[a], **Vigo-4**	NE Atlantic, Spain	Bay of Vigo	1/04/04
Cn-172	NW Atlantic, Canada	Cardigan River, Prince Edward Island	5/09/02
Cn-181, Cn-184	NW Atlantic, Canada	Oak Point, Miramichi Bay, New Brunswick	23/09/02
Cn-193	NW Atlantic, Canada	Deadmans Hbr., Bay of Fundy, New Brunswick	9/10/02
Cn-196, Cn-200	NW Atlantic, Canada	Egg Island, Miramichi Bay, New Brunswick	28/10/02
Cn-201, Cn-202	NW Atlantic, Canada	Egg Island, Miramichi Bay, New Brunswick	15/09/03
Cn-204, **Cn-205**	NW Atlantic, Canada	Malpeque Bay, Prince Edward Island	27/10/03
Cn-213, Cn-214	NW Atlantic, Canada	Brudenell River, Prince Edward Island	7/09/04
Cn-215, **Cn-216**	NW Atlantic, Canada	Cardigan River, Prince Edward Island	7/09/04
Cn-217, **Cn-218**	NW Atlantic, Canada	Boughton River, Prince Edward Island	7/09/04
US-77	NE Pacific, USA	La Push, WA	18/09/01
US-93	NE Pacific, USA	Eld Inlet, Mud Bay, WA	17/06/02
US-94[a], **US-96**	NE Pacific, USA	Sequim Bay State Park, WA	8/08/02
US-115, **123**[a], 125, 126, **132**, 134, **135**, 136	NE Pacific, USA	ECOHAB I (coastal Washington)	/06/03
Jp-1	NW Pacific, Japan	Ofunato Bay, Iwate Prefecture	7/08/00
Jp-11	NW Pacific, Japan	Ofunato Bay, Iwate Prefecture	25/06/01
Jp-14	NW Pacific, Japan	Ofunato Bay, Iwate Prefecture	3/12/01
Jp-01	NW Pacific, Japan	Okkirai Bay, Iwate Prefecture	25/07/00
Jp-02	NW Pacific, Japan	Okkirai Bay, Iwate Prefecture	28/07/00
NZ-49	S Pacific, New Zealand	Steels Reef, North Island	/10/00
NZ-67	S Pacific, New Zealand	Big Glory Bay, Stewart Island	/02/03
NZ-74	S Pacific, New Zealand	Taylor's Mistake, South Island	2004
DQ166533[b]	NW Pacific, Vietnam	Hai Phong	
AY544769[b]	NW Pacific, China		
DQ0626665 (KBH2)[b]	NW Pacific, Vietnam	Khan Hoa Bay	
AY257846 (Mex 18)[b]	Gulf of Mexico, Mexico	Near Tuxpam	

Strains that were sequenced are shown in bold.
[a] Strains of which PCR products were cloned.
[b] Strains for which no live cells but only sequences were available.

4.0*beta test Version 10 (Swofford, 2002). Maximum parsimony (MP) analyses were carried out using heuristic search options with random stepwise addition of taxa (1000 repetitions) and the tree bisection-reconnection branch-swapping algorithm. Gaps were treated as missing data. Maximum Likelihood (ML) and Neighbor Joining (NJ) analyses were performed using a Hasegawa–Kishino–Yano nucleotide substitution model (Hasegawa et al., 1985), as determined by the Akaike Information Criterion (AIC) using Modeltest Version 3.06 (Posada and Crandall, 1998). ML analyses were performed using the same heuristic search settings as in the MP analyses. Bootstrap analyses (1000 replicates) were performed to test the robustness of tree topologies (Felsenstein, 1985).

P. multiseries (Hasle) Hasle was used to root all trees since it has always been recovered with high bootstrap support as the sister taxon of *P. pungens* (Lundholm et al., 2003; Hasle and Lundholm, 2005).

2.4. Secondary structures of rDNA ITS1 and ITS2

Common secondary structural motifs of the ITS1 and ITS2 sequences of *P. pungens* were initially recognized by folding each sequence using the Mfold software (http://www.bioinfo.rpi.edu/; Zuker, 2003). Foldings were conducted at 25 °C using a search within 10% of thermodynamic suboptimality. Paired regions were then recognized by comparing the ITS secondary structures of different *Pseudo-nitzschia* taxa [including *P. australis* Frenguelli, *P. decipiens* Lundholm et Moestrup, *P. galaxiae* Lundholm et Moestrup, *P. multiseries*, *P. obtusa* (Hasle) Hasle and Lundholm and *P. seriata* (Cleve) Peragallo] and by identifying compensatory base changes (CBCs: change of the nucleotides at both sides of a double-stranded helix, preserving the pairing) and hemi-CBCs (change of a nucleotide at one side of a stem, but still preserving the pairing) as proposed by Mai and Coleman (1997). The common structural patterns were then annotated in a manual alignment with DCSE v. 2.60 (De Rijk and De Wachter, 1993). This DCSE file was used to create the secondary structures diagrams with RnaViz (De Rijk et al., 2003). The helices of ITS2 were numbered according to Mai and Coleman (1997).

2.5. Mating experiments

All crosses were carried out in the Laboratory of Protistology and Aquatic Ecology (Ghent University, Belgium). Crosses were performed by inoculating pairs of clones (in exponential growth phase; see Davidovich and Bates, 1998) into wells of 24-well Repli plates (Greiner Bio-One, Kremsmuenster, Austria). The mixed cultures were examined daily for 6–8 days, using a Zeiss Axiovert 135 inverted microscope, until they reached the stationary phase of growth and started to die. Previous investigations (Chepurnov et al., 2005) had shown that North Sea *P. pungens* is heterothallic and that clones of opposite mating type differed in the behavior of gametes they produced. Clones can be designated as PNp^+ ('PNp' is an acronym representing the genus and species; '+' indicates that the gametangia produce two active gametes) or PNp^- (the gametangia produce two passive gametes). As in many other diatoms (e.g. Geitler, 1932; Drebes, 1977; Chepurnov et al., 2004), induction of sexuality in North Sea *P. pungens* clones was size-dependent, the upper threshold for sexual induction being about 115 μm (Chepurnov et al., 2005).

Out of the 24 strains from 2002 to 2003, six reference clones of known sexuality (i.e. clones 1, 14, 19 of mating type PNp^- and clones 2, 6 and 18 of opposite mating type PNp^+) were selected to cross with other strains available (supplementary Table S1). Crosses between clones derived from various geographic areas were also performed (Tables 3 and 4).

2.6. Morphological analysis

The combination of a few morphological characters, including cell size, robustness and curvature of cell chains and the extent of overlap of cells in the 'stepped' colonies (Chepurnov et al., 2005), is sufficient to distinguish *P. pungens* from other co-occurring *Pseudo-nitzschia* species [e.g. *P. turgidula* (Hustedt) Hasle, *P. delicatissima* (Cleve) Heiden and *P. fraudulenta* (Cleve) Hasle] under an inverted microscope. After isolation, every clone was assessed for valve ornamentation and the stria and fibula densities characteristic of *P. pungens* via light microscopy; this was possible even in live cells in water mounts, without cleaning the frustule by oxidation (see also Hasle and Syvertsen, 1996). In addition, however, frustules were cleaned by oxidation with hydrogen peroxide and glacial acetic acid and washed repeatedly with distilled water before being mounted in Naphrax (PhycoTech, St. Joseph, USA). Light microscopical (LM) observations were carried out using a Zeiss Axioplan 2 Universal microscope (Zeiss, Jena, Germany). Scanning electron microscopy (SEM) was performed using a JEOL JSM5600LV (Tokyo, Japan) on cleaned material dried onto aluminium stubs and coated with gold–palladium.

Detailed morphological analyses had already been performed for the 24 clones isolated from the North Sea, including the reference clones (Chepurnov et al., 2005). In addition, morphometric data for 11 clones (7 from clade I and 4 from clade II) are presented in Table 5.

Statistical analyses (*t*-tests) were performed using STATISTICA Version 5.0 for Windows (StatSoft, Tulsa, USA).

