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HIGH LEVELS OF GENETIC DIVERSITY AND LOW LEVELS OF GENETIC DIFFERENTIATION IN NORTH SEA *PSEUDO-NITZSCHIA PUNGENS* (BACILLARIOPHYCEAE) POPULATIONS[1]

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Six microsatellite markers were used to investigate the genetic structure of North Sea *Pseudo-nitzschia pungens* (Grunow *ex* P. T. Cleve) Hasle populations. Isolates were collected on 42 separate occasions from waters surrounding the German islands of Helgoland and Sylt over the course of three sampling periods: spring 2002, spring 2003, and autumn 2003. In total, 464 isolates were genotyped, of which 453 were different (i.e. clonal diversity was 98%). The numbers of alleles per locus ranged from 6 to 24 and the observed heterozygosities from 0.59 to 0.87 (mean H_o and H_e were 0.73); there were no significant departures from Hardy-Weinberg equilibrium at any of the six loci. Sexual reproduction therefore appears to be important in the production of genetic variation. Over the temporal and spatial scales sampled (18 months and 100 km), weak genetic differentiation was detected both within and between sampling periods (significant F_{ST} values ranged from 0.0018 to 0.0389), suggesting that the German North Sea supports a single largely unstructured population of *P. pungens*.

Key index words: **diatom; genetic differentiation; genetic diversity; genetic structure; genetic variation; microsatellites; population genetics; *Pseudo-nitzschia pungens***

Abbreviation: **HWE, Hardy-Weinberg equilibrium**

Few studies have investigated the genetic structures of marine phytoplankton populations on a local rather than a biogeographical scale (Gallagher 1980, Skov et al. 1997, Rynearson and Armbrust 2000, 2004, Hayes et al. 2002, Orsini et al. 2004, Shankle et al. 2004), and most of these have one or more associated drawbacks. For example, some used a small number of isolates (Skov et al. 1997, Rynearson and Armbrust 2000); some used molecular methods that have a number of well-known shortcomings (Gallagher 1980, Skov et al. 1997, Hayes et al. 2002, Orsini et al. 2004), and some investigated species that have subsequently been reported to belong to cryptic species complexes (Skov et al. 1997). Consequently, our understanding of levels of genetic diversity and its partitioning, both spatially and temporally, within phytoplankton species is limited: very few studies have been conducted that use both powerful enough molecular markers to detect genetic variation within populations of members of the same species and a sufficiently large sample size.

One notable exception is the study of Rynearson and Armbrust (2004) into the genetic structure of populations of the diatom *Ditylum brightwellii* (West) Grunow growing in two connected estuaries in Washington: Puget Sound and the Strait of Juan de Fuca. Using three microsatellite markers to genotype 265 isolates, they reported very high levels of genetic diversity and identified three genetically distinct populations associated with the different water bodies; the degree of genetic differentiation between populations was correlated with neither spatial nor temporal separation. Their investigation demonstrated that despite the potential for widespread dispersal in the phytoplankton, populations with distinct genetic and physiological characteristics can be maintained over periods of at least two years. Further such studies are needed if we are to understand the ecological and evolutionary implications of genetic diversity in the phytoplankton, for example, how genetic diversity within a population affects its ability to bloom and also how genetic diversity is related to physiological diversity, including toxin production.

Our aim was to genotype, using microsatellite markers, a large number of diatom isolates from the North Sea to determine whether there was also evidence of genetic structure over small scales in an open-water

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environment, where there are no obvious barriers to mixing. We chose a member of the genus *Pseudo-nitzschia* H. Peragallo because *Pseudo-nitzschia* species form an important component of the plankton throughout the world's seas (Hasle 2002). In addition, *Pseudo-nitzschia* species are of particular interest because ten of the approximately 30 species so far described have the ability to produce the potent neurotoxin domoic acid (Bates 2000, Lundholm et al. 2003). *Pseudo-nitzschia multiseries* (Hasle) Hasle was the first documented case of a domoic acid–producing diatom (Bates et al. 1989), but we chose its closest relative, the generally nontoxic *P. pungens* (Grunow *ex* P. T. Cleve) Hasle (Rhodes et al. 1996, Trainer et al. 1998b) for two reasons. First, *P. pungens* is the dominant *Pseudo-nitzschia* species in the North Sea off the German coast. Second, unlike several other *Pseudo-nitszchia* species (Lundholm et al. 2003), ongoing research on North Sea populations of *P. pungens* using internal transcribed spacer sequence data has so far not revealed any evidence of cryptic speciation (G. Casteleyn, personal communication). For this analysis we used a set of six microsatellite markers whose suitability for population genetics studies had already been established (Evans and Hayes 2004).

MATERIALS AND METHODS

Sampling sites and collection of isolates. *Pseudo-nitzschia* cells were isolated during three different time periods and from two different sites approximately 100 km apart in the North Sea (Helgoland and Sylt; Fig. 1) between spring 2002 and autumn 2003. Helgoland waters were sampled during spring 2002 (15 April to 7 May) and spring 2003 (16 April to 8 May), and Sylt waters were sampled during autumn 2003 (18 September to 4 October).

