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MICROSATELLITE MARKER DEVELOPMENT AND GENETIC VARIATION IN THE TOXIC MARINE DIATOM *PSEUDO-NITZSCHIA MULTISERIES* (BACILLARIOPHYCEAE)[1]

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The genetic structure of phytoplankton populations is largely unknown. In this study we developed nine polymorphic microsatellite markers for the domoic acid–producing marine diatom *Pseudo-nitzschia multiseries* (Hasle) Hasle. We then used them in the genotyping of 25 physiologically diverse field isolates and six of their descendants: 22 field isolates originated from eastern Canadian waters, two from European waters, and one from Russian waters. The numbers of alleles per locus ranged from three to seven and the observed heterozygosities from 0.39 to 0.70. A substantial degree of genetic variation was observed within the field isolates, with 23 different genotypes detected. The Russian isolate was the most genetically distinct, although there was also evidence of genetic differentiation at a more local scale. Mating experiments demonstrated that alleles were inherited in a Mendelian manner. *Pseudo-nitzschia multiseries* primer pairs were tested on DNA from four congeners: *P. calliantha* Lundholm, Moestrup et Hasle; *P. fraudulenta* (P. T. Cleve) Hasle; *P. pungens* (Grunow *ex* P. T. Cleve) Hasle; and *P. seriata* (P. T. Cleve) H. Peragallo. Cross-reactivity was only observed in *P. pungens*. Our results are a first step in understanding the genetic variation present at the *Pseudo-nitzschia* "species" level and in determining the true biogeographic extent of *Pseudo-nitzschia* species.

Key index words: **algae; diatoms; domoic acid; genetic diversity; genetic variation; microsatellites; population genetics; *Pseudo-nitzschia*; sexual reproduction**

Abbreviation: **DA, domoic acid**

Harmful algal blooms can cause severe economic losses to aquaculture, fishery, and tourism industries and pose a significant risk to human health (Hallegraeff 2003). Diatoms in the genus *Pseudo-nitzschia* H. Peragallo are one of the main groups of toxin-producing algal species. Members of this pennate diatom genus are a common component of the marine phytoplankton worldwide, occurring in polar, temperate, subtropical, and tropical waters (Hasle 2002). The first documented case of toxicity within this genus was in 1987, when a bloom of *P. multiseries* (Hasle) Hasle caused an amnesic shellfish poisoning event, which led to the deaths of three people and over 100 illnesses in Canada (Bates et al. 1989). Subsequent investigations found that about a third of the 30 or so *Pseudo-nitzschia* species so far described are producers of domoic acid (DA), the neurotoxin implicated in such toxic blooms (Lundholm et al. 1994, 2003, Bates et al. 1998, Bates 2000). Because of their potential adverse impacts, the occurrence of these species in coastal waters has been surveyed and subsequently monitored in many parts of the world (Hallegraeff 1994, Hernández-Becerril 1998, Parsons et al. 1999, Trainer et al. 2000, Cho et al. 2002).

Despite such intense interest, the distributions of *Pseudo-nitzschia* species, and in some cases their taxonomy, is far from resolved (Hasle 2002, Lundholm et al. 2003, Fehling et al. 2004, Kaczmarska et al. 2004). This is partly because of the close morphological similarity between many species and the fact that both nontoxic and toxic strains are present within the same morphospecies, for example, *P. pungens* (Grunow *ex* P. T. Cleve) Hasle; *P. seriata* (P. T. Cleve) H. Peragallo; *P. delicatissima* (P. T. Cleve) Heiden; *P. fraudulenta* (P. T. Cleve) Hasle; *P. pseudodelicatissima* (Hasle) Hasle emend. Lundholm, Hasle et Moestrup; and *P. calliantha* Lundholm, Moestrup et Hasle (Bates et al. 1989, 1998, Lundholm et al. 1994, 2003, Orsini et al. 2004). Molecular species-specific probes have been developed

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for several *Pseudo-nitzschia* species to rapidly identify them for early detection (Miller and Scholin 1996, 1998, 2000, Scholin et al. 1996, 1999, Medlin and Eller, unpublished data). However, because of the high levels of genetic diversity present within certain species and because the original probes were designed from sequences of isolates collected over a limited geographic area, they have not always been sufficiently specific when applied elsewhere (Parsons et al. 1999, Orsini et al. 2002). If a trustworthy reliable taxonomy for this genus is to be achieved, then molecular studies must be conducted to investigate the degree of genetic variation present at the "species" level; only then will a clear idea of their true biogeographic extent be known and an understanding of their population dynamics be achieved.

We have developed microsatellite markers for *Pseudo-nitzschia* species and are now in a position to quantify genetic diversity and to determine its distribution at different spatial and temporal scales. We chose two *Pseudo-nitzschia* species, the toxic *P. multiseries* and its closest congener, the generally nontoxic *P. pungens* (Rhodes et al. 1996, Trainer et al. 1998). Because of their morphological similarity, it was not until 1995 that *P. multiseries* was given species status (Hasle 1995); previously it was described as a form of *P. pungens*. This change in taxonomic status was based on detailed morphological measurements of its silica frustule, physiological differences related to toxin production, immunofluorescence reactions, and rDNA sequence divergence (Bates et al. 1993, Douglas et al. 1994, Hasle 1995, Manhart et al. 1995). The development of microsatellite loci in *P. pungens* has been described elsewhere (Evans and Hayes 2004). Here we report the development of nine polymorphic microsatellite markers for *P. multiseries* and their subsequent use in a small-scale investigation of genetic diversity and patterns of inheritance in a group of physiologically diverse field isolates and their descendants.

MATERIALS AND METHODS

Pseudo-nitzschia *spp. isolates*. A total of 31 *P. multiseries* clonal isolates was used in this study; 25 were isolated from the field (Table 1) and a further six were representatives of the F1 generation of two field isolates (see below). The field isolates were collected from various locations between 1993 and 2002. Of these 25 isolates, 22 were collected from seven different locations on the east coast of Canada (CCMP1660 and isolates labeled "CL"; Fig. 1). Two isolates were collected from European waters: one from the North Sea, off the German west coast (Sylt), and the other from the Baltic Sea, off the Danish east coast (Orø13). The one isolate from Russian waters (PM-02) was from the Sea of Japan, near Vladivostok, Russia.

Canadian field isolates CL-45 and CL-47 were mated, using the method of Davidovich and Bates (1998), to obtain the six F1 generation clones included in this study. In brief, 0.45 mL of each of the CL-45 and CL-47 parent clones taken from exponentially growing cultures (day 4) was mixed in borosilicate Petri dishes (5 cm diameter) containing 15 mL of f/2 medium plus 107 μM silica (Guillard 1975). After 2–3 days, gametes were produced, which combined to form auxospores within which large initial cells were created. Single large cells or single chains of cells from these initial cells were isolated by micropipette and placed in 5 cm-diameter Petri dishes containing f/2 + Si medium. Viable F1 cultures (labeled "CLN") were transferred into 50 mL f/2 + Si and grown, as were all the other isolates, at 20° C and at an incident irradiance of 100 μmol photons $\cdot m^{-2} \cdot s^{-1}$ (12:12-h light:dark cycle). Other *Pseudo-nitzschia* spp. clonal isolates used to assess the cross-reactivity of microsatellite primers (see below) are shown in Table 2; these clones were isolated from either the North Sea or from eastern Canadian waters.