3. Results

3.1. ITS diversity

ITS1–5.8S–ITS2 rDNA sequences were obtained by direct sequencing of PCR products for 54 *P. pungens* strains representing different localities and seasons (Table 1). For six strains the PCR products were cloned;

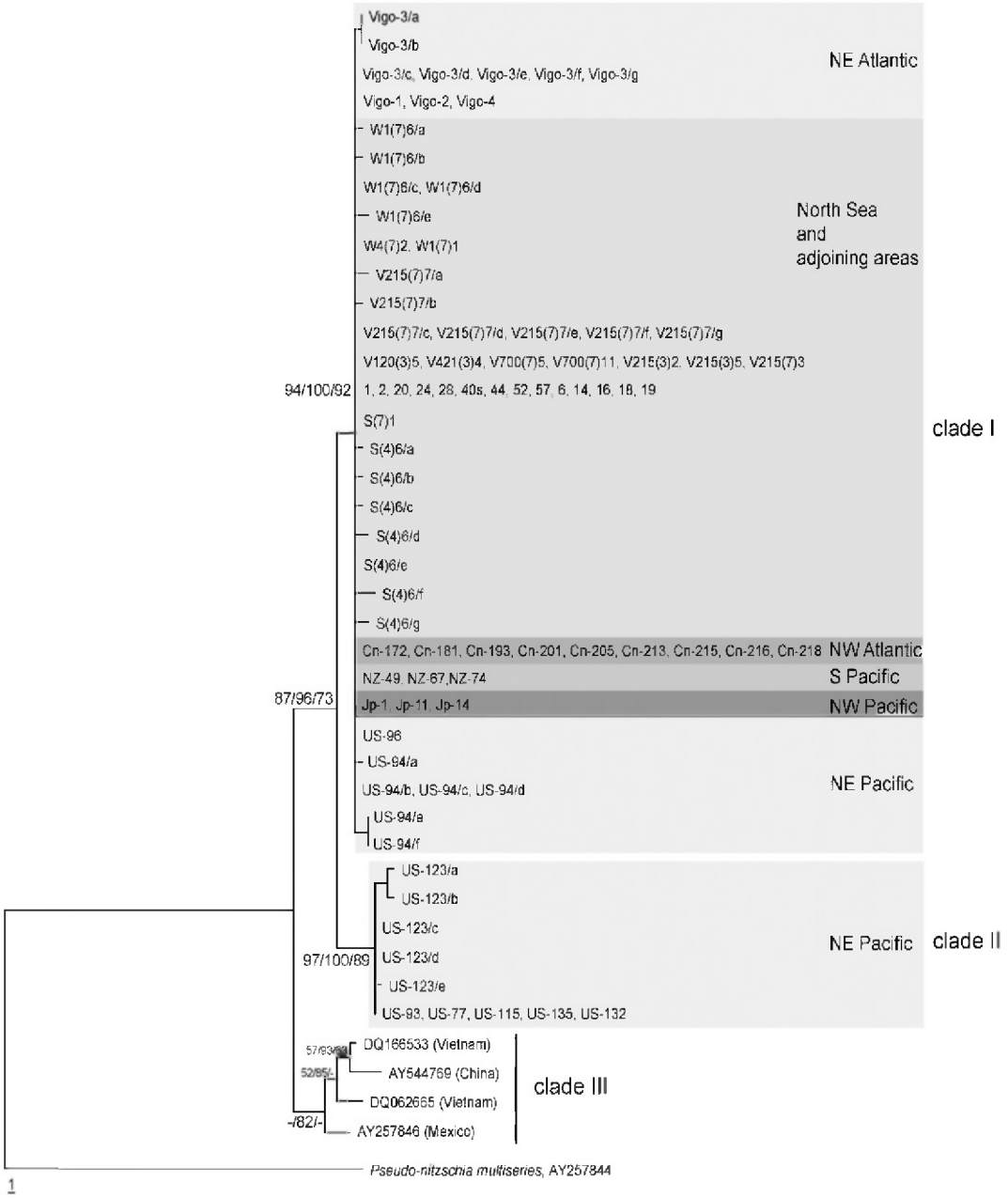

Fig. 2. *Pseudo-nitzschia pungens*. Maximum parsimony tree inferred from ITS1–5.8S–ITS2 sequence data. Bootstrap values (MP/NJ/ML) are indicated above branches.

37 sequences were obtained (Table 1 and supplementary Table S1). All newly obtained sequences of *P. pungens* were identical in length, with ITS1: 260 bp, 5.8S: 171 bp and ITS2: 264 bp. The complete alignment, including *P. pungens* sequences from GenBank and *P. multiseries* as the out-group, comprised 718 characters, of which 144 positions were variable and 36 parsimony-informative. However, many of these sites reflect the separation between *P. pungens* and *P. multiseries* and the total number of variable sites in the in-group was 61, among which 32 were parsimony-informative. Comparison of the measure of skewedness with the empirical threshold values in Hillis and Huelsenbeck (1992) revealed that the data contained significant phylogenetic signal [g1 = −1.50; threshold value g1 = −0.19 ($p = 0.01$) for 15 taxa and 50 variable characters].

Intra-isolate sequence variation, studied in six strains by cloning of PCR products, was extremely low (0–0.7%). Changes involved autapomorphic point mutations that were not phylogenetically informative, except at two sites in US-123 (US-123/a and b) and one site in Vigo-3 (Vigo-3/a and b) and US-94 (US-94/e and f), where partially homogenized mutations were found.

Maximum parsimony (MP) analysis resulted in three most parsimonious trees [tree length = 165, consistency index (CI) = 0.94]. Neighbor Joining and Maximum Likelihood analysis (for ML, −log likelihood = 1835.13) yielded similar tree topologies. One of the MP trees is illustrated in Fig. 2. Two sister clades (I and II) were always recovered with high bootstrap support, whereas the basal branches either clustered in a clade (NJ, one MP tree, Fig. 2) or formed a grade (ML and two of the MP trees). Analyses with in-group sequences only (data not shown) always recovered the three clades (NJ; ML; MP: 1 tree found, CI = 1) with very high bootstrap support. Clade I included the largest number of clones, representing all of the major geographic areas investigated, i.e. the North Sea (Belgium, the Netherlands, northern France) and adjoining areas (Westerscheldt and Spuikom), NE Atlantic (Spain), NW Atlantic (Canada), NW Pacific (Japan), S Pacific (New Zealand) and two clones (US-94 and US-96) from the NE Pacific (USA). Clade II exclusively comprised strains from the NE Pacific (USA). Clade III contained sequences found in GenBank from Vietnam, China and Mexico.

Sequence divergences (uncorrected *p*-values), both within and between the three clades, are shown in Table 2. Between clades I and II, whole ITS1–5.8S–ITS2 region differed by 1.3% (only direct sequences included), corresponding to three substitutions (one

Table 2
Sequence divergence (uncorrected *p*-values) within and amongst the three clades shown in Fig. 2

	Clade I			Clade II			Clade III		
	ITS1–5.8S–ITS2	ITS1	ITS2	ITS1–5.8S–ITS2	ITS1	ITS2	ITS1–5.8S–ITS2	ITS1	ITS2
Clade I ($n = 76$)	0–0.007 (0.001)	0–0.019 (0.001)	0–0.008 (0.001)						
Clade II ($n = 10$)	0.013–0.022 (0.014)	0.012–0.031 (0.014)	0.023–0.034 (0.024)	0–0.006 (0.002)	0–0.012 (0.003)	0–0.008 (0.002)			
Clade III ($n = 4$)	0.027–0.039 (0.031)	0.023–0.050 (0.030)	0.045–0.053 (0.048)	0.032–0.044 (0.035)	0.027–0.050 (0.034)	0.053–0.064 (0.056)	0–0.019 (0.013)	0–0.023 (0.015)	0–0.019 (0.014)

Shown as: minimum–maximum (average). Note the three clades are characterized by a different number of sequences, preventing precise comparisons of genetic variability among them.