During each sampling period, individual cells or, more often, chains of cells (cells forming single chains are clonal) were isolated by micropipette from plankton samples collected using either a 20-μm or 80-μm mesh net. Isolates established from Helgoland waters were labeled Hel- and those from Sylt waters were labeled Sy-. During spring 2002, cells were mostly isolated from plankton samples collected the previous day; during the latter two sampling periods, cells were isolated on the day of sample collection. This was to minimize the likelihood of sampling clones that had undergone asexual reproduction during the previous 24-h period. Isolated cells were transferred into either individual wells of a 24-well culture plate or into 2-mL Eppendorf tubes, both receptacles containing approximately 1.5 mL sterile f/2 + Si medium (Guillard 1975), before being transported to Bristol, United Kingdom and transferred to glass tubes containing 5 mL of medium. Isolates were grown for approximately two weeks at 15° C and at 20–35 μmol photons $\cdot m^{-2} \cdot s^{1}$. During this time, isolates were checked microscopically for contamination with other algal species; contaminated cultures were either discarded or cells were reisolated into fresh medium. Unialgal *Pseudo-nitzschia* cultures were transferred to flasks containing 50 mL of medium. After at least a further two weeks, but still during the exponential growth phase, cultures were harvested by centrifugation at 10,000*g* for 20 min and the pellets stored at −70° C.

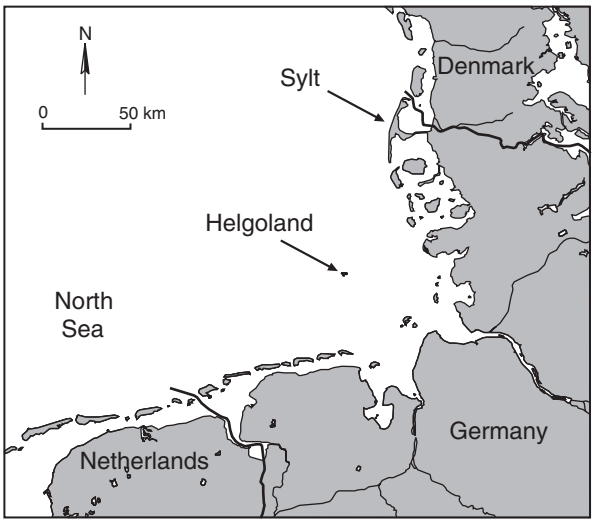

FIG. 1. Map showing the location of the North Sea islands Helgoland and Sylt. Helgoland lies 53 km off the German north coast and Sylt lies 5 km off the German west coast, approximately 65 km to the northeast of Helgoland. The two sampling sites are separated by approximately 100 km.

In addition to the *Pseudo-nitzschia* samples isolated from the North Sea, Dr. Stephen Bates provided three *P. pungens* isolates (CL-180, CL-193, and CL-200) collected from eastern Canadian waters (Miramichi Bay and the Bay of Fundy) in autumn 2002.

Identification of isolates. Under the light microscope, it is difficult and in some cases impossible to distinguish between members of the genus *Pseudo-nitzschia*. This is particularly so for *P. pungens* and *P. multiseries* (Hasle 1995, Manhart et al. 1995). Although both species are found in the North Sea, *P. pungens* occurs more frequently. A subset of the *Pseudo-nitzschia* samples isolated from the North Sea and the three Canadian isolates were identified by SEM, using the species descriptions of Hasle and Syvertsen (1996).

DNA extraction, microsatellite development, and genotyping. Genomic DNA was extracted from pelleted cells using a DNeasy Plant Mini Kit (Qiagen, Crawley, West Sussex, UK), with the modifications described by Rynearson and Armbrust (2000). Extracted DNA from a single isolate (Hel-149) was enriched for CA and CT repeats following a protocol based on Edwards et al. (1996); this library yielded the six polymorphic microsatellite loci described in Evans and Hayes (2004). These loci were amplified in separate PCR reactions (Evans and Hayes 2004) using 5′ fluorescently labeled forward primers (6-FAM, HEX, or TET; MWG Biotech, Milton Keynes, Buckinghamshire, UK) to enable post-PCR multiplexing. To remove salts from the PCR reactions, mixtures of three different fluorescently labeled PCR products (10 μL of each) were passed through Sephadex columns (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). The desalted PCR products were size fractionated on a MegaBACE 1000 automated sequencer (Amersham Biosciences) using a fluorescently labeled (ROX) 550-bp internal size standard (Amersham Biosciences). The length of each PCR product was determined using GENETIC PROFILER 1.5 software (Amersham Biosciences) and was used to construct a multilocus genotype for each isolate.

Similar to the other two microsatellite-based diatom population genetics studies (Evans et al. 2004, Rynearson and Armbrust 2004), several of the six *P. pungens* loci are complex in structure (Evans and Hayes 2004). At locus PP3, just 1 bp separates a number of distinct alleles (e.g. three of these alleles are 222, 223, and 224 bp in length). It has been demonstrated that the allele length detected during genotyping by capillary electrophoresis is temperature dependent, with a 5° C change resulting in a difference of up to 0.7 bp (Davison and Chiba

TABLE 1. Locations and dates of sample collections, numbers of days on which samples were collected, numbers of clones isolated, numbers of isolates that survived to the DNA extraction stage, and numbers and percentages of isolates that were successfully genotyped.