Identification of the isolates. The identification of all Canadian, Danish, German, and Russian *Pseudo-nitzschia* spp. isolates was confirmed by SEM (Kaczmarska et al. 2004) using the species descriptions of Hasle and Syvertsen (1996) and Lundholm et al. (2003).

Microsatellite development. *Pseudo-nitzschia* isolates were grown in batch culture for approximately three weeks and harvested by centrifugation at 10,000 *g* for 20 min; the pellets were frozen at −70° C. Initially, genomic DNA was extracted from the North Sea *P. multiseries* isolate (Sylt) following the protocol described in Rynearson and Armbrust (2000). Extracted DNA from this single isolate was enriched for CA and CT repeats following a protocol based on Edwards et al. (1996) and used to generate a microsatellite-enriched library. In brief, the DNA was digested with *Alu* I or *Rsa* I, ligated to a *Mlu* I adapter, amplified using one of the adapter oligonucleotides, and hybridized to CA and CT dinucleotide repeat oligonucleotides immobilized on Hybond N^+ filter paper (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). The enriched microsatellites were reamplified as above and cloned into pJV1 vector (Edwards et al. 1996). Cloned DNA fragments were amplified using M13 forward and reverse primers. Sequencing was performed using an Amersham Biosciences sequencing kit and carried out on a MegaBACE 1000 automated sequencer (Amersham Biosciences). Sequences containing suitable microsatellites were exported to the PRIMER3 software package (Rozen and Skaletsky 2000) for primer design. Primer pairs were tested for their ability to amplify the corresponding microsatellite loci using a PTC-200 gradient thermal cycler (MJ Research, Waltham, MA, USA). The 20-μL reactions contained 100–400 ng of template DNA, 10 mM Tris-HCl, 50 mM KCl, 0.1% Triton X-100, 2 mM $MgCl_2$, 0.2 mM dNTPs, 75 ng of each primer, and 0.5 units of Taq polymerase (Promega, Southampton, UK). After 3 min at 94° C, 35 cycles were performed: 1 min at 94° C, 1 min at 48–65° C, 30 s at 72° C, and a final extension step at 72° C for 10 min. For the primer pairs that generated amplification products of the expected size, 5′ fluorescently labeled forward primers (6-FAM) were used in repeat amplification reactions with several *P. multiseries* isolates at the optimal annealing temperatures; the products were sized on a MegaBACE 1000.

Analysis of microsatellite data. Because of the limited number of field samples (25), their diverse geographic origins, and the time span over which they were collected (between 1993 and 2002), analysis of the microsatellite data was largely limited to determining the attributes of the markers we had developed, examining the overall intraspecific genetic variation present, and tracing the inheritance of alleles at each of the loci, from the two parental isolates through to the six representatives of the F1 generation. In addition, a small number of tests for genetic differentiation between isolates from different sites were conducted.

GENEPOP 3.3 (Raymond and Rousset 1995) was used to detect deviations from Hardy-Weinberg equilibrium at each locus (by comparing the observed number of heterozygotes, H_o, with the expected number of heterozygotes, H_e) and to test for linkage disequilibrium between pairs of loci. Pair-wise genic tests in GENEPOP 3.3 were used to test for significant differentiation between isolates from the ten different locations (seven Canadian sites, two European sites, and one Russian site).

TABLE 1. Geographic origins and genotypes (length of PCR amplicons [alleles] in base pairs) of the 25 clonal field isolates of *Pseudo-nitzschia multiseries*: 22 from eastern Canadian waters, 2 from European waters, and 1 from Russian waters.

			Genotype								
Isolate name	Geographic origin	Date collected	PM1	PM2	PM3	PM4	PM5	PM6	PM7	PM8	PM9
Sylt	Sylt, North Sea	October 2001			293/293		563/563				
Orø13	Orø, Baltic Sea	August 2001	190/205	434/434	293/293	425/425	549/563		381/381	293/312	242/242
PM-02	Vladivostok, Sea of Japan	1 November 2002	185/190	434/434	291/293	421/423	549/551	201/207	367/375	300/320	254/256
CCMP1660	Cardigan Bay, PEI	2 December 1993	185/185	434/434	293/293	419/425	549/549	209/209	377/377	306/306	242/344
CL-45	New London Bay, PEI	26 November 1996	187/195	434/434	293/297	425/425	549/549	209/209	377/377	291/293	242/242
CL-47	New London Bay, PEI	26 November 1996	185/185	434/441	297/315	419/425	549/549	209/209	377/377	291/306	242/246
CL-48	New London Bay, PEI	26 November 1996	185/195	434/441	293/297	419/425	549/549	209/211	377/381	293/312	246/246
CL-84	Cardigan Bay, PEI	28 October 1998	185/189	434/434	293/297	425/425	549/563	209/209	377/381	293/306	242/242
CL-90	Cardigan Bay, PEI	24 November 1998	185/195	434/441	293/297	419/425	563/563	209/209	377/377	306/312	242/246
CL-91	Cardigan Bay, PEI	24 November 1998	187/187	441/441	293/293	419/419	549/549	187/209	381/381	293/293	242/242
CL-93	Cardigan Bay, PEI	24 November 1998	185/195	434/441	293/293	419/425	549/549	209/209	377/377	291/306	242/246
CL-125	Mill River, PEI	23 September 2000		434/441	293/293		549/563		377/381		
CL-142	Little Harbour, NS	20 December 2001	201/201	434/434	293/293	425/425	549/563	209/209	381/381	293/312	242/242
CL-143	Little Harbour, NS	20 December 2001	185/189	434/441	293/315	419/425	549/549	209/211	381/381	306/306	242/246
CL-147	Caribou Harbour, NS	20 December 2001	195/195	434/441	293/293	419/425	549/563	209/211	377/381	293/306	246/344
CL-148	Caribou Harbour, NS	20 December 2001	185/189	434/434	297/297	425/425	549/563		377/381	306/312	242/246
CL-150	Caribou Harbour, NS	20 December 2001	185/185	434/441	293/297	419/425	549/549	209/209	377/377	293/306	242/242
CL-155	Caribou Harbour, NS	3 January 2002	185/185	441/441	293/315	419/419	549/549	209/211	377/381	306/306	246/246
CL-156	Caribou Harbour, NS	3 January 2002	201/201	434/434	293/293	425/425	549/563	209/209	381/381	293/312	242/242
CL-157	Caribou Harbour, NS	3 January 2002	185/195	441/441	293/297	419/419	549/563	211/211	377/381	293/306	242/246
CL-158	Caribou Harbour, NS	3 January 2002	187/189	434/434	293/293	425/425	549/549	209/211	377/381	306/312	242/246
CL-174	Cardigan Bay, PEI	5 September 2002	185/185	434/441	297/297	419/425	563/563	209/209	377/377	291/291	242/246
CL-191	Deadmans Harbour, Bay of Fundy, NB	9 October 2002	189/195	434/441	295/315	419/425	563/563	209/211	381/381	306/306	242/246
CL-195	Deadmans Harbour, Bay of Fundy, NB	9 October 2002	185/189	439/439	295/295	419/425	563/563	209/211	377/381	306/312	242/246
CL-197	Egg Island, Miramichi Bay, NB	28 October 2002	185/189	434/439	293/295	419/425	549/563	209/209	381/387	312/312	242/242

NB, New Brunswick; NS, Nova Scotia; PEI, Prince Edward Island. Blank cells indicate missing data.