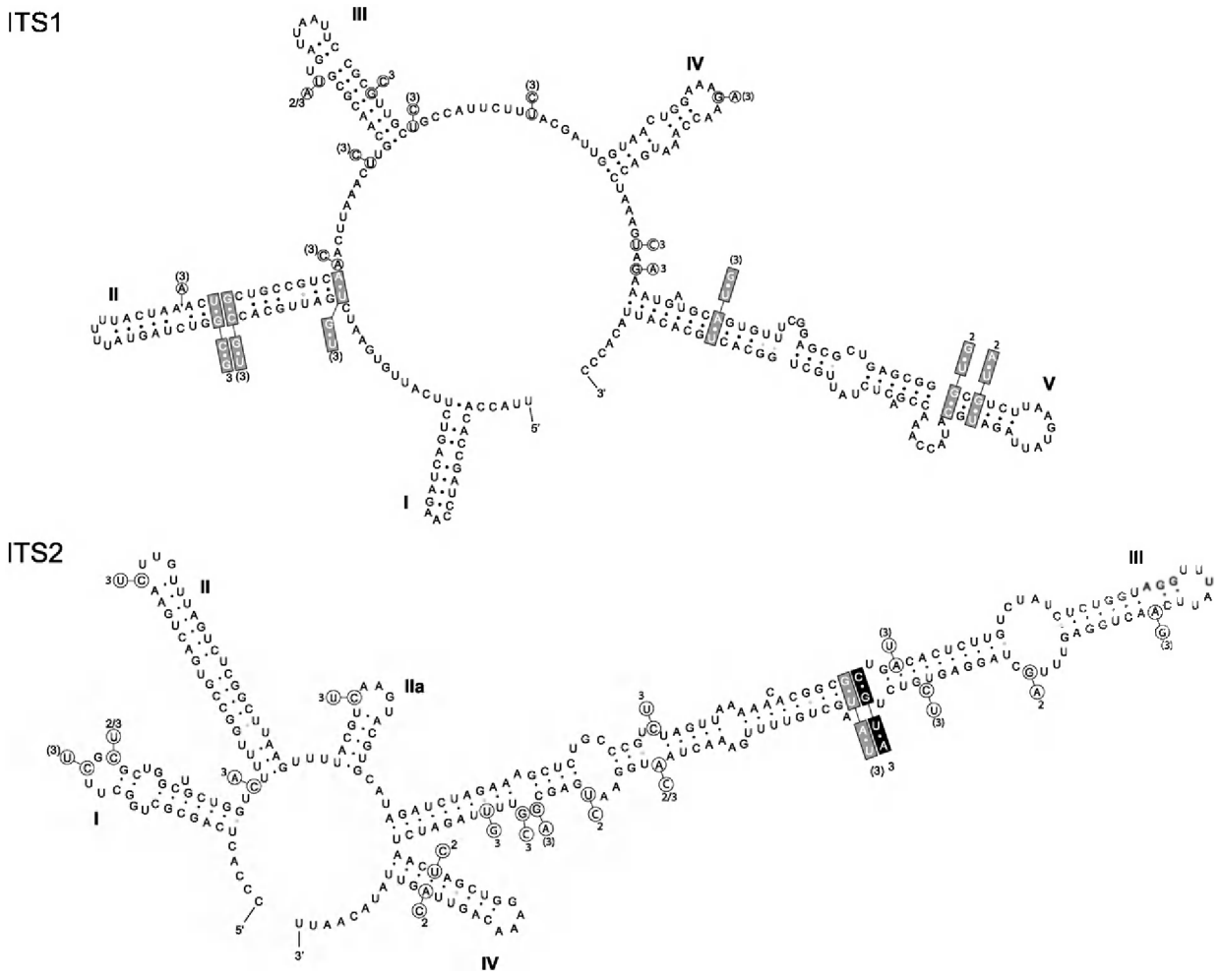

Fig. 3. Diagrams of secondary structures of the ITS1 and ITS2 transcripts of *Pseudo-nitzschia pungens* (clone 1, EMBL accession number AM778733) derived by comparisons among seven *Pseudo-nitzschia* species. Base changes between the different genotypes of *P. pungens* are indicated: the base pair marked in a black box indicates a CBC; base pairs marked in grey boxes indicate HCBCs; single base changes are marked in circles. The numbers next to the boxes and circles specify the *P. pungens* clade in which the base changes occurred; numbers in brackets indicate that the base change occurred in only some sequences of the clade.

transversion and two transitions) in ITS1 and 6 substitutions (all transitions) in ITS2. Within each of clades I and II, the direct sequences were all identical and the differences seen were entirely due to intraclonal variation (<0.7%). The divergence between clade III and the rest of the in-group was 2.7–4.4%.

3.2. ITS1 and ITS2 secondary structures

In the seven *Pseudo-nitzschia* species compared (*P. australis, P. decipiens, P. galaxiae, P. multiseries, P. obtusa, P. pungens*, and *P. seriata*), a common overall organization of the ITS1 secondary structure could be identified, despite large sequence divergence and considerable variation in secondary structural motifs; this common structure comprised a large loop and five paired regions (helices I–V) (Fig. 3). Within *P. pungens*, the ITS1 secondary structures of all genotypes had an identical organization. Eight base changes among the three *P. pungens* clades were situated in unpaired regions, thus having no effect on the secondary structure of the ITS1 RNA molecule. Six HCBCs could be identified. Three were in helix II and occurred between clade I and some strains of clade III, and three others were in helix V, occurring between clade I and clades II and III.

The ITS2 secondary structures were also organized similarly in all *Pseudo-nitzschia* species, with a loop and five helices (I, II, IIa, III and IV). Within *P. pungens*, the organization of all ITS2 secondary structures was

Table 3
Pseudo-nitzschia pungens: Canadian clones were crossed between themselves and selected clones from distant geographic areas

			Cn-172	Cn-195	Cn-204	Cn-213	Cn-214	Cn-184	Cn-193	Cn-200	Cn-201	Cn-202	Cn-205	Cn-215	Cn-217	Cn-218	Cn-181	Cn-216
Clade I	PNp$^+$	Cn-172	[0]															
ns	PNp$^+$	Cn-195	0	[0]														
ns	PNp$^+$	Cn-204	0	0	[0]													
Clade I	PNp$^+$	Cn-213	0	0	0	[0]												
ns	PNp$^+$	Cn-214	0	0	0	0	[0]											
ns	PNp$^-$	Cn-184	+	+	+	+	+	[0]										
Clade I	PNp$^-$	Cn-193	+	+	+	+	+	0	[0]									
ns	PNp$^-$	Cn-200	+	+	+	+	+	0	0	[0]								
Clade I	PNp$^-$	Cn-201	+	+	+	+	+	0	0	0	[0]							
ns	PNp$^-$	Cn-202	+	+	+	+	+	0	0	0	0	[0]						
Clade I	PNp$^-$	Cn-205	+	+	+	+	+	0	0	0	0	0	[0]					
Clade I	PNp$^-$	Cn-215	+	+	+	+	+	0	0	0	0	0	0	[0]				
ns	PNp$^-$	Cn-217	+	+	+	+	+	0	0	0	0	0	0	0	[0]			
Clade I	PNp$^-$	Cn-218	+	+	+	+	+	0	0	0	0	0	0	0	0	[0]		
Clade I	?	Cn-181	0	0	0	0	0	0	0	0	0	0	0	0	0	0	[0]	
Clade I	?	Cn-216	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	[0]
Clade I	PNp$^-$	Jp-11	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0
Clade I	PNp$^-$	NZ-67	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0
Clade I	PNp$^-$	US-94	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0
Clade I	PNp$^-$	Vigo-1	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0
Clade I	PNp$^-$	1	+	+	+	+	+	0	0	0	0	0	0	0	0	0	0	0
Clade I	PNp$^+$	Jp-14	0	0	0	0	0	+	+	+	+	+	+	+	+	+	0	0
Clade I	PNp$^+$	NZ-74	0	0	0	0	0	+	+	+	+	+	+	+	+	+	0	0
Clade II	PNp$^+$	US-93	0	0	0	0	0	+	+	+	+	+	+	+	+	+	0	0
Clade I	PNp$^+$	Vigo-2	0	0	0	0	0	+	+	+	+	+	+	+	+	+	0	0
Clade I	PNp$^+$	2	0	0	0	0	0	+	+	+	+	+	+	+	+	+	0	0
Clade II	?	US-123	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Clade II	?	US-132	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ns	?	US-134	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

+, Sexual reproduction occurred in mixed culture; 0, sexual reproduction absent in mixed culture; [0], sexual reproduction absent in monoclonal culture; ns, no sequence available. Shaded cells show successful crosses between clade I and clade II clones.

identical. Most of the base changes were situated in unpaired regions. No CBCs or HCBCs were found between clades I and II, but one CBC and one HCBC, both in helix III, were recognized between clade III and the other two.

3.3. Crossing experiments

All 193 isolated *P. pungens* clones – from both clade I and clade II – were involved in breeding tests with at least some other clones. None of the clones exhibited intraclonal sexual reproduction. The six selected reference clones from the North Sea were used to determine the mating types of other clones, revealing no contradictory results (i.e. each clone behaved consistently, either as PNp$^+$ or PNp$^-$). Where sex was induced, it occurred exclusively in mixed cultures of clones that belonged to opposite mating types (Tables 3 and 4; supplementary Table S1 and Fig. S1).