Location	Sampling period	No. days	No. isolates	No. DNA	No. genotyped	% genotyped
Helgoland	15 April to 7 May 2002	7	213	97	81	38.0
Helgoland	16 April to 8 May 2003	20	360	190	160	44.4
Sylt	18 Sept. to 4 Oct. 2003	15	477	261	223	46.8
	Total	42	1050	548	464	44.2

2003). Although genotyping was carried out in a temperature-controlled room, for locus PP3 in particular, multiple positive controls were used and replicate reactions were performed to ensure accurate designation of alleles. The primers for locus PP4 often yield only low amounts of product, and so replicate reactions were also included for this locus. To further reduce the occurrence of genotyping errors, negative controls were used and the same person carried out all scoring of alleles (Bonin et al. 2004).

Analysis of microsatellite data. Basic descriptive population genetics statistics (numbers of alleles, allele frequencies, expected heterozygosity or gene diversity, H_e, and observed heterozygosity, H_o) were calculated for each locus using GENEPOP 3.4 (Raymond and Rousset 1995). The GENEPOP program was also used to detect deviations from the Hardy-Weinberg equilibrium (HWE) at each locus (by comparing the observed proportion of heterozygotes, H_o, with the expected proportion of heterozygotes, H_e) and to test for linkage disequilibrium between pairs of loci to determine whether the loci represent independent samples of the *P. pungens* genome. If loci are in HWE (i.e. there is neither an excess nor a deficit of heterozygotes), this suggests no null alleles, that the population to which the isolates belong is large, that mating is random, and that alleles are selectively neutral.

To assess the resolving power of the microsatellite markers, GIMLET (Valière 2002) was used to calculate the probability of identity, $P_{(ID)}$, and the probability of identity between sibs, $P_{(ID)sib}$, for each locus and all six loci together (Waits et al. 2001). These values, particularly $P_{(ID)sib}$, provide a conservative upper bound for the probability of observing identical multilocus genotypes between two isolates sampled from a population. Waits et al. (2001) suggested that $P_{(ID)}$ or $P_{(ID)sib}$ values between 0.01 and 0.0001 indicate that the markers possess sufficient resolving power. The GIMLET program was also used to calculate the matching probabilities of each genotype.

To avoid the misinterpretation of tests for population differentiation (Balloux and Lugon-Moulin 2002), population genetic structure was assessed visually by plotting frequency distributions of alleles (when sample size >50 isolates) as well as statistically by using both allelic tests of differentiation in GENEPOP and Weir and Cockerham's (1984) estimates of Wright's (1951) *F*-statistics in FSTAT 2.9.3.2 (Goudet 2001). The F_{ST} values indicate the degree of reduction in heterozygosity in subpopulations due to nonrandom mating, relative to the total population size. Values of F_{ST} range between zero, if there is no genetic differentiation between populations, and a theoretical maximum of one, as allele frequencies diverge. Permutation tests were used to determine the significance of statistical values; *P* values are presented without correction for multiple tests and so we interpret their significance with caution.

RESULTS

Collection of isolates. During spring 2002, a total of 213 single-chain isolations was performed; corresponding figures for spring 2003 and autumn 2003 were 360 and 477, respectively (Table 1). On individual days, between 11 and 73 isolations were performed: in total the populations were sampled on 42 separate occasions (Table 1).

Identification of isolates. SEM confirmed the identity of 20 isolates examined from Helgoland and eight isolates examined from Sylt as *P. pungens*. A representative SEM image of Hel-244 is shown in Figure 2. Measurements and characteristics of cells of these 28 *Pseudo-nitzschia* isolates were consistent with those described for *P. pungens* (Hasle and Syvertsen 1996): an absence of a central interspace, two rows of poroids between the striae, cell length 74–142 μm (up to 164 μm in the North Sea isolates), cell width 3–4.5 μm (2.9–5.4 μm in the North Sea isolates), 9–15 striae per 10 μm, and 9–15 fibulae per 10 μm.

Genotyping of isolates. In total, 548 of the 1050 North Sea *Pseudo-nitzschia* isolates collected were contaminant free and grew sufficiently well to be harvested for DNA extraction (Table 1). Altogether, 464 North Sea *P. pungens* isolates were successfully genotyped (44.2%): 81 from spring 2002, 160 from spring 2003, and 223 from autumn 2003 (Table 1). Including the three Canadian *P. pungens* samples, 467 isolates were genotyped. The attrition of isolates (approximately 55%) was partly due to their contamination (approximately 7%) and the non-amplifiability of DNA (approximately 8%), however, by far the largest contributing factor was the death of isolates before material could be harvested (approximately 40%; Table 1).

At locus PP3, all isolates possessing alleles that differed in length by just 1 bp were genotyped on at least two (and up to four) separate occasions. Overall, 67% of isolates were genotyped more than once at this locus. At locus PP4, 76% of isolates were genotyped at least twice. At the other four loci, approximately 50% of isolates were genotyped on more than one occasion. Overall, 98% of replicate amplifications yielded identical genotypes, thus confirming the reproducibility of the microsatellite technique. In the small number of cases where conflicting genotypes were produced, additional amplification reactions were performed and the majority rule was used to assign the final genotype.