FIG. 1. Map showing the location of the sites on the east coast of Canada from where the Canadian *Pseudo-nitzschia* spp. clones were isolated. 1, Oak Island (Miramichi Bay), NB; 2, Egg Island (Miramichi Bay), NB; 3, Baie-Ste.-Anne (Miramichi Bay), NB; 4, Mill River, PEI; 5, New London Bay, PEI; 6, Rustico Bay, PEI; 7, Tracadie Bay, PEI; 8, Cardigan Bay, PEI; 9, Caribou Harbour, NS; 10, Little Harbour, NS; 11, Deadmans Harbour (Bay of Fundy), NB. Heavy circles indicate the seven sites from where the 22 Canadian *P. multiseries* clones were isolated.

Cross-reactivity of primers. To assess the cross-reactivity of the primers in other *Pseudo-nitzschia* species, DNA of four congeners (*P. calliantha*, *P. fraudulenta*, *P. pungens*, and *P. seriata*; Table 2) was amplified with primers for the microsatellite loci identified in *P. multiseries*; generic eukaryote 18S-rDNA primers (EukF and EukR; DeLong 1992) were used to check that their DNA was amplifiable. In addition, four of the six primer pairs developed for *P. pungens* (Evans and Hayes 2004) were tested using DNA from four isolates of *P. multiseries* (CL-45, CL-47, CL-48, and CL-84).

Toxin analysis. DA concentrations in stationary-phase cultures (days 21–28) of 21 Canadian *P. multiseries* field isolates (excluding CCMP1660), six F1 generation clones, and the Russian isolate PM-02 grown in the above f/2 medium were determined using HPLC of the fluorenylmethoxycarbonyl derivative (Pocklington et al. 1990). Total DA was measured in whole-culture samples (cells plus medium) (Bates et al. 1991). Cells were first disrupted by sonication for 1 min, filtered through a 0.2-μm disposable acrodisc (25 mm surfactant-free cellulose acetate membrane, Nalgene, Rochester, NY, USA) to remove cell debris, and then frozen at -20° C before analysis.

RESULTS

Identification of the isolates. SEM confirmed that the identity of each of the 22 isolates from eastern Canadian waters, the North Sea Sylt isolate, the Baltic Sea Orø13 isolate, and the Russian PM-02 isolate was *P. multiseries*. Representative SEM images of two of these isolates (CL-158 and PM-02) are shown in Figure 2. The cells were characterized by an absence of a central interspace, three to four rows of poroids between the striae, a mean length of 107.1 ± 21.9 μm, a mean width of 3.7 ± 0.3 μm, 13–15 striae per 10 μm, 13–14 fibulae per 10 μm, and five to six poroids per μm, typical of the species (Hasle and Syvertsen 1996). The identity of all other *Pseudo-nitzschia* spp. (Table 2) was also confirmed by SEM (not shown).

Microsatellites in Pseudo-nitzschia multiseries. Of the initial 24 cloned DNA fragments sequenced from each of the CA- and CT-enriched libraries, the CA library yielded eight microsatellites, an enrichment of approximately 33%, and the CT library yielded none. Consequently, further sequencing was only performed on additional DNA fragments from the enriched CA library. In total, 191 cloned DNA fragments were sequenced, of which 61 (32%)

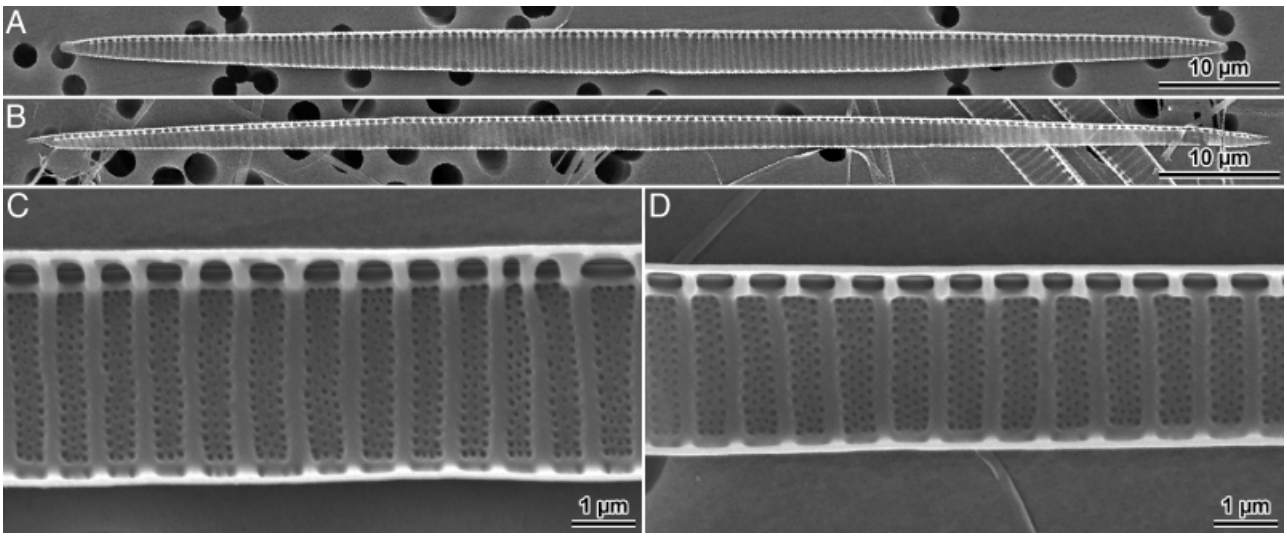

FIG. 2. SEM images of *P. multiseries* clones CL-158, isolated from Caribou Harbour, NS, Canada (A and C), and PM-02, isolated from offshore Vladivostok (Sea of Japan), Russia (B and D).

TABLE 2. Geographic origins of the clonal field isolates of *Pseudo-nitzschia* spp. from eastern Canadian waters (Fig. 1) and the North Sea used to test for primer cross-reactivity.