Most of the new clones isolated from the North Sea and adjoining waters in 2004 (the V, W and S series of clones) that had cells smaller than 115 μm were able to reproduce sexually with the reference clones. However, a few clones that were well below 115 μm long (the threshold size for sexual reproduction estimated by Chepurnov et al., 2005) did not exhibit any sign of sexualization (see supplementary Table S1). Cells that were longer than 115 μm did not reveal any signs of sexuality when crossed with the reference strains (supplementary Table S1).

Sex was induced in all clones from Spain (4), Japan (5) and New Zealand (3). Sexual reproduction was successfully initiated in mixed cultures of opposite mating type clones, both within and between each of

Table 4
Pseudo-nitzschia pungens: results of crosses between the clones originating from Washington State, Japan, New Zealand, Spain and the Netherlands

			US-123	US-132	US-134	US-135	US-115	US-94	US-96	US-125	US-126	US-136	US-77	US-93	Jp-11	Jp-21	Jp-22	Jp-01	Jp-14	NZ-49	NZ-67	NZ-74	Vigo-1	Vigo-4	Vigo-2	Vigo-3
Clade II	?	US-123	[0]																							
Clade II	?	US-132	0	[0]																						
ns	?	US-134	0	0	[0]																					
Clade II	?	US-135	0	0	0	[0]																				
Clade II	PNp^-	US-115	0	0	0	0	[0]																			
Clade I	PNp^-	US-94	0	0	0	0	0	[0]																		
Clade I	PNp^-	US-96	0	0	0	0	0	0	[0]																	
ns	PNp^-	US-125	0	0	0	0	0	0	0	[0]																
ns	PNp^-	US-126	0	0	0	0	0	0	0	0	[0]															
ns	PNp^-	US-136	0	0	0	0	0	0	0	0	0	[0]														
Clade II	PNp^+	US-77	0	0	0	0	0	+	+	+	+	+	[0]													
Clade II	PNp^+	US-93	0	0	0	0	+	+	+	+	+	+	0	[0]												
Clade I	PNp^-	Jp-11	0	0	0	0	0	0	0	0	0	0	+	+	[0]											
Clade I	PNp^-	Jp-21	0	0	0	0	0	0	nt	nt	nt	nt	nt	+	0	[0]										
Clade I	PNp^-	Jp-22	0	0	0	0	0	0	nt	nt	nt	nt	nt	+	0	0	[0]									
Clade I	PNp^+	Jp-01	0	0	0	0	0	+	nt	+	+	+	0	0	+	nt	nt	[0]								
Clade I	PNp^+	Jp-14	0	0	0	0	0	+	+	+	+	+	0	0	+	+	+	0	[0]							
Clade I	PNp^-	NZ-49	0	0	nt	0	0	nt	nt	nt	nt	0	nt	nt	nt	nt	nt	nt	nt	[0]						
Clade I	PNp^-	NZ-67	0	0	0	0	0	0	0	0	0	0	+	+	0	0	0	+	+	0	[0]					
Clade I	PNp^+	NZ-74	0	0	0	0	0	+	+	+	+	+	0	0	+	+	+	0	0	nt	+	[0]				
Clade I	PNp^-	Vigo-1	0	0	0	0	0	0	0	0	0	0	+	+	0	0	0	+	+	0	0	+	[0]			
Clade I	PNp^-	Vigo-4	0	0	0	0	0	0	nt	nt	nt	nt	+	+	nt	nt	nt	nt	nt	0	0	nt	0	[0]		
Clade I	PNp^+	Vigo-2	0	0	0	0	0	+	+	+	+	+	0	0	+	+	+	0	0	+	+	0	+	+	[0]	
Clade I	PNp^+	Vigo-3	0	0	0	0	0	+	nt	nt	nt	nt	0	0	nt	nt	nt	nt	nt	+	+	nt	+	+	0	[0]
Clade I	PNp^-	1	0	0	0	0	0	0	0	0	0	0	+	+	0	0	0	+	+	0	0	+	0	0	+	+
Clade I	PNp^-	19	0	0	0	0	0	0	0	0	0	0	+	nt	0	0	0	+	+	nt	0	+	0	0	+	+
Clade I	PNp^+	2	0	0	0	0	0	+	+	+	+	+	0	0	+	+	+	0	0	+	+	0	+	+	0	0
Clade I	PNp^+	6	0	0	0	0	0	+	+	+	+	+	0	nt	+	+	+	0	0	nt	+	0	+	+	0	0

+, Sexual reproduction occurred in mixed culture; 0, sexual reproduction absent in mixed culture; [0], sexual reproduction absent in monoclonal culture; nt, cross not made; ns, no sequence available. Shaded cells show successful crosses between clade I and clade II clones.

these three geographical groups (Tables 3 and 4). These clones also proved to be sexually compatible with the reference clones from the North Sea (Tables 3 and 4; supplementary Table S1 and Fig. S1). The characteristics of sexual reproduction (which involved physiological anisogamy) were identical to those that were reported previously (Chepurnov et al., 2005).

Among 16 clones collected in the NW Atlantic (Canada), 14 were capable of sexual reproduction. Each exhibited compatibility with the clones of corresponding mating type from this region and geographically distant locations (Table 3 and S1). Sex could not be initiated in two Canadian strains, Cn-181 and Cn-216, which were at opposite ends of the size spectrum. When used in mating experiments, cells of Cn-216, were 122–129 μm long and hence above the upper sexual threshold (~115 μm) for *P. pungens* clones from the North Sea. Intriguingly, however, two other large-celled Canadian clones, Cn-202 and Cn-215, were readily capable of sexual reproduction when crossed with various clones of opposite mating type, even though they were 118–124 and 126–131 μm long, respectively. The other sexually recalcitrant clone, Cn-181, had abnormally curved, small cells (only 36–56 μm long) and multiplied very slowly (at least twice as slowly as clones with larger cells). Furthermore, Cn-181 cells were typically solitary, only rarely forming the stepped colonies characteristic of *Pseudo-nitzschia* species and then of only a few cells.

All of the above clones, from the seas bordering Atlantic Canada, Europe, Japan and New Zealand, belonged to molecular clade I. Clade II clones were present only among the NE Pacific clones from Washington State, USA, and breeding tests involving 12 clones from this area gave more 'irregular' and ambiguous results, compared to those among isolates

from the other five regions. Three clones belonging to clade II (US-123, US-132 and US-135), plus US-134, for which we have no molecular data, did not show any signs of sexualization in any of the crosses tried (Table 4 and S1), although all four were well below the 115-μm sexual threshold determined for *P. pungens* from the North Sea. Another clade II clone that we had expected to become sexual – US-115 (89–90 μm) – became sexual only once, when mated with clone US-93. The remaining clones examined, comprising US-77 and US-93 (clade II), US-94 and US-96 (clade I), and US-125, US-126, and US-136 (all three of unknown ribotype), were crossed successfully with each other, regardless of the clade attribution. They could also mate with reference cultures from the North Sea and clones from other distant geographic areas (Table 4 and S1).

In all mixed cultures where sexual reproduction occurred, auxospores successfully developed into viable initial cells, regardless of whether the clones originated from the same or different regions. The F1 cells were capable of further vegetative divisions, without any visible signs of growth impairment.

3.4. Morphology

To assess possible morphological differentiation between the clades, the frustule morphology of 11 global strains from clades I (7) and II (4) was investigated in detail using LM and SEM. For the third clade, we had no access to material for morphological analysis. Morphometric measurements made with LM (Table 5) showed that, although length did not differ between clades I and II (*t*-test, $p = 0.15$), there were some subtle but significant differences in width ($p = 0.005$) and fibula density ($p = 0.004$). There may also be a trend towards a higher stria density in clade II ($p = 0.09$). Under SEM, the differences between the clades were more pronounced (Fig. 4). All three girdle bands (copulae) of clade I cells (Fig. 4A and B) had one row of simple large oval poroids (Fig. 4A and B, see also Fig. 6 in Chepurnov et al., 2005). In clade II clones, the poroids of the valvocopulae were typically square or rectangular and were always occluded by rotae (usually consisting of four radial bars per poroid) (Fig. 4C). The valvar striae of strains of both clades were composed of two rows of poroids and sometimes included a third incipient row. However, the poroid density in 1 μm (measured for two strains of clade II and four strains of clade I) was significantly higher ($p \ll 0.01$) in clones of clade II (Table 5; Fig. 4D) than in clade I (Fig. 4B).