In total, 429 of the 464 genotyped North Sea *P. pungens* isolates (92.5%) were genotyped at all six loci. Between one (at loci PP5 and PP6) and 23 isolates (at locus PP4) could not be genotyped at individual loci

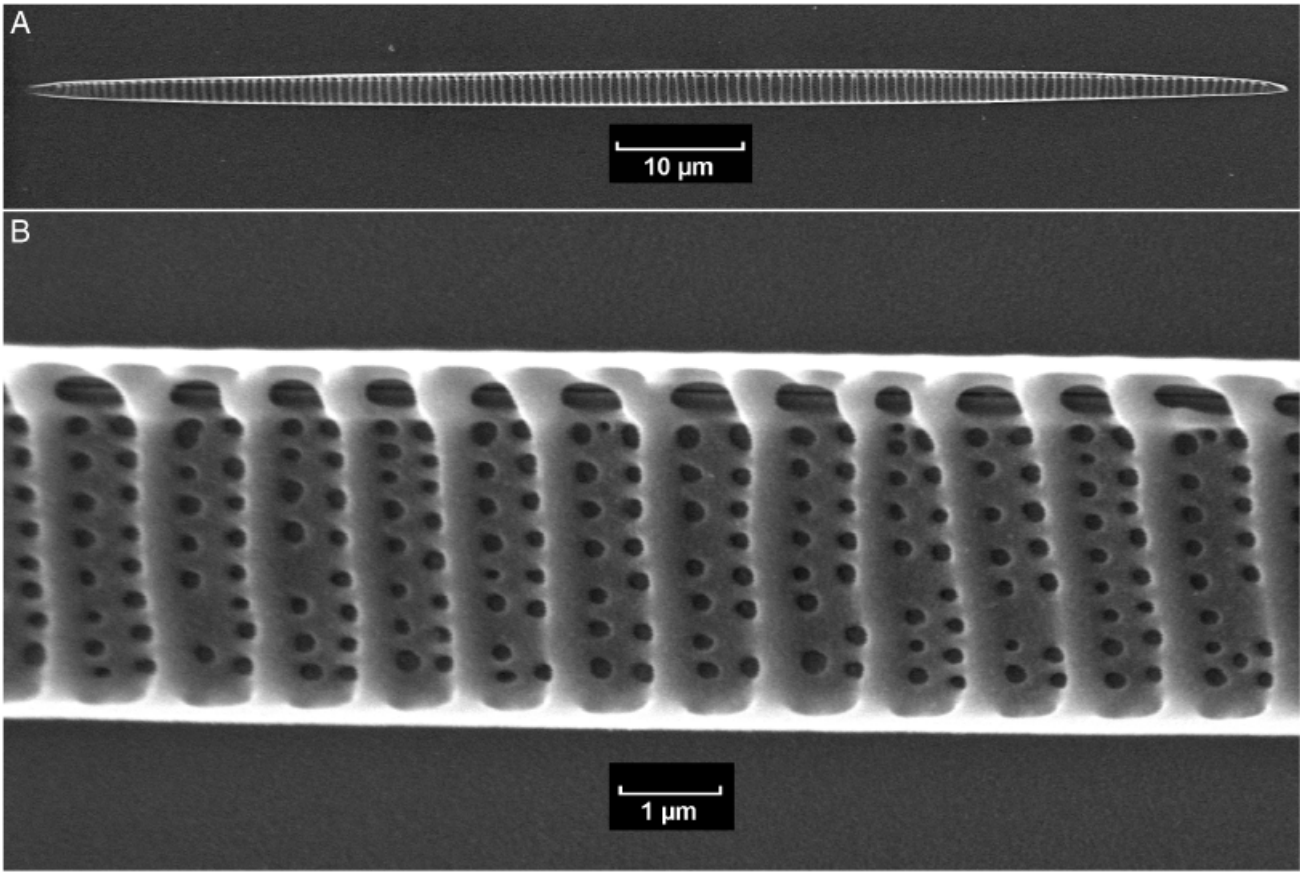

FIG. 2. SEM images of *Pseudo-nitzschia pungens* clone Hel-244, isolated from waters off Helgoland in the North Sea. (A) Entire valve. (B) Details of the valve inner surface.

(Table 2); 29 isolates were missing data at one locus and six isolates were missing data at two loci. Of the 464 genotypes, 453 were different, that is, clonal diversity was 98%. Isolates sharing a genotype were generally isolated during the same sampling period: of the 81 isolates genotyped from Helgoland in spring 2002, two pairs of isolates shared one genotype and four isolates shared another genotype; just two of the 160 isolates genotyped from Helgoland in spring 2003 were identical and these were isolated on the same day (i.e. 1 May); and three pairs of isolates of the 223 genotyped from Sylt in autumn 2003 shared a genotype, with two pairs being isolated on 22 September and the other pair being isolated on 24 September. The two cases of isolates from different sampling periods sharing a genotype were Hel-181 and Sy-140 and Sy-660 and Hel-194.