Isolate name	Species name	Geographic origin	Date collected
CL-190	*P. calliantha*	Baie-Ste.-Anne, Miramichi Bay, NB	30 September 2002
CL-192	*P. fraudulenta*	Deadmans Harbour, Bay of Fundy, NB	9 October 2002
CL-194	*P. fraudulenta*	Deadmans Harbour, Bay of Fundy, NB	9 October 2002
CL-172	*P. pungens*	Cardigan Bay, PEI	5 September 2002
CL-180	*P. pungens*	Oak Point, Miramichi Bay, NB	23 September 2002
CL-193	*P. pungens*	Deadmans Harbour, Bay of Fundy, NB	9 October 2002
CL-200	*P. pungens*	Egg Island, Miramichi Bay, NB	28 October 2002
Hel-760	*P. pungens*	Helgoland, North Sea	6 May 2003
Hel-761	*P. pungens*	Helgoland, North Sea	6 May 2003
Hel-762	*P. pungens*	Helgoland, North Sea	6 May 2003
Hel-764	*P. pungens*	Helgoland, North Sea	6 May 2003
Hel-768	*P. pungens*	Helgoland, North Sea	6 May 2003
Hel-771	*P. pungens*	Helgoland, North Sea	7 May 2003
CL-162	*P. seriata*	Tracadie Bay, PEI	10 April 2002
CL-167	*P. seriata*	Rustico Bay, PEI	15 April 2002

NB, New Brunswick; PEI, Prince Edward Island.

contained microsatellites. Primer pairs were designed to flank 18 cloned CA repeats; 13 of these primer pairs yielded amplification products of the expected size. To determine whether these 13 loci were polymorphic, amplifications were repeated using several *P. multiseries* isolates and 5′ fluorescently labeled forward primers (6-FAM). Of these 13 primer pairs, nine yielded scoreable and polymorphic microsatellites. Primer pairs for these nine polymorphic loci were tested on between 21 and 25 clonal field isolates. The core repeat sequences, primer sequences, optimal annealing temperatures, size ranges of the alleles, numbers of alleles observed, and heterozygosity values for these loci are listed in Table 3. Amplification of microsatellite loci generated highly reproducible genotypes between duplicate PCRs (97% of reactions). For the small number of reactions where there was ambiguity between duplicates (3% of reactions), additional PCRs were performed and the majority rule was used to assign the final genotype. It is not known why a small number of PCRs gave variable results, but it is likely to be due to small variations in reaction conditions between duplicates.

Genetic and physiological variation in Pseudo-nitzschia multiseries. For 21 of the 25 field isolates, genotypes were obtained at all nine loci; for the remaining four isolates, data were missing at one locus for both Orø13 and CL-148, at five loci for CL-125, and at seven loci for Sylt (Table 1). The observed numbers of alleles per locus ranged from three to seven, and the observed heterozygosities from 0.39 to 0.70 (Table 3), suggesting a high degree of genetic diversity within the 25 *P. multiseries* field isolates. A significant ($P < 0.05$) deficit of heterozygotes was

TABLE 3. Attributes of nine microsatellite loci for a North Sea isolate (Sylt) of *Pseudo-nitzschia multiseries*.

Locus	n	Core sequence[a]	Primer sequences (5′–3′)	T_a	bp	N_A	H_o	H_e
PM1 (AY486322)	23	$(CA)_{16}GA(CA)_7GACGA\ (CA)_5$	F: CGAGAATGAATCGAGGTATC R: TGTCGCGTAAACCTATCCG	52	185–205	7	0.61	0.76
PM2 (AY486323)	24	$(CA)_{12}N_{63}(CT)_6(CA)_2TAGCG(CA)_{11}$	F: GCCTCGCAAAAAGAAAACAA R: TGGTACAAACCACCGGTACA	62	434–441	3	0.46	0.53
PM3 (AY486324)	25	$(CA)_9(A)_2(CA)_5GCG(A)_4TG(A)_6(TC)_9$ $(AC)_6AGCG\ (A)_4GT(A)_8CT(CA)_{13}$	F: CTGTCAGGCCCAGTAGCAAT R: GTTCGATGGAATGTTCGACCG	62	291–315	5	0.48	0.59
PM4 (AY486325)	23	$(CA)_{16}GCGGA(CA)_4GCG$ $(A)_2(CA)_4GC(A)_3(CA)_{10}$	F: ACGGCACTGGTGTAGAAAGC R: GCCCCTGCAAAGCACTAATA	60	419–425	4	0.57	0.54
PM5 (AY486326)	25	$(CA)_{11}CNGC(A)_5G(CA)_9GA(CA)_5$	F: CAAACCACCGCTACATGAAA R: CTCCGATGGAGTGCCTAAAG	62	549–563	3	0.39	0.50
PM6 (AY486327)	21	$(CA)_{11}TAC(A)_3(CA)_2CTTT(AC)_2(CA)_{13}$	F: ATTCAGCAAGGGGGCGA R: AGTAGAAAATAGTAGCAAAAC	52	187–211	5	0.43	0.45
PM7 (AY486328)	24	$(CA)_{15}TA(CA)_4(TA(CA)_2)_2TA(CA)_4$	F: CTCAGACGCACTTGGAGGAT R: CCCGCTCTGGATCGTGGAT	62	367–387	5	0.46	0.57
PM8 (AY486329)	23	$(GT)_5(T)_2(GT)_{23}(T)_2((GT)_3(T)_2)_2(GT)_2$	F: CTCCGCATTGTCGCTGGAAA R: GATCATACCTTAGACGAGAGCT	65	291–321	6	0.70	0.75
PM9 (AY486330)	23	$(CA)_9(TA(CA)_4)_2T(AC)_2T$ $(CA)_2T(AC)_2T(CA)_{13}TA\ (CA)_7TA(CA)_3TA(CA)_4$	F: CTAGGAGGACGCCATCAA R: GGCTGGAGTAGGCATATTG	60	242–344	5	0.57	0.56

The number of individuals tested (excluding F1 clones; n), annealing temperature (T_a: ° C), allele size range (bp), number of alleles (N_A), and observed (H_o) and expected (H_e) heterozygosities are given. GenBank accession numbers are given in parentheses below each locus.

[a]N indicates either an ambiguity or bases (usually T or G) that are within the core but that do not form part of a repeat unit

TABLE 4. Domoic acid concentrations of stationary-phase (days 21–28) clonal field isolates of *Pseudo-nitzschia multiseries* from eastern Canadian waters and Russian waters (see Table 1 and Fig. 1).

Isolate name	Geographic origin	Domoic acid ($ng \cdot mL^{-1}$)	Culture age (d)	Isolate age (d)
PM-02	Vladivostok, Sea of Japan	450	28	433
CL-45	New London Bay, PEI	2220	26	1883
CL-47	New London Bay, PEI	240	26	1883
CL-48	New London Bay, PEI	370	28	1883
CL-84	Cardigan Bay, PEI	ND	26	1478
CL-90	Cardigan Bay, PEI	20.5	26	64
CL-91	Cardigan Bay, PEI	22.8	26	64
CL–93	Cardigan Bay, PEI	20.5	26	64
CL-125	Mill River, PEI	350	26	409
CL-142	Little Harbour, NS	12	24	77
CL-143	Little Harbour, NS	11.2	24	77
CL-147	Caribou Harbour, NS	8.1	24	77
CL-148	Caribou Harbour, NS	51.7	24	77
CL-150	Caribou Harbour, NS	4.4	24	77
CL-155	Caribou Harbour, NS	308	28	710
CL-156	Caribou Harbour, NS	12.2	21	39
CL-157	Caribou Harbour, NS	7.1	21	39
CL-158	Caribou Harbour, NS	268	28	710
CL-174	Cardigan Bay, PEI	49.2	28	489
CL-191	Deadmans Harbour, NB	42.6	28	287
CL-195	Deadmans Harbour, NB	ND	28	54
CL-197	Egg Island, NB	200	28	433

Culture age (days) is the time since inoculation and isolate age (days) is the time since isolation. NB, New Brunswick; ND, domoic acid not detected ($< 1 ng \cdot mL^{-1}$); NS, Nova Scotia; PEI, Prince Edward Island.

observed at just two loci, PM1 and PM2, and only one pair of loci was in linkage disequilibrium ($P < 0.001$), PM2 and PM4.