Table 5
Morphometric measurements (dimensions and stria, fibula and poroid densities) of selected clones

Clone	Clade	Apical length (μm)	Width (μm)	Striae (in 10 μm)	Fibulae (in 10 μm)	Poroids[a] (in 1 μm)
US-132 ($n = 10$)	Clade II	108.2–110.8 (109.4 ± 0.74)	3.4–4.2 (3.8 ± 0.27)	10–13 (11.6 ± 0.88)	11–15 (12.8 ± 1.40)	4.0–5.0 (4.34 ± 0.47)
US-115 ($n = 10$)	Clade II	87.9–91.1 (89.9 ± 1.02)	3.8–4.4 (4.1 ± 0.22)	11–13 (11.75 ± 0.68)	11.5–14 (12.5 ± 1.03)	3.0–5.0 (3.96 ± 0.46)
US-123 ($n = 11$)	Clade II	98.6–102.4 (100.2 ± 1.21)	3.5–4.7 (3.9 ± 0.28)	10–12 (11.2 ± 0.68)	11–15 (12.5 ± 1.29)	
US-135 ($n = 10$)	Clade II	103.5–108.7 (106.7 ± 1.35)	3.6–4.2 (3.9 ± 0.19)	11–13 (12.0 ± 0.69)	11.5–14 (12.9 ± 0.82)	
US-94 ($n = 10$)	Clade I	83.9–93.1 (88.3 ± 3.76)	3.2–3.8 (3.5 ± 0.22)	10.5–12 (11.3 ± 0.49)	11–14 (11.7 ± 0.97)	2.0–4.0 (2.89 ± 0.54)
NZ-67 ($n = 10$)	Clade I	68.2–75.3 (72.7 ± 2.97)	2.4–3.2 (2.7 ± 0.28)	10.5–12 (11.2 ± 0.63)	11–13 (11.9 ± 0.52)	2.0–4.0 (3.0 ± 0.52)
NZ-49 ($n = 10$)	Clade I	24.4–28.8 (27.4 ± 1.27)	2.8–3.7 (3.2 ± 0.24)	9–12 (10.7 ± 1.16)	10–14 (12.0 ± 1.25)	
Vigo-2 ($n = 10$)	Clade I	77.6–78.8 (78.2 ± 0.35)	2.6–2.9 (2.7 ± 0.14)	10–11 (10.8 ± 0.42)	10.5–12 (11.4 ± 0.57)	3.0–4.0 (3.3 ± 0.38)
Vigo-3 ($n = 10$)	Clade I	84.7–86.7 (85.8 ± 0.69)	2.8–3.5 (3.3 ± 0.19)	10–11.5 (10.7 ± 0.54)	10–14 (12.1 ± 1.21)	
Vigo-4 ($n = 10$)	Clade I	81.5–83.3 (82.7 ± 0.56)	2.6–3.2 (3.0 ± 0.17)	11–12 (11.7 ± 0.47)	11–13 (11.9 ± 0.70)	
Cn-216 ($n = 10$)	Clade I	117.3–121.0 (118.8 ± 1.12)	3.4–4.2 (3.8 ± 0.25)	11–13 (11.8 ± 0.68)	11–14 (12.5 ± 0.85)	2.0–4.0 (2.98 ± 0.45)
Clade I (average ± S.D.)		79.1 ± 8.7	3.2 ± 0.4	11.1 ± 0.5	11.9 ± 0.3	3.0 ± 0.5
Clade II (average ± S.D.)		101.5 ± 27.2	3.9 ± 0.1	11.6 ± 0.3	12.7 ± 0.2	4.2 ± 0.5

Range (average ± S.D.).
[a] Fifty measurements per clone.

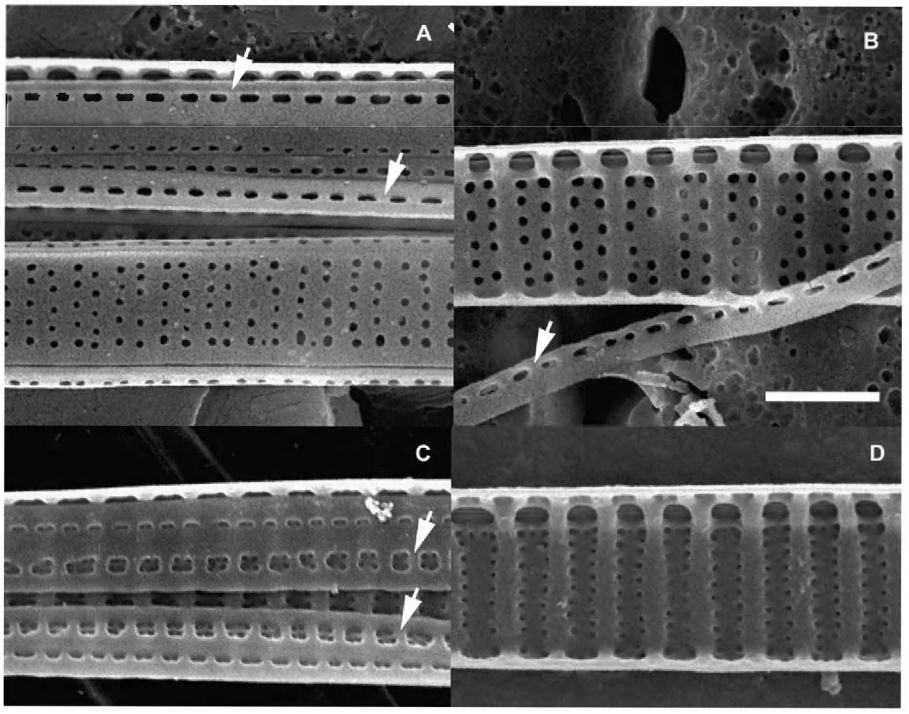

Fig. 4. *Pseudo-nitzschia pungens* frustules. SEM (valvocopulae are marked with an arrow). (A) Clade I (clone Vigo-2): valve face in external view (lower cell) and girdle bands (copulae) on internal valve face (upper cell). (B) Clade I (clone NZ-67): valve face in internal view and valvocopula. (C) Clade II (clone US-115): copulae lying on internal face of valve. (D) Clade II (clone US-132): valve face in internal view. Scale bar, 2 μm.

4. Discussion

4.1. Mating behavior

The extent of our crossing experiments probably exceeds that in any other diatom study to date. The data obtained were unambiguous in relation to heterothally and, in the vast majority of crosses, the dependence of sexual inducibility on cell size. However, a few cases were aberrant and require comment and further study. First, some clones did not reveal sexual activity when crossed with the reference clones of both mating types, even though the clones were below the 115-μm upper threshold for the sexually inducible size range of North Sea *P. pungens* (Chepurnov et al., 2005). These clones included several of clade II, but also some belonging to clade I. Some other clones, although clearly unisexual (we found no evidence in the present study for bisexual clones like those present in *Achnanthes*: Chepurnov and Mann, 1997), mated with only some of the clones of opposite mating type with which they were mixed: these included clones 14 and 28 (clade I) from the North Sea studied by Chepurnov et al. (2005) and clone US-115 in clade II (Table 3). Neither molecular nor morphological data suggested the existence of any other specific differences between these 'atypical' clones and others belonging to the same ITS clade and isolated from the same geographic locations.

Lack of sexualization where it was expected suggests that something essential is still missing in our knowledge of the genetic control or experimental initiation of sex in *P. pungens*. Indeed, there is remarkably little information on how the environment affects the induction of sexual reproduction in diatoms, once the cells are of the 'right' size and the partner of opposite sex is present (Chepurnov et al., 2004). Nor are there any data on the molecular genetic mechanisms underpinning sex in heterothallic pennate diatoms in general, and *Pseudo-nitzschia* in particular.

The behavior of clones Cn-202 and Cn-215 shows that the sexual size threshold, specified as the apical cell length (though it may be cell volume or the cell volume: nuclear volume ratio or some other parameter that is actually perceived by the cell), can vary among closely related diatoms. In these clones, sexualization occurred at lengths of 118–124 and 126–131 μm, respectively, whereas none of the clones from the North Sea has ever auxosporulated above the 115-μm threshold, even though numerous large-celled cultures have been checked in mating experiments (supplementary Table

S1). It is possible, therefore, that Canadian *P. pungens* are genetically differentiated from other clone I populations with respect to size range, despite sharing the same ribotype.