Analysis of microsatellite data. The numbers of alleles amplified at each locus for the 464 North Sea *P. pungens* isolates ranged from 6 (PP6) to 24 (PP3; Table 2). Altogether, 77 alleles were recorded (an average of 12.5 per locus), 11 more than reported

TABLE 2. Numbers of North Sea *Pseudo-nitzschia pungens* isolates genotyped (n); annealing temperatures (T_a, ° C); size ranges of the alleles (bp); numbers of alleles observed (N_a); numbers of possible genotypes (G_p); numbers of genotypes observed (G_o); and observed (H_o) and expected (H_e) heterozygosities for each of the six microsatellite loci.

Locus	n	T_a	bp	N_a	G_p	G_o	H_o	H_e	$P_{(ID)}$	$P_{(ID)sib}$
PP1	457	58	221–249	11	66	40	0.74	0.75	9.07×10^{-3}	3.96×10^{-1}
PP2	459	60	171–259	10	55	19	0.63	0.63	2.09×10^{-1}	4.88×10^{-1}
PP3	460	58	195–266	24	300	79	0.86	0.86	3.32×10^{-2}	3.28×10^{-1}
PP4	441	55	136–220	17	153	70	0.87	0.85	3.68×10^{-2}	3.34×10^{-1}
PP5	463	55	189–205	7	28	16	0.59	0.59	2.04×10^{-1}	5.06×10^{-1}
PP6	463	55	199–241	6	21	12	0.69	0.67	1.82×10^{-1}	4.64×10^{-1}
									8.60×10^{-7}	4.97×10^{-3}

The probabilities of identity ($P_{(ID)}$ and $P_{(ID)sib}$) are listed for each locus and across all six loci.

previously (Evans and Hayes 2004), largely because of the increased number of isolates genotyped. The three Canadian isolates only introduced two private alleles, one at locus PP5 and one at locus PP6.

The observed heterozygosities per locus ranged from 0.59 to 0.87 (Table 2), indicating a very high degree of genetic diversity within the 464 North Sea *P. pungens* isolates (both mean H_o and H_e were 0.73). Although Evans and Hayes (2004) reported a deficit of heterozygotes at locus PP1, the increased sample size per locus in the current study provided no evidence for significant ($P < 0.05$) heterozygote deficiency or excess at any of the six loci, that is, when all 464 North Sea isolates were considered together, all six loci were in HWE. When isolates from the three sampling periods were considered separately, a significant heterozygote deficiency was detected only at locus PP4 in the isolates collected during autumn 2003 ($P = 0.043$), and a significant excess of heterozygotes was only detected at locus PP4 in isolates collected during spring 2003 ($P = 0.003$). Global tests indicated that overall, there was neither a deficit nor an excess of heterozygotes in any of these three groups.

Of the 15 tests between pairs of loci for genotypic disequilibrium, only loci PP3 and PP4 were significantly linked ($P < 0.001$). When the isolates were split according to the period during which they were isolated, significant linkage ($P < 0.001$) was only detected between loci PP1 and PP3 in the spring 2002 samples, suggesting that genotypic disequilibrium is not a particularly important characteristic of this data set.

Theoretically, this level of polymorphism in the North Sea isolates meant that the six microsatellite markers could distinguish between 9.8×10^{10} different genotypes (Table 2). However, in this study, one or more alleles dominated at each locus; for example, six of the 17 alleles at locus PP4 and two of the seven alleles at locus PP5 were encountered fewer than 10 times (with a uniform distribution of alleles at these loci, each allele should be encountered 52 and 133 times, respectively; Fig. 3). Consequently, the $P_{(ID)}$ value across all six loci indicated a lower resolving power (8.6×10^{-7}; Table 2), albeit clearly within the guidelines of suitability suggested by Waits et al. (2001). The matching probabilities for isolates with identical genotypes from the same sampling period (with the exception of Hel-181, which had data missing at two loci, and Hel-152), strongly suggest that these isolates were the product of asexual reproduction; for example, the matching probabilities for the four identical spring 2002 isolates, Hel-240, Hel-244, Hel-248, and Hel-252, were $P_{(ID)} = 2.0 \times 10^{-7}$ and $P_{(ID)sib} = 5.9 \times 10^{-3}$. In the cases of the isolates with identical genotypes from different sampling periods, that is, Hel-181 and Sy-140, and Sy-660 (data missing at one locus) and Hel-194, the probabilities that these pairs of isolates are truly clonal are lower: $P_{(ID)} = 1.8 \times 10^{-3}$ and $P_{(ID)sib} = 7.3 \times 10^{-2}$, $P_{(ID)} = 3.1 \times 10^{-5}$ and $P_{(ID)sib} = 1.4 \times 10^{-2}$, respectively. The values are greater than the upper limit suggested by Waits et al. (2001), and so it is not possible to conclude with confidence that they were the result of asexual division.

To assess population genetic structure, isolates were assigned to groups. In total, isolates were obtained from 42 days of sampling over the three sampling periods: seven days during spring 2002, 20 days during spring 2003, and 15 days during autumn 2003 (Table 1). The numbers of isolates genotyped from each day of sampling varied from zero to 32. When looking for evidence of genetic structure, groups containing fewer than 10 isolates were excluded from the analyses.