Measurements of DA concentrations of stationary-phase cultures of *P. multiseries* clonal field isolates (Table 4) demonstrated a high degree of variation in DA production, from below the limit of detection ($< 1 ng\ DA \cdot mL^{-1}$) for isolates CL-84 and CL-195, up to $2220 ng\ DA \cdot mL^{-1}$ for isolate CL-45.

Twenty-three genotypes were represented in the collection of field isolates. CL-142 and CL-156 were identical at each of the nine loci and also had similar toxicities (Table 4). Isolates CL-125 and CL-147 were also identical; however, data were missing at five of the nine loci for CL-125 and these two isolates differed in their toxicity (Table 4).

From the genotype data for each F1 individual and their parents (Table 5), it is apparent that all six members of the F1 generation were indeed the result of sexual reproduction, there being evidence of genetic recombination at one of the nine microsatellite loci in CLN-17, CLN-18, CLN-19, CLN-20, and CLN-21 and at three of the nine microsatellite loci in CLN-16 (data were missing at one locus in CLN-18 and at four loci in CLN-19; Table 5). F1 samples CLN-17 and CLN-18 had identical genotypes, but different toxicities (Table 5).

Pair-wise genic tests conducted across all nine loci showed significant differences in allele distribution between certain geographical locations: between Cardigan Bay, PEI and Deadmans Harbour, NB ($P < 0.01$); between Cardigan Bay and Sea of Japan ($P < 0.001$); between Caribou Harbour, NS and Deadmans Harbour ($P < 0.05$); and between Caribou Harbour and Sea of Japan ($P < 0.001$) (Fig. 1).

Cross-reactivity of primers. DNA from all the congeners used to test the cross-reactivity of *P. multiseries* primers was amplifiable with the generic eukaryote 18S-rDNA primers, EukF and EukR (DeLong 1992).

TABLE 5. Genotypes (length of PCR amplicons [alleles] in base pairs) and domoic acid concentrations (days 26–28 in stationary phase) of the two parental field isolates of *P. multiseries* (CL-45 and CL-47) and the six F1 clones.

	Genotype									
Isolate name	PM1	PM2	PM3	PM4	PM5	PM6	PM7	PM8	PM9	Domoic acid ($ng \cdot mL^{-1}$)
CL-45	187/195	434/434	293/297	425/425	549/549	209/209	377/377	291/293	242/242	2220
CL-47	185/185	434/441	297/315	419/425	549/549	209/209	377/377	291/306	242/246	240
CLN-16	**185/195**	434/434	**293/315**	425/425	549/549	209/209	377/377	**293/306**	242/242	525
CLN-17	**185/195**	434/441	297/315	419/425	549/549	209/209	377/377	291/293	242/242	180
CLN-18	**185/195**	434/441	297/315	419/425	549/549	209/209	377/377	291/293		320
CLN-19				425/425	549/549	209/209	377/377	**293/306**		370
CLN-20	**185/195**	434/434	297/315	425/425	549/549	209/209	377/377	291/293	242/242	360
CLN-21	**185/195**	434/434	297/315	425/425	549/549	209/209	377/377	291/293	242/246	130

Blank cells indicate missing data. Genotypes in bold type are those where it is evident that recombination has taken place.

Cross-reactivity was only observed when the primer pairs for loci PM1, PM5, and PM6 were used to amplify DNA from clonal isolates of *P. pungens* (up to 10 clonal isolates in total). In the case of locus PM1, one allele distinct from those in *P. multiseries* was observed (amplicon length, 228 bp); for locus PM5, three distinct alleles were observed (amplicon lengths, 136 bp, 180 bp, and 201 bp); and for locus PM6, one distinct allele (amplicon length, 141 bp) was observed. When the four pairs of *P. pungens* primers (Evans and Hayes 2004) were tested on four isolates of *P. multiseries*, cross-reactivity was observed at loci PP5 and PP6. At locus PP5, two alleles distinct from those in *P. pungens* were observed (amplicon lengths, 155 bp and 263 bp), and at locus PP6, one distinct allele was observed (amplicon length, 239 bp).

DISCUSSION

The complex *P. multiseries* microsatellite repeats are similar to those identified in other diatoms (Rynearson and Armbrust 2000, Evans and Hayes 2004). Compared with these previous studies, however, the *P. multiseries* loci appear to be less polymorphic, with just three to seven alleles per locus; genotyping more individuals will undoubtedly identify further alleles, particularly if these individuals are drawn from a wide geographical area. For example, isolate PM-02, the first documented toxic *P. multiseries* isolate from Russian waters, was the most genetically distinct in our collection, introducing 11 new alleles at six loci.

The primers developed for *P. multiseries* exhibited limited cross-reactivity when tested with DNA from four congeners (*P. calliantha*, *P. fraudulenta*, *P. pungens*, and *P. seriata*). Only the DNA from *P. pungens* isolates yielded amplification products (at loci PM1, PM5, and PM6). Two primer pairs developed for *P. pungens* (PP5 and PP6; Evans and Hayes 2004) successfully amplified DNA from *P. multiseries* isolates. In all instances, the alleles amplified were distinct from those amplified in the species for which the primers were developed. These findings are consistent with the 28S rDNA based phylogeny of *Pseudo-nitzschia* (Lundholm et al. 2002), where *P. pungens* is the closest relative to *P. multiseries*, these two species forming a well-supported clade. Further cross-reactivity may be observed if more individuals and more species are tested and if amplification conditions are optimized for each new species–primer combination.

Within the 25 field isolates of *P. multiseries*, there was a substantial degree of genetic variation, 23 different genotypes being recorded. Canadian isolates CL-142 and CL-156 were identical at all nine loci; isolates CL-125 and CL-147 were also identical, although data were missing at five loci for CL-125. For CL-142 and CL-156, it is likely that they are clonal, despite having been isolated two weeks apart from locations 15 km distant. For clones CL-125 and CL-147, isolated 15 months apart and from locations over 200 km distant, it is much less likely that they are in fact clonal; the relatively low levels of polymorphism and the dominance of one or two alleles at most loci results in many nonclonal field samples sharing genotypes at four loci (Table 1). It therefore appears necessary to genotype isolates at all nine loci to identify clonal individuals, although fewer loci may be needed to differentiate between isolates from geographically distant locations; just two loci revealed that isolate Sylt (North Sea) is genetically distinct.

A number of the F1 isolates also appeared to be clonal, for example CLN-17 and CLN-18. This is most likely due to the high levels of homozygosity in the parents (CL-45 is homozygous at six loci and CL-47 is homozygous at four loci), which decrease the chances of novel genotypes arising.