4.2. Variation in molecular, breeding and morphological data

Although ITS rDNA sequences have proven to be a valuable phylogenetic marker at the species level and below, their use has also been criticized (Alvarez and Wendel, 2003). The prevalent complication for phylogenies is the existence of extensive sequence variation, arising from ancient or recent array duplication events, genomic harboring of pseudogenes in various states of decay, and/or incomplete intra- or inter-array homogenization. These phenomena can create a network of paralogous sequence relationships that can potentially confound phylogenetic reconstruction. We tested six of our strains for intraclonal ITS variation. The degree of variation found was very low (maximum 0.7%), and most changes involved autapomorphic point mutations, except at a few sites where partially homogenized mutations were found. These point mutations may represent recently arisen mutations that the homogenization process has not yet excluded or promoted; the partially homogenized mutations are apparently in the process of homogenization (Dover et al., 1993). When the different paralogues of a strain were included in the phylogenetic analysis (Fig. 2), the sequence variation was too minor to affect the division in three separate clades. We therefore regard the ITS1–5.8S–ITS2 region as a useful tool to investigate relationships between *P. pungens* strains. In addition, our sequencing results never revealed any ambiguities through direct sequencing (see above). Homogenization of ribosomal cistrons seems sufficiently complete within *P. pungens* as to offer no hindrance to utilizing direct sequencing results.

Our data show that at least three distinct ITS entities exist within *P. pungens*. Mating tests demonstrated sexual compatibility among the strains of clade I, regardless of their geographic origin (North Sea region, Spain, Canada, Japan, New Zealand or the Pacific coast of North America) (Fig. 2). Some clones of clade II (US-93 and US-77) provided unambiguous evidence that clones from clades I and II are sexually compatible in the laboratory and can produce viable hybrid offspring. The molecular differences between these two ITS clades are correlated with slight but consistent morphological differences. At present, nothing can be said about morphology and mating behavior for clade III clones because only ITS data were available.

In *Pseudo-nitzschia*, differences between well-supported ITS clades, when accompanied by morphological differences, have been suggested to be of taxonomic significance at the species level and have led to the description of new species (Manhart et al., 1995; Lundholm et al., 2003, 2006). Mostly, sequence divergence between these clades was high (>10%). However, some studies of *Pseudo-nitzschia* have discriminated species on the basis of a much lower ITS sequence divergence. For example, Hasle and Lundholm (2005) raised *P. seriata* f. *obtusa* to species level (*P. obtusa*) with ITS divergence of only 6%, and the morphologically distinguishable *P. seriata* and *P. australis* have an ITS sequence divergence as low as 2.6% (Fehling et al., 2004; Hasle and Lundholm, 2005). In comparison, the divergence between *P. pungens* clades I and II (1.3%) is lower than the variation found between any currently accepted congeneric species, while the divergence between clade III and the rest of the in-group (2.7–4.4%) is comparable with the ITS separation between *P. seriata* and *P. australis*.

Although ITS sequence comparisons have been used several times to help delimit diatom species in cases where morphological differences are subtle or difficult to interpret (e.g. Zechman et al., 1994; Behnke et al., 2004; Beszteri et al., 2005; Godhe et al., 2006), including examples in *Pseudo-nitzschia* (see above), diatom studies that combine breeding tests with molecular analyses are still scarce (Mann, 1999; Behnke et al., 2004; Vanormelingen et al., 2007). In the freshwater diatoms *Sellaphora pupula* (Kützing) Mereschkowsky (Behnke et al., 2004) and *Eunotia bilunaris* (Ehrenberg) Mills (Vanormelingen et al., 2007), a correlation has been found between sexual compatibility and ITS sequence divergence. Organisms capable of interbreeding in the laboratory show low variation in ITS sequences (at most 7.3% in *S. pupula* and 4.3% in *E. bilunaris*), whereas in those that cannot interbreed, the ITS sequence divergence is much higher. To date, mating tests have been used alongside both morphological and molecular data in only one taxonomic study of *Pseudo-nitzschia*, of the *P. delicatissima* and *P. pseudodelicatissima* (Hasle) Hasle species complexes in the Gulf of Naples (Amato et al., 2007). In that study, only ITS2 divergence was a consistently good discriminator for reproductively isolated groups. The present study is the first for the genus that combines molecular approaches and multiple crossing experiments on a global scale.

A correlation between the evolution of ITS2 secondary structure and sexual compatibility has been shown for green algae of the order Volvocales and for the ciliate *Paramecium aurelia* Ehrenberg (e.g. Fabry et al., 1999; Coleman, 2000, 2005). These studies demonstrated that if two organisms mate successfully, they are found to have no CBCs or HCBCs, whereas the converse is not always true: some pairs of *Paramecium* syngens are reproductively isolated, despite having identical sequences (Coleman, 2005). The *P. delicatissima*-like and *P. pseudodelicatissima*-like strains from the Gulf of Naples fit this hypothesis (Amato et al., 2007): reproductively isolated strains belonged to different CBC-clades (group of organisms where there are no CBCs). This appears to be true also in *Sellaphora* where ITS2 sequences of the 'rectangular' and 'pseudocapitate' demes of *S. pupula* differ only in HCBCs and can mate in the laboratory (Behnke et al., 2004). In the present study, sexual compatibility was illustrated between clades I and II, and ITS2 secondary structure comparisons revealed no CBCs and HCBCs between these clades. On the other hand, a CBC and HCBC were detected between clades {I + II} and III. Thus, if Coleman's idea is correct, i.e. that CBCs act as a marker for the cessation of gene flow, we can predict that members of clade III will be reproductively isolated from clades I and II.

In the present study, the ITS difference between clades I and II was accompanied by morphological dissimilarities. The morphological features of strains belonging to clade II correspond to *P. pungens* var. *cingulata* Villac as described by Villac and Fryxell (1998) from Monterey Bay (California), in the NE Pacific, along the same coast (dominated by the California Current System) where the isolates of clade II were found. By contrast, clade I clones correspond to the morphology of the nominate form of *P. pungens*, as observed in previous studies (Hasle, 1995; Hasle and Syvertsen, 1996; Chepurnov et al., 2005). In addition, there may be differences between clades I and II in the upper sexual size threshold, or in the external factors that permit sexualization, judging by the surprising lack of sexual response in clones US-123, US-132, US-134, US-135 and US-115.

In the present study, no correlation was found between domoic acid production and ITS ribotype, either because no toxic strains (non-detectable for DA) were included in this study or because toxicity was not tested (for North Sea strains). If such a correlation were to be found, then the ITS differences could be used for development of molecular probes for monitoring of toxic *P. pungens* strains, because toxic clones of *P. pungens* have been reported from New Zealand (Rhodes et al., 1996), Washington State (Trainer et al., 1998) and Monterey Bay, California (Bates et al., 1998).

Taken together, our evidence suggests that clades I and II could represent slightly differentiated populations of the same species (consistent perhaps with Villac and Fryxell's use of the variety category). However, members of both clades occur sympatrically in Washington State waters. We found no signs of mixtures of ITS genotypes (after cloning to detect intra-isolate ITS variation), indicating no hybridization between clades (cf. Coleman, 2002), but the number of isolates studied is still too small to allow definite conclusions concerning gene flow in nature. There is apparently little or no intrinsic pre-zygotic isolation between the two ITS clades, but the existence of post-zygotic reproductive isolation (inability to produce an F2 or backcross to parental strains) cannot yet be excluded. Various forms of post-zygotic isolation have been demonstrated in other organisms, ranging from hybrid sterility to a decrease in fitness of hybrids to even non-viability of hybrids (Coyne and Orr, 2004).

Speciation in this group might be ongoing and very recent, so that pre-zygotic isolation is not yet complete. Populations can diverge genetically despite their potential for gene exchange. Studies in fungi have shown that genetic isolation always precedes morphological or reproductive isolation (Taylor et al., 2006). Even if there is no intrinsic barrier to hybridization, it is still possible that interbreeding between clades I and II is rare or absent in nature, even where they co-occur. Clades I and II strains are still able to recognize each other but assortative mating could account for the absence of hybrids in nature (Coyne and Orr, 2004). Another explanation could be that populations of the clades are temporally separated, blooming in different seasons while maintaining a low concentration throughout the remainder of the year, as shown for sympatric *P. delicatissima* ITS types in parts of the Mediterranean (Orsini et al., 2004). There could also be some ecological selection against hybrids in nature (Schluter, 2001). Amato et al. (2007) have suggested that small morphological differences may be ecologically relevant in *Pseudo-nitzschia* species and this could apply also in *P. pungens*. The significance of pseudocryptic variation is better understood in foraminifera, where it has been shown that morphologically very similar species can have quite different ecological requirements and geographical distributions, with significant implications for the use of these species in paleooceanographical reconstruction (e.g. Kucera and Darling, 2002).