First, isolates were split into three groups according to the sampling period during which they were isolated: 81 isolates from spring 2002, 160 isolates from spring 2003, and 223 isolates from autumn 2003. There were no marked differences between the three groups in the frequency distributions of alleles at any of the six loci (data shown only for loci PP4 and PP5; Fig. 3). Although 16 unique alleles were detected (i.e. found in just one of the three groups), all were introduced by just one isolate. Samples isolated during spring 2002 accounted for only one unique allele, suggesting that the occurrence of unique alleles was largely a function of sample size. The significance of allelic tests and F_{ST} values between the three groups are listed in Table 3. Both statistical tests indicated significant genetic differentiation ($P < 0.05$) only between those isolates collected during spring 2003 and those collected during autumn 2003 (allelic test, $P = 0.0128$; $F_{ST} = 0.0018$, $P = 0.0167$).

Second, tests of genetic differentiation were conducted between days in the different sampling periods represented by ten or more genotyped isolates (20 days out of the 42 sampled): 3 days during spring 2002 (11–26 isolates per day), 8 days during spring 2003 (10–23 isolates per day), and 9 days during autumn 2003 (14–32 isolates per day). In total, 123 different comparisons were performed; 21 allelic tests and 17 F_{ST} values were significant ($P < 0.05$; only results where one or both of the tests were significant are listed; Supplemental Table 4, http://www.blackwellpublishing.com/products/journals/suppmat/JPY/JPY04149/JPY04149sm.htm). Seventeen of the 22 significant between-day comparisons were between sites (i.e. Helgoland vs. Sylt) and five were between years at Helgoland (i.e. spring 2002 vs. spring 2003). Half of the significant tests were attributable to the 15 isolates collected on 4 May 2003 from Helgoland waters (10 of the 21 allelic tests and 9 of the 17 F_{ST} values).

Third, samples were split into two groups according to the location of isolation: 241 isolates from Helgoland (spring 2002 and 2003) and 223 isolates from Sylt (autumn 2003). There were no marked differences between the two groups in the frequency distributions of alleles at each of the six loci (data not shown). The significances of the allelic test and F_{ST} value between the two groups are listed in Table 3. Although the allelic test indicated significant genetic differentiation between isolates collected from the two locations ($P = 0.0219$), the F_{ST} value between the two groups was not quite significant ($F_{ST} = 0.0016$, $P = 0.05$).

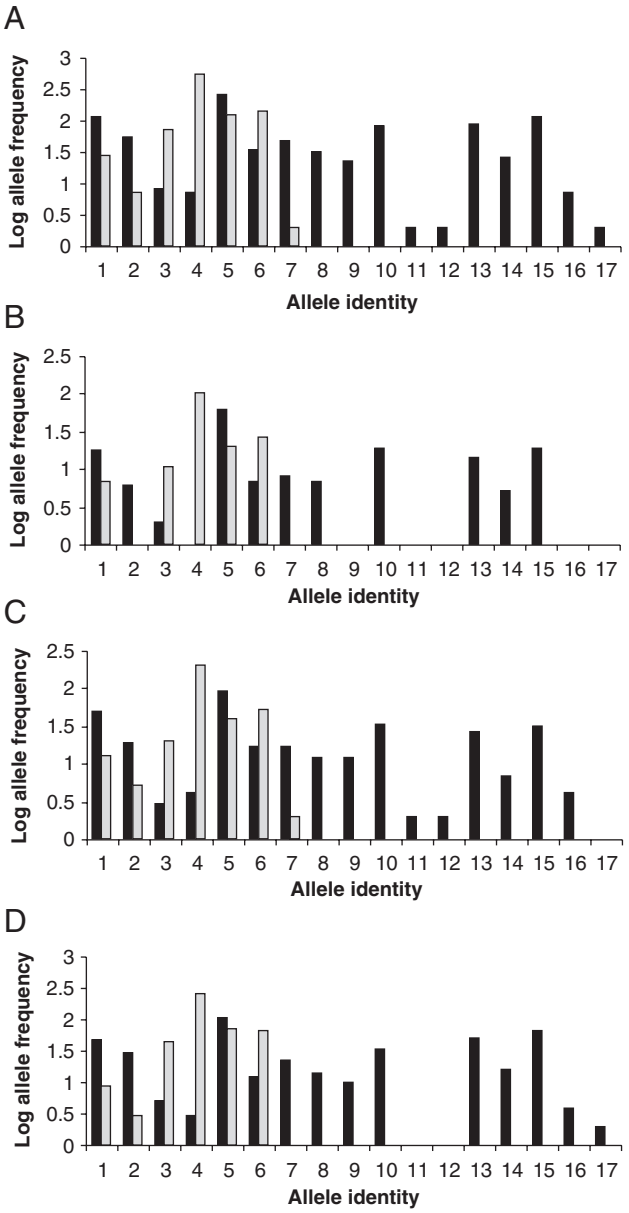

FIG. 3. Allele frequency distributions (logarithmic scales) at loci PP4 (black bars) and PP5 (gray bars): (A) all isolates considered together, (B) isolates from spring 2002, (C) isolates from spring 2003, and (D) isolates from autumn 2003. To allow determination of the log of the allele abundance, all numbers were incremented by one; this was necessary because not all alleles are represented in every sample.