A high level of variation in DA production within the Canadian *P. multiseries* isolates has been noted previously (Bates et al. 1989, 1998, 1999). Although there is undoubtedly great interclonal variability in DA production, not all the variation will be attributable to genetic differences. It is known that the presence of bacteria (either epiphytic or free-living) can greatly affect the level of DA production (Douglas and Bates 1992, Bates et al. 1995, 2004). It is also known that DA production changes as a function of both the time since isolation and the time since inoculation. For example, DA production increases rapidly during the stationary phase in batch culture (Bates et al. 1991, 1998); this is also a time when bacterial abundance and diversity increase (Kaczmarska and Bates, unpublished data). In contrast, there is a decrease in the toxicity of most *P. multiseries* clonal isolates over a period of a year or more (Villac et al. 1993, Bates et al. 1998, 1999). This may be related to the decrease in apical cell length of these diatoms as they divide (Bates et al. 1999), to changes in the number and diversity of bacterial species as the clones age, or to the removal of the selection pressure needed to maintain DA production. Without further advances in our understanding of factors affecting DA production, it is not possible to compare genotypic and physiological data with confidence. Characterizing the bacterial diversity and abundance within *P. multiseries* cultures of known genotype will help to resolve the degree of genetic versus bacterial influence on toxicity.

Two previous studies also reported high levels of genetic variation in populations of *Pseudo-nitzschia* species. In a small-scale isozyme-based study of *P. calliantha* (previously called *P. pseudodelicatissima*), 10 clones isolated from the Skagerrak (North Sea) were distinct (Skov et al. 1997). In a larger-scale microsatellite-based study, preliminary results show that of 121 North Sea *P. pungens* clones, only two isolates are clonal (unpublished data).

Further studies have investigated genetic structures of other phytoplankton populations (Gallagher 1980, 1982, Medlin et al. 1996, Bolch et al. 1999, Rynearson and Armbrust 2000, 2004, Hayes et al. 2002, Shankle et al. 2004). Most of these studies used either isozymes

or RAPDs, which have a number of well-known associated drawbacks (Karp et al. 1996, Müller-Starck 1998), undoubtedly resulting in an underestimation of genetic diversity.

Gallagher (1980) conducted the first large-scale study to quantify genetic variation in phytoplankton populations, in this case the marine diatom *Skeletonema costatum* (Grev.) Cleve. Using five isozyme loci to genotype 457 clonal isolates established over a two-year period from Narragansett Bay, Rhode Island, she found that despite a lack of morphological variation, both physiological and genetic variation were present. Effectively though, only two dominant genotypes were detected: summer and winter. Physiological variation between individuals with the same genotype suggested that genetic variation was underestimated (Gallagher 1982).

Rynearson and Armbrust (2000) were the first to use microsatellite markers to investigate the genetic diversity of phytoplankton populations. In another study, three genetically and physiologically distinct populations of *Ditylum brightwellii* (West) Grunow could be identified in two connected estuaries: two in the Strait of Juan de Fuca and one in Puget Sound (Rynearson and Armbrust 2004). This illustrates that despite the potential for widespread dispersal in the phytoplankton, populations with distinct genetic and physiological characteristics can be maintained (most likely by differential selection) over periods of at least two years (Rynearson and Armbrust 2004).

The genetic distinctiveness of the Russian *P. multiseries* isolate (PM-02) and the significant genetic differentiation between it and many of the Canadian isolates suggests that discrete populations exist, albeit from this evidence, on a large geographical scale. Recent attempts to mate the Russian clone with Canadian clones CL-147 or CL-191 (as well as with six others) have so far been unsuccessful, even though these same two Canadian clones are able to interbreed (Bates and Léger, unpublished data).

At a more local scale, significant genetic differences were observed between *P. multiseries* isolates from different locations on the east coast of Canada (Fig. 1): between the six clones isolated from Cardigan Bay and the two from Deadmans Harbour and between the seven clones isolated from Caribou Harbour and the two from Deadmans Harbour. Clones CL-147 and CL-191, from Caribou Harbour and Deadmans Harbour, respectively, were nevertheless able to interbreed. Without more extensive sampling, it is impossible to draw definite conclusions about the distribution of genetic variation over different spatial and temporal scales in this species. However, the apparent genetic differences between the two samples from Deadmans Harbour and the other Canadian samples are not unexpected; Deadmans Harbour is the only sampling site outside the Gulf of St. Lawrence. The two identical clones (CL-142 and CL-156) were both isolated from the Gulf of St. Lawrence, from locations only 15 km apart. The isolation of genetically identical individuals from distinct sites within the same hydrographic system is consistent with the findings of Rynearson and Armbrust (2004).

Field evidence from the Cardigan Bay bloom of *P. multiseries* in December 1987 suggests that blooms originate within embayments on the eastern Canadian coast (Bates et al. 1989). Further genotyping of isolates from these waters may demonstrate that isolates from different embayments are genetically distinct, lending further support to the notion that phytoplankton populations can be genetically differentiated, even over small geographical scales, if the prevailing hydrographic conditions do not promote significant mixing.

In recent years, the debate over whether most microorganisms are cosmopolitan or more restricted in their distributions has resurfaced (Mann 1999, 2001, Finlay 2002). Hasle (2002) reported that most DA-producing *Pseudo-nitzschia* species are cosmopolites. However, it is impossible to know whether this is really the case without an improved understanding of the levels of genetic variation present at the species level over the supposed species' range. Some diatoms are notoriously difficult to identify to species level (Mann 1999), and molecular studies suggest that there are large numbers of new species waiting to be described (Medlin et al. 1995, Larsen and Medlin 1997, Pace 1997, Lange et al. 2002). For example, the summer and winter genotypes of *S. costatum* could represent a seasonal succession of different species (Gallagher 1982); studies based on variable large subunit rRNA data of many *S. costatum* clones support the division of this species into four molecular clades (Medlin et al., unpublished data). Similarly, Rynearson and Armbrust (2004) suggested that the two most divergent populations of *D. brightwellii* could be different species. It is also likely that the genetic variation seen in the isozyme study of *P. calliantha* clones (Skov et al. 1997) was confounded by the lack of sufficiently detailed species descriptions (Lundholm et al. 2003). Hence, it is only through an integrated approach, that is, through morphological descriptions, molecular characterization, and breeding experiments, that the true diversity present within the Bacillariophyta will become evident.

At present, the population dynamics of *P. multiseries* are very poorly understood. For example, its abundance on the east coast of Canada has fluctuated greatly in the last 15 years or so; in 1987 it was very abundant, between 1991 and 1993 it was drastically reduced, and then in 2000 it was again the dominant *Pseudo-nitzschia* species (Hasle 2002). The microsatellite markers we developed provide a powerful tool that will help us to begin to understand the bloom dynamics of *P. multiseries* and the genetic diversity that allows the cells to respond to changing environmental conditions (Shankle et al. 2004). It is only when we fully understand the biology of toxic algae that we will have confidence in our ability to predict when and where blooms might occur. In addition, the availability of microsatellite markers will make it possible to identify the sources of introductions via ballast water or contaminated shellfish stocks.