4.3. Geographic distribution

Recently, the debate has been resurrected over whether most microorganisms are cosmopolitan, because their vegetative cells or propagules may have virtually unlimited dispersal potential (Finlay, 2002; Fenchel and Finlay, 2004). Traditionally, tentative judgments about whether a species is cosmopolitan or has a restricted geographic distribution have been based purely on morphological data (e.g. Hasle, 2002; Finlay, 2002). In *Pseudo-nitzschia*, most species have been reported worldwide, while others seem to be restricted to certain latitudinal zones, e.g. *P. seriata* only occurs in the north Atlantic and *P. obtusa* is restricted to Artic regions (Hasle, 2002; Hasle and Lundholm, 2005). With the introduction of molecular approaches, many morphologically defined 'cosmopolitan' protist species, including members of the genus *Pseudo-nitzschia*, are now known to be composed of multiple (semi- or pseudo-) cryptic species, whose distribution is unknown and may be restricted (Lundholm et al., 2003, 2006; Amato et al., 2007).

Like most *Pseudo-nitzschia* species, the morphologically distinct entity currently known as '*Pseudo-nitzschia pungens*' has been reported worldwide (Hasle, 2002). However, the three ITS clades within *P. pungens* have different geographic distributions. Clade III strains have hitherto been isolated only from warm waters (annual mean sea surface temperatures ~25–30 °C), whereas the other two *P. pungens* clades were all derived from more temperate waters (annual mean sea surface temperatures ~10–20 °C). The second ITS clade has been found so far only in the NE Pacific, whereas clade I was found in the North Sea, the eastern and western N Atlantic, and the NW, NE and S Pacific. While we have insufficient data to make any conclusive statements about the biogeography of clades II and III, the data for clade I are highly significant. Clade I is a lineage with a very broad oceanic distribution, perhaps with no barriers to global dispersal. In addition, crossing experiments demonstrated sexual compatibility among the strains of clade I, regardless of their geographic origin: this is important because uniformity in ITS regions does not always correspond to reproductive compatibility (e.g. *Paramecium*; Coleman, 2005). Clade I is therefore widespread within temperate zones in both hemispheres and supplies the most conclusive evidence so far for the global distribution of a biologically, genetically and morphologically defined diatom species.

It is yet unclear if this distribution is natural or anthropogenic. A few other HAB taxa found globally have also been reported with very low intraspecific genetic diversity in the ITS region, e.g. the raphidophytes *Heterosigma akashiwo* and *Fibrocapsa japonica* (Connell, 2000; Kooistra et al., 2001). In these, the apparent lack of genetic differentiation has been suggested to reflect geologically recent spreading between oceanic regions, possibly mediated by humans. Viable cells of *Pseudo-nitzschia* species, and of *P. pungens* in particular, have been found in ballast water samples (Gollasch et al., 2000; Rhodes et al., 1998) and so it is possible that *P. pungens* could be spread around the world through shipping. The co-occurrence of clades I and II in the NE Pacific could therefore reflect recent breakdown of a previously effective geographical separation by land (Pacific–Atlantic) and sea (NW–NE Pacific) barriers. To test this further, genetic differentiation between widely separated populations needs to be explored with other genetic markers that evolve faster than ITS. Six microsatellite markers developed for *P. pungens* by Evans et al. (2005) revealed weak genetic differentiation over the temporal and spatial scales sampled (18 months and 100 km) and suggested that *P. pungens* in the German part of the North Sea exists as a single, largely unstructured population. We are now using such markers to explore the genetic differentiation among widely separated *P. pungens* populations and to discover possible dispersion routes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.hal.2007.08.004.

References

Agapow, P.M., Bininda-Emonds, O.R.P., Crandall, K.A., Gittleman, J.L., Mace, G.M., Marshall, J.C., Purvis, A., 2004. The impact of species concept on biodiversity studies. Q. Rev. Biol. 79, 161–179.

Amato, A., Kooistra, W.H.C.F., Levialdi Ghiron, J.H., Mann, D.G., Pröschold, T., Montresor, M., 2007. Reproductive isolation among sympatric cryptic species in marine diatoms. Protist 158, 193–207.

Alvarez, I., Wendel, J.F., 2003. Ribosomal ITS sequences and plant phylogenetic inference. Mol. Phylogenet. Evol. 29, 417–434.

Bates, S.S., 2000. Domoic-acid-producing diatoms: another genus added! J. Phycol. 36, 978–983.

Bates, S.S., Garrison, D.L., Horner, R.A., 1998. Bloom dynamics and physiology of domoic-acid-producing Pseudo-nitzschia species. In: Anderson, D.M., Cembella, A.D., Hallegraeff, G.M. (Eds.), Physiological Ecology of Harmful Algal Blooms. Springer-Verlag, Heidelberg, pp. 267–292.

Bates, S.S., Trainer, V.L., 2006. The ecology of harmful diatoms. In: Granéli, E., Turner, J. (Eds.), Ecology of Harmful Algae. Springer-Verlag, Heidelberg, pp. 81–93.

Behnke, A., Friedl, T., Chepurnov, V.A., Mann, D.G., 2004. Reproductive compatibility and rDNA sequence analyses in the *Sellaphora pupula* species complex (Bacillariophyta). J. Phycol. 40, 193–208.

Beszteri, B., Ács, É., Medlin, L.K., 2005. Ribosomal DNA sequence variation among sympatric strains of the *Cyclotella meneghiniana* complex (Bacillariophyceae) reveals cryptic diversity. Protist 156, 317–333.

Cangelosi, G.A., Hamlin, A.M., Marin III, R., Scholin, C.A., 1997. Detection of stable pre-rRNA in toxigenic *Pseudo-nitzschia* species. Appl. Environ. Microbiol. 63, 4859–4865.

Chepurnov, V.A., Mann, D.G., 1997. Variation in the sexual behavior of natural clones of *Achnanthes longipes*. Eur. J. Phycol. 32, 147–154.

Chepurnov, V.A., Mann, D.G., Sabbe, K., Vannerum, K., Casteleyn, G., Verleyen, E., Peperzak, L., Vyverman, W., 2005. Sexual reproduction, mating system, chloroplast dynamics and abrupt cell size reduction in *Pseudo-nitzschia pungens* from the North Sea (Bacillariophyta). Eur. J. Phycol. 40, 379–395.

Chepurnov, V.A., Mann, D.G., Sabbe, K., Vyverman, W., 2004. Experimental studies on sexual reproduction in diatoms. Int. Rev. Cytol. 237, 91–154.

Coleman, A.W., 2000. The significance of a coincidence between evolutionary landmarks found in mating affinity and a DNA sequence. Protist 151, 1–9.

Coleman, A.W., 2002. Comparison of *Eudorina/Pleodorina* ITS sequences of isolates from nature with those from experimental hybrids. Am. J. Bot. 89, 1523–1530.

Coleman, A.W., 2005. *Paramecium aurelia* revisited. J. Eukaryot. Microbiol. 52, 68–77.

Connell, L.B., 2000. Nuclear ITS region of the alga *Heterosigma akashiwo* (Chromophyta; Raphidophyceae) is identical in isolates from Atlantic and Pacific basins. Mar. Biol. 136, 953–960.

Coyne, J.A., Orr, H.A., 2004. Speciation. Sinauer Associates, Inc., Sunderland, MA, USA, pp. 545.

Davidovich, N.A., Bates, S.S., 1998. Sexual reproduction in the pennate diatoms *Pseudo-nitzschia multiseries* and *P. pseudodelicatissima* (Bacillariophyceae). J. Phycol. 34, 126–137.

De Rijk, P., De Wachter, R., 1993. DCSE, an interactive tool for sequence alignment and secondary structure research. Comput. Appl. Biosci. 9, 735–740.

De Rijk, P., Wuyts, J., De Wachter, R., 2003. RnaViz2: an improved representation of RNA secondary structure. Bioinformatics 19, 299–300.

Dettman, J.R., Jacobson, D.J., Turner, E., Pringle, A., Taylor, J.W., 2003. Reproductive isolation and phylogenetic divergence in *Neurospora*: comparing methods of species recognition in a model eukaryote. Evolution 57, 2721–2741.

Dover, G.A., Linares, A.R., Bowen, T., Hancock, J.M., 1993. Detection and quantification of concerted evolution and molecular drive. Methods Enzymol. 224, 525–541.

Drebes, G., 1977. Sexuality. In: Werner, D. (Ed.), The Biology of Diatoms. Blackwell, Oxford, pp. 250–283.

Evans, K.M., Kuhn, S.F., Hayes, P.K., 2005. High levels of genetic diversity and low levels of genetic differentiation in North Sea *Pseudo-nitzschia pungens* (Bacillariophyceae) populations. J. Phycol. 41, 506–514.