Finally, isolates were split into groups to determine whether there was evidence of genetic differentiation within a sampling period. Isolates collected during spring 2002 were split into two groups collected 14 days apart. The first group included the 56 isolates collected during the first four days of the sampling period (15–18 April), and the second group included the 25 isolates collected toward the end of the sampling period (3–7 May). Isolates collected during spring 2003 and autumn 2003 were split into the same daily groups as detailed in the second analysis (i.e. eight and nine groups, respectively).

TABLE 3. Significance of allelic tests and F_{ST} values between isolates collected during each of the three sampling periods (spring 2002, spring 2003, and autumn 2003) and between isolates collected from Helgoland (includes spring 2002 and 2003) and Sylt (autumn 2003).

Comparison	Allelic test P value	F_{ST} value	F_{ST} P value
Spring 2002 vs. spring 2003	0.4499	0.0007	0.1833
Spring 2002 vs. autumn 2003	0.1499	0.0017	0.1000
Spring 2003 vs. autumn 2003	0.0128[a]	0.0018	0.0167[a]
Helgoland vs. Sylt	0.0219[a]	0.0016	0.0500

[a]Indicates significance at $P < 0.05$.

For these analyses, as before, only those statistical tests that were significant ($P < 0.05$) are reported (Supplemental Table 5, http://www.blackwellpublishing.com/products/journals/suppmat/JPY/JPY04149/JPY04149sm.htm). For those isolates collected during spring 2002, only the allelic test between isolates from the two groups sampled was significant ($P = 0.0342$; Supplemental Table 5). Only a fraction of comparisons between the eight groups of isolates collected during spring 2003 was significant, six of the 28 allelic tests and two of the 28 F_{ST} values (Supplemental Table 5). Most of these significant tests again involved the 15 isolates collected on 4 May 2003 (five of the six allelic tests and both F_{ST} values). Even fewer tests of genetic differentiation between the nine groups of isolates collected during autumn 2003 were significant; only one of the 36 allelic tests and two of the 36 F_{ST} values (Supplemental Table 5.).

Overall, these results indicated that there were neither marked spatial nor temporal patterns of genetic structure within the North Sea *P. pungens* population. Therefore, a similar degree of genetic differentiation between years at one site as between sites in a given year would be expected. This hypothesis was tested in FSTAT by comparing the F_{ST} of group 1 (spring 2002 and 2003, Helgoland) to group 2 (spring 2003, Helgoland, and autumn 2003, Sylt). The one-tailed probability obtained after 10,000 permutations that the F_{ST} value of group 1 was greater than that of group 2 was $P = 0.828$ (i.e. the hypothesis was supported); the degree of temporal genetic differentiation was no different to the degree of spatial genetic differentiation.

DISCUSSION

The use of powerful molecular markers and a large sample size revealed very high degrees of genetic and clonal diversity in the 464 North Sea isolates of *P. pungens*, with evidence of only weak genetic structure over the spatial (100 km) and temporal (18 months) scales sampled.

Approximately 45% of the samples isolated into clonal culture were successfully genotyped (464 of 1050 isolates), with several factors contributing to the lack of success for the remaining 55%: loss of culture

viability, unamplifiable DNA, and contamination by other microalgal species. Probably the most important factor contributing to the loss of culture viability was the 36-h journey (in the dark and without temperature control) from the North Sea islands to the laboratory in Bristol, United Kingdom. In addition, samples collected toward the ends of periods of population growth, particularly those in autumn, may have higher incidences of parasitoid infection (personal observation). For the unamplifiable DNAs, it is possible that either the quality of the extracted DNA was poor or a different *Pseudo-nitzschia* species was isolated into culture; *P. delicatissima* (P. T. Cleve) Heiden, *P. multiseries*, and *P. fraudulenta* (P. T. Cleve) Hasle also occur in the North Sea (Hasle 2002), and previous studies have demonstrated low levels of cross-amplifiability of microsatellite primer pairs even between closely related *Pseudo-nitzschia* species (Evans et al. 2004, Evans and Hayes 2004). Overall, the proportion of isolates genotyped in the current study is greater than those reported for other studies; for example, Rynearson and Armbrust (2004) genotyped 57% of *D. brightwellii* isolates collected (but only 23% at all three loci), and Shankle et al. (2004) genotyped 22% of *Prorocentrum micans* Ehrenberg isolates collected.

Loss of cultures in phytoplankton population genetics studies can lead to an underestimation of genetic and clonal diversities (Gallagher 1980, Medlin et al. 2000). Although this does not seem to have been a particularly relevant issue in the current study, the impact of culture loss and the time required to maintain them can be minimized by developing culture-independent methods (Hayes and Barker 1997). Preliminary experiments conducted by Evans and Hayes (unpublished data) and by Orsini et al. (2004) demonstrate that PCR amplification from single *Pseudo-nitzschia* chains is possible, although further experiments are needed to refine the technique.