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Bates, S. S. 2000. Domoic-acid-producing diatoms: another genus added! *J. Phycol.* 36:978–85.

Bates, S. S., Bird, C. J., de Freitas, A. S. W., Foxall, R., Gilgan, M., Hanic, L. A., Johnson, G. R., McCulloch, A. W., Odense, P., Pocklington, R., Quilliam, M. A., Sim, P. G., Smith, J. C., Subba Rao, D. V., Todd, E. C. D., Walter, J. A. & Wright, J. L. C. 1989. Pennate diatom *Nitzschia pungens* as the primary source of domoic acid, a toxin in shellfish from eastern Prince Edward Island, Canada. *Can. J. Fish. Aquat. Sci.* 46:1203–15.

Bates, S. S., de Freitas, A. S. W., Milley, J. E., Pocklington, R., Quilliam, M. A., Smith, J. C. & Worms, J. 1991. Controls on domoic acid production by the diatom *Nitzschia pungens* f. *multiseries* in culture: nutrients and irradiance. *Can. J. Fish. Aquat. Sci.* 48:1136–44.

Bates, S. S., Douglas, D. J., Doucette, G. J. & Léger, C. 1995. Enhancement of domoic acid production by reintroducing bacteria to axenic cultures of the diatom *Pseudo-nitzschia multiseries*. *Nat. Toxins* 3:428–35.

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 Algae Blooms*. Springer-Verlag, Heidelberg, pp. 267–92.

Bates, S. S., Gaudet, J., Kaczmarska, I. & Ehrman, J. M. 2004. Interaction between bacteria and the domoic-acid-producing diatom *Pseudo-nitzschia multiseries* (Hasle) Hasle; can bacteria produce domoic acid autonomously? *Harmful Algae* 3:11–20.

Bates, S. S., Hiltz, M. F. & Léger, C. 1999. Domoic acid toxicity of large new cells of *Pseudo-nitzschia multiseries* resulting from sexual reproduction. *In* Martin, J. L. & Haya, K. [Eds.] *Proceedings of the Sixth Canadian Workshop on Harmful Marine Algae. Can. Tech. Rep. Fish. Aquat. Sci.* 2261, pp. 22–7.

Bates, S. S., Léger, C., Keafer, B. A. & Anderson, D. M. 1993. Discrimination between domoic-acid-producing and nontoxic forms of the diatom *Pseudo-nitzschia pungens* using immunofluorescence. *Mar. Ecol. Prog. Ser.* 100:185–95.

Bolch, C. J. S., Blackburn, S. I., Hallegraeff, G. M. & Vaillancourt, R. E. 1999. Genetic variation among strains of the toxic dinoflagellate *Gymnodinium catenatum* (Dinophyceae). *J. Phycol.* 35:356–67.

Cho, E. S., Hur, H. J., Byun, H. S., Lee, S. G., Rhodes, L. L., Jeong, C. S. & Park, J. G. 2002. Monthly monitoring of domoic acid producer *Pseudo-nitzschia multiseries* (Hasle) Hasle using species-specific DNA probes and WGA lectins and abundance of *Pseudo-nitzschia* species (Bacillariophyceae) from Chinae Bay, Korea. *Bot. Mar.* 45:364–72.

Davidovich, N. A. & Bates, S. S. 1998. Sexual reproduction in the pennate diatoms *Pseudo-nitzschia multiseries* and *P. pseudodelicatissima* (Bacillariophyceae). *J. Phycol.* 34:126–37.

DeLong, E. F. 1992. Archaea in coastal marine environments. *Proc. Natl. Acad. Sci. USA* 89:5685–9.

Douglas, D. J. & Bates, S. S. 1992. Production of domoic acid, a neurotoxic amino acid, by an axenic culture of the marine diatom *Nitzschia pungens* f. *multiseries* Hasle. *Can. J. Fish. Aquat. Sci.* 49:85–90.

Douglas, D. J., Landry, D. & Douglas, S. E. 1994. Genetic relatedness of two toxic and non-toxic isolates of the marine pennate diatom *Pseudo-nitzschia* (Bacillariophyceae): phylogenetic analysis of 18S rRNA sequences. *Nat. Toxins* 2:166–74.

Edwards, K. J., Barker, J. H. A., Daly, A., Jones, C. & Karp, A. 1996. Microsatellite libraries enriched for several microsatellite sequences in plants. *Biotechniques* 20:758–60.

Evans, K. M. & Hayes, P. K. 2004. Microsatellite markers for the cosmopolitan marine diatom *Pseudo-nitzschia pungens*. *Mol. Ecol. Notes* 4:125–6.

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.* 40:622–30.

Finlay, B. J. 2002. Global dispersal of free-living microbial eukaryote species. *Science* 296:1061–3.

Gallagher, J. C. 1980. Population genetics of *Skeletonema costatum* (Bacillariophyceae) in Narragansett Bay. *J. Phycol.* 16:464–74.

Gallagher, J. C. 1982. Physiological variation and electrophoretic banding patterns of genetically different seasonal populations of *Skeletonema costatum* (Bacillariophyceae). *J. Phycol.* 18:148–62.

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 Press, New York, pp. 29–60.

Hallegraeff, G. M. 1994. Species of the diatom genus *Pseudonitzschia* in Australian waters. *Bot. Mar.* 37:397–411.

Hallegraeff, G. M. 2003. Harmful algal blooms: a global overview. *In* Hallegraeff, G. M., Anderson, D. M. & Cembella, A. D. [Eds.] *Manual on Harmful Marine Microalgae*. UNESCO, Paris, pp. 25–49.

Hasle, G. R. 1995. *Pseudo-nitzschia pungens* and *P. multiseries* (Bacillariophyceae): nomenclatural history, morphology, and distribution. *J. Phycol.* 31:428–35.

Hasle, G. R. 2002. Are most of the domoic acid-producing species of the diatom genus *Pseudo-nitzschia* cosmopolites? *Harmful Algae* 1:137–46.

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.

Hayes, P. K., Barker, G. L. A., Batley, J., Beard, S. J., Handley, B. A., Vacharapiyasophon, P. & Walsby, A. E. 2002. Genetic diversity within populations of cyanobacteria assessed by analysis of single filaments. *Antonie Van Leeuwenhoek* 81:197–202.

Hernández-Becerril, D. U. 1998. Species of the planktonic diatom genus *Pseudo-nitzschia* of the Pacific coasts of Mexico. *Hydrobiologia* 379:77–84.

Kaczmarska, I., LeGresley, M. M., Martin, J. L. & Ehrman, J. 2004. Diversity of the diatom genus *Pseudo-nitzschia* Peragallo in the Quoddy region of the Bay of Fundy, Canada. *Harmful Algae* (in press).

Karp, A., Seberg, O. & Buiatti, M. 1996. Molecular techniques in the assessment of botanical diversity. *Ann. Bot.* 78:143–9.