Fabry, S., Kohler, A., Coleman, A.W., 1999. Intraspecies analysis: comparison of ITS sequence data and gene intron sequence data with breeding data for a worldwide collection of *Gonium pectorale*. J. Mol. Evol. 48, 94–101.

Fehling, J., Green, D.H., Davidson, K., Bolch, C.J., Bates, S.S., 2004. Domoic acid production by *Pseudo-nitzschia seriata* (Bacillariophyceae) in Scottish waters. J. Phycol. 40, 622–630.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39, 783–791.

Fenchel, T., Finlay, B.J., 2004. The ubiquity of small species: patterns of local and global diversity. Bioscience 54, 777–784.

Finlay, B.J., 2002. Global dispersal of free-living microbial eukaryote species. Science 296, 1061–1063.

Friedl, T., 1996. Evolution of the polyphyletic genus *Pleurastrum* (Chlorophyta): inferences from nuclear-encoded ribosomal DNA sequences and motile cell ultrastructure. Phycologia 35, 456–469.

Geitler, L., 1932. Der Formwechsel der pennaten Diatomeen. Arch. Protistenk. 78, 1–226.

Godhe, A., McQuoid, M.R., Karunasagar, I., Karunasagar, I., Rehnstam-Holm, A.S., 2006. Comparison of three common molecular tools for distinguishing among geographically separated clones of the diatom *Skeletonema marinoi* Sarno et Zingone (Bacillariophyceae). J. Phycol. 42, 280–291.

Gollasch, S., Lenz, J., Dammer, M., Andres, H.G., 2000. Survival of tropical ballast water organisms during a cruise from the Indian Ocean to the North Sea. J. Plankton Res. 22, 923–937.

Guillard, R.R.L., 1975. Culture of phytoplankton for feeding marine invertebrates. In: Smith, W.L., Chanley, M.H. (Eds.), Culture of Marine Invertebrate Animals. Plenum, New York, pp. 29–60.

Hasegawa, M., Kishino, H., Yano, T.A., 1985. Dating of the human ape splitting by a molecular clock of mitochondrial DNA. J. Mol. Evol. 22, 160–174.

Hasle, G.R., 1995. *Pseudo-nitzschia pungens* and *P. multiseries* (Bacillariophyceae)-nomenclatural history, morphology and distribution. J. Phycol. 31, 428–435.

Hasle, G.R., 2002. Are most of the domoic acid-producing species of the diatom genus *Pseudo-nitzschia* cosmopolites? Harmful Algae 1, 137–146.

Hasle, G.R., Syvertsen, E.E., 1996. Marine diatoms. In: Tomas, C.R. (Ed.), Identifying Marine Diatoms and Dinoflagellates. Academic Press, San Diego, pp. 5–385.

Hasle, G.R., Lundholm, N., 2005. *Pseudo-nitzschia seriata* f. *obtusa* (Bacillariophyceae) raised in rank based on morphological, phylogenetic and distributional data. Phycologia 44, 608–619.

Hillis, D.M., Huelsenbeck, J.P., 1992. Signal, noise, and reliability in molecular phylogenetic analyses. J. Hered. 83, 189–195.

Kooistra, W.H.C.F., de Boer, M.K., Vrieling, E.G., Connell, L.B., Gieskes, W.W.C., 2001. Variation along ITS markers across strains of *Fibrocapsa japonica* (Raphidophyceae) suggests hybridisation events and recent range expansion. J. Sea Res. 46, 213–222.

Kucera, M., Darling, K.F., 2002. Cryptic species of planktonic foraminifera: their effect on palaeoceanographic reconstructions. Philos. Trans. R. Soc. Lond. A 360, 695–719.

Lundholm, N., Moestrup, Ø., Hasle, G.R., Hoef-Emden, K., 2003. A study of the *Pseudo-nitzschia pseudodelicatissima/cuspidata* complex (Bacillariophyceae): what is *P. pseudodelicatissima*? J. Phycol. 39, 797–813.

Lundholm, N., Moestrup, Ø., Kotaki, Y., Hoef-Emden, K., Scholin, C., Miller, P., 2006. Inter- and intraspecific variation of the *Pseudo-nitzschia delicatissima* complex (Bacillariophyceae) illustrated by rRNA probes, morphological data and phylogenetic analyses. J. Phycol. 42, 464–481.

Mai, J.C., Coleman, A.W., 1997. The Internal Transcribed Spacer 2 exhibits a common secondary structure in green algae and flowering Plants. J. Mol. Evol. 44, 258–271.

Manhart, J.R., Fryxell, G.A., Villac, C., Segura, L.Y., 1995. *Pseudo-nitzschia pungens* and *P. multiseries* (Bacillariophyceae): nuclear ribosomal DNAs and species differences. J. Phycol. 31, 421–427.

Mann, D.G., 1999. The species concept in diatoms (Phycological Reviews 18). Phycologia 38, 437–495.

Orsini, L., Procaccini, G., Sarno, D., Montresor, M., 2004. Multiple rDNA ITS-types within the diatom *Pseudo-nitzschia delicatissima* (Bacillariophyceae) and their relative abundances across a spring bloom in the Gulf of Naples. Mar. Ecol. Prog. Ser. 271, 87–98.

Posada, D., Crandall, K.A., 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics 14, 817–818.

Rhodes, L.L., White, D., Syhre, M., Atkinson, M., 1996. *Pseudo-nitzschia* species isolated from New Zealand coastal waters: domoic acid production in vitro and links with shellfish toxicity. In: Yasumoto, T., Oshima, Y., Fukuyo, Y. (Eds.), Harmful and Toxic Algal Blooms. Intergov. Oceanogr. Comm. UNESCO, Paris, pp. 155–158.

Rhodes, L.L., Scholin, C., Garthwaite, I., Haywood, A., Thomas, A., 1998. Domoic acid producing *Pseudo-nitzschia* species educed by whole cell DNA probe-based and immunochemical assays. In: Reguera, B., Blanco, J., Fernández, M.L., Wyatt, T. (Eds.), Harmful Algae. Xunta de Galicia and IOC of UNESCO, pp. 274–277.

Schluter, D., 2001. Ecology and the origin of species. Trends Ecol. Evol. 16, 372–380.

Swofford, D.L., 2002. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.

Taylor, J.W., Turner, E., Townsend, J.P., Dettman, J.R., Jacobson, D., 2006. Eukaryotic microbes, species recognition and the geographic limits of species: examples from the kingdom Fungi. Philos. Trans. Soc. B 361, 1947–1963.

Trainer, V.L., Wekell, J.C., Horner, R.A., Hatfield, C.L., Stein, J.E., 1998. Domoic acid production by *Pseudo-nitzschia* pungens. In: Reguera, B., Blanco, J., Fernández, M.L., Wyatt, T. (Eds.), Harmful Algae. Xunta de Galicia and the IOC of UNESCO, Paris, pp. 337–340.

Vanormelingen, P., Chepurnov, V.A., Mann, D.G., Cousin, S., Sabbe, K., Vyverman, W., 2007. Congruence of morphological, reproductive and ITS rDNA sequence data in some Australasian *Eunotia bilunaris*. Eur. J. Phycol. 41, 61–79.

Villac, M.C., Fryxell, G.A., 1998. *Pseudo-nitzschia pungens* var. *cingulata* var. nov. (Bacillariophyceae) based on field and culture observations. Phycologia 37, 269–274.

Vrieling, E.G., Koeman, R.P., Scholin, C.A., Scheerman, P., Peperzak, L., Veenhuis, M., Gieskes, W.W.C., 1996. Identification of a domoic acid-producing *Pseudo-nitzschia* species (Bacillariophyceae) in the Dutch Wadden Sea with electron microscopy and molecular probes. Eur. J. Phycol. 31, 333–340.

White, T.J., Bruns, T., Lee, S., Taylor, J.W., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innes, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (Eds.), PCR Protocols: A Guide to Methods and Applications. Academic Press, New York, pp. 315–322.

Zechman, F.W., Zimmer, E.A., Theriot, E.C., 1994. Use of ribosomal DNA internal transcribed spacers for phylogenetic studies in diatoms. J. Phycol. 30, 507–512.

Zuker, M., 2003. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. 31, 3406–3415.

Zwart, G., Huismans, R., van Agterveld, M.P., Van de Peer, Y., De Rijk, P., Eenhoorn, H., Muyzer, G., van Hannen, E.J., Gons, H.J., Laanbroek, H.J., 1998. Divergent members of the bacterial division Verrucomicrobiales in a temperate freshwater lake. FEMS Microbiol. Ecol. 25, 159–169.