Lange, M., Chen, Y.-Q. & Medlin, L. K. 2002. Molecular genetic delineation of *Phaeocystis* species (Prymnesiophyceae) using coding and non-coding regions of nuclear and plastid genomes. *Eur. J. Phycol.* 37:77–92.

Larsen, A. & Medlin, L. K. 1997. Inter- and intraspecific genetic variation in twelve *Prymnesium* (Haptophyceae) clones. *J. Phycol.* 33:1007–15.

Lundholm, N., Daugbjerg, N. & Moestrup, Ø. 2002. Phylogeny of the Bacillariaceae with emphasis on the genus *Pseudo-nitzschia* (Bacillariophyceae) based on partial LSU rDNA. *Eur. J. Phycol.* 37:115–34.

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., Skov, J., Pocklington, R. & Moestrup, Ø. 1994. Domoic acid, the toxic amino acid responsible for amnesic shellfish poisoning, now in *Pseudonitzschia seriata* (Bacillariophyceae) in Europe. *Phycologia* 33:475–8.

Manhart, J. R., Fryxell, G. A., Villac, M. C. & Segura, L. Y. 1995. *Pseudo-nitzschia pungens* and *P. multiseries* (Bacillariophyceae): nuclear ribosomal DNAs and species differences. *J. Phycol.* 31:421–7.

Mann, D. G. 1999. The species concept in diatoms. *Phycologia* 38:437–95.

Mann, D. G. 2001. Book review: freshwater algae. *Phycologia* 40:387–9.

Medlin, L. K., Barker, G. L. A., Campbell, L., Green, J. C., Hayes, P. K., Marie, D., Wrieden, S. & Vaulot, D. 1996. Genetic characterisation of *Emiliania huxleyi* (Haptophyta). *J. Mar. Syst.* 9:13–31.

Medlin, L. K., Lange, M., Barker, G. L. A. & Hayes, P. K. 1995. Can molecular techniques change our ideas about the species concept? *In* Joint, I., Falkowski, P. & Carr, N. [Eds.] *Molecular Ecology of Aquatic Microbes*. NATO ASI Series, Springer-Verlag, Berlin, Heidelberg, pp. 133–52.

Miller, P. E. & Scholin, C. A. 1996. Identification of cultured *Pseudo-nitzschia* (Bacillariophyceae) using species-specific LSU rRNA-targeted fluorescent probes. *J. Phycol.* 32:646–55.

Miller, P. E. & Scholin, C. A. 1998. Identification and enumeration of cultured and wild *Pseudo-nitzschia* (Bacillariophyceae) using species-specific LSU rRNA-targeted fluorescent probes and filter-based whole cell hybridization. *J. Phycol.* 34: 371–82.

Miller, P. E. & Scholin, C. A. 2000. On detection of *Pseudo-nitzschia* (Bacillariophyceae) species using whole cell hybridization: sample fixation and stability. *J. Phycol.* 36:238–50.

Müller-Starck, G. 1998. Isozymes. *In* Karp, A., Isaac, P. G. & Ingram, D. S. [Eds.] *Molecular Tools for Screening Biodiversity*. Chapman and Hall, London, pp. 75–81.

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.

Orsini, L., Sarno, D., Procaccini, G., Poletti, R., Dahlmann, J. & Montresor, M. 2002. Toxic *Pseudo-nitzschia multistriata* (Bacillariophyceae) from the Gulf of Naples: morphology, toxin analysis and phylogenetic relationships with other *Pseudo-nitzschia* species. *Eur. J. Phycol.* 37:247–57.

Pace, N. R. 1997. A molecular view of microbial diversity and the biosphere. *Science* 276:734–40.

Parsons, M. L., Scholin, C. A., Miller, P. E., Doucette, G. J., Powell, C. L., Fryxell, G. A., Dortch, Q. & Soniat, T. M. 1999. *Pseudo-nitzschia* species (Bacillariophyceae) in Louisiana coastal waters: molecular probe field trials, genetic variability, and domoic acid analyses. *J. Phycol.* 35:1368–78.

Pocklington, R., Milley, J. E., Bates, S. S., Bird, C. J., de Freitas, A. S. W. & Quilliam, M. A. 1990. Trace determination of domoic acid in seawater and phytoplankton by high-performance liquid chromatography of the fluorenylmethoxycarbonyl (FMOC) derivative. *Int. J. Environ. Anal. Chem.* 38: 351–68.

Raymond, M. & Rousset, F. 1995. GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *J. Hered.* 86:248–9.

Rhodes, 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*. Intergovernmental Oceanographic Commission, UNESCO, Paris, pp. 155–8.

Rozen, S. & Skaletsky, H. J. 2000. PRIMER3 on the WWW for general users and for biologist programmers. *In* Krawetz, S. & Misener, S. [Eds.] *Bioinformatics Methods and Protocols: Methods in Molecular Biology*. Humana Press, Totowa, NJ, pp. 365–86.

Rynearson, T. A. & Armbrust, E. V. 2000. DNA fingerprinting reveals extensive genetic diversity in a field population of the centric diatom *Ditylum brightwellii*. *Limnol. Oceanogr.* 45:1329–40.

Rynearson, T. A. & Armbrust, E. V. 2004. Genetic differentiation among populations of the planktonic marine diatom *Ditylum brightwellii* (Bacillariophyceae). *J. Phycol.* 40:34–43.

Scholin, C. A., Buck, K. R., Britschgi, T., Cangelosi, G. & Chavez, F. P. 1996. Identification of *Pseudo-nitzschia australis* (Bacillariophyceae) using rRNA-targeted probes in whole cell and sandwich hybridization formats. *Phycologia* 35:190–7.

Scholin, C. A., Marin, R. III, Miller, P. E., Doucette, G. J., Powell, C. L., Haydock, P., Howard, J. & Ray, J. 1999. DNA probes and a receptor-binding assay for detection of *Pseudo-nitzschia* (Bacillariophyceae) species and domoic acid activity in cultured and natural samples. *J. Phycol.* 35:1356–67.

Shankle, A. M., Mayali, X. & Franks, P. J. S. 2004. Temporal patterns in population genetic diversity of *Prorocentrum micans* (Dinophyceae). *J. Phycol.* 40:239–47.

Skov, J., Lundholm, N., Pocklington, R., Rosendahl, S. & Moestrup, Ø. 1997. Studies on the marine planktonic diatom *Pseudo-nitzschia*. 1. Isozyme variation among isolates of *P. pseudodelicatissima* during a bloom in Danish coastal waters. *Phycologia* 36:374–80.

Trainer, V. L., Adams, N. G., Bill, B. D., Stehr, C. M., Wekell, J. C., Moeller, P., Busman, M. & Woodruff, D. 2000. Domoic acid production near California coastal upwelling zones, June 1998. *Limnol. Oceanogr.* 45:818–33.

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 Intergovernmental Oceanographic Commission, UNESCO, Santiago de Compostella, pp. 337–40.

Villac, M. C., Roelke, D. L., Chavez, F. P., Cifuentes, L. A. & Fryxell, G. A. 1993. *Pseudo-nitzschia australis* Frenguelli and related species from the west coast of the U.S.A.: occurrence and domoic acid production. *J. Shellfish Res.* 12:457–65.