The very high degree of clonal diversity detected (98%) is similar to that recorded by Rynearson and Armbrust (2004); just two pairs of 265 *D. brightwellii* isolates possessed identical genotypes. In contrast, studies that investigated the genetic structure of populations of *P. delicatissima* (Orsini et al. 2004) and the cyanobacterim *Nodularia* (Barker et al. 2000, Hayes et al. 2002) documented reduced levels of clonal diversity related to bloom formation, suggesting that selection occurred during population development. The nature of the North Sea may prevent the dominance of small numbers of clones, even during bloom periods when asexual reproduction presumably dominates.

The nine instances where identical genotypes were detected within the North Sea *P. pungens* isolates were generally restricted to the same sampling period. The exceptions were two pairs of isolates collected during spring 2002 and autumn 2003. However, because of missing data, $P_{(ID)sib}$ values were higher than the upper limit (0.01) suggested by Waits et al. (2001), and so it is not possible to say with confidence that these isolates were truly clonal. In contrast, the $P_{(ID)sib}$ values for the identical isolates collected within sampling periods were all lower than 0.007 (with the exception of the one isolate with missing data), indicating that these isolates are highly likely to be true clones. The higher proportion of identical isolates from the first sampling period is possibly because plankton samples were kept in the laboratory for a 24-h period before isolations were carried out, thus giving time for postsampling cell division to occur.

Although it is evident that asexual reproduction occurs in field populations of *P. pungens*, the low levels of linkage between pairs of loci (1 of 15 pairs), the high clonal diversity, the high levels of gene diversity and observed heterozygosity (mean H_e and $H_o = 0.73$), and the fact that loci were in HWE suggest that sexual reproduction is also important, perhaps even during periods of rapid population growth. Considering the estimated length of time (3 years) between sexual events in *P. multiseries* clonal lineages (Davidovich and Bates 1998), the closest relative to *P. pungens*, the fact that all loci were in HWE is intriguing. Rynearson and Armbrust (2004), in contrast, detected deviations from HWE (heterozygote deficiencies) at the majority of loci in populations of *D. brightwellii*, a result more in line with expectation for a predominantly asexually reproducing organism. However, because so little is known about the timing and magnitude of sexual reproduction in field populations of phytoplankton species, including *Pseudo-nitzschia* (Davidovich and Bates 1998), it is very difficult to quantify its importance in contributing to the observed levels of genetic variation. Propagule banks also have the potential to influence patterns of genetic variation in phytoplankton populations, although to date no resting stages have ever been recorded for *Pseudo-nitzschia* species (Itakura et al. 1997, McQuoid 2002). Instead, it is perhaps more likely that during nonbloom periods, vegetative cells persist; several studies found healthy *Pseudo-nitzschia* cells at depth at very low irradiance (Trainer et al. 1998a, Rines et al. 2002). Again though, lack of relevant knowledge makes it impossible to quantify the importance of vegetative cells or resting stages (if they exist) in influencing the genetic variability of the population.

Overall, only a small fraction of the tests conducted between groups of *P. pungens* isolates for genetic differentiation was significant ($P < 0.05$; Table 3 and Supplemental Tables 4 and 5, http://www.blackwellpublishing.com/products/journals/suppmat/JPY/JPY04149/JPY04149sm.htm): 31 of the 192 allelic tests (16%) and 22 of the 192 F_{ST} values (11%) were significant, but the values ranged from just 0.0018 to 0.0389, indicating weak genetic structure (Wright 1978). For the sample groups that included 50 or more isolates (i.e. comparisons between sampling periods and locations), visual examination of allele frequency distributions confirmed that there were no marked differences between groups, unique alleles being represented by just one (in the case of the comparison between the three sampling periods) or a few isolates (in the case of the comparison between the two sampling locations).

For the comparisons between sampling days, it is relevant that due to smaller sample sizes (10–32 isolates per group), there were likely to be significant skews in the distributions of alleles between groups, leading to more significant allelic tests than significant F_{ST} values, and that because corrections for multiple tests were not made to P values, results that are not highly significant (i.e. $P > 0.01$) should be interpreted with caution.

One further factor to consider is that because isolates were collected from different locations and at different times, due to the movement of water bodies, it is difficult to apportion the observed genetic structure accurately to its spatial and temporal components. This is particularly true for those groups of isolates collected many months apart and from different locations; this factor may partly account for the nonsignificance of the test to determine the relative importance of temporal versus spatial genetic differentiation. Between sampling periods and locations there was evidence of significant but weak genetic differentiation between isolates collected during spring 2003 and isolates collected during autumn 2003 and between isolates ori ginating from Helgoland waters and isolates originating from Sylt waters. The long-term anticlockwise circulation in the North Sea may explain the low level of genetic differentiation between sampling periods, because it is expected that the population sampled from Helgoland waters will disperse to Sylt but over a long enough time scale for there to be a detectable difference in genetic structure.

Significant but weak genetic differentiation was also demonstrated between individual days belonging to the different sampling periods, although a high proportion of significant tests was attributable to the 15 isolates collected on 4 May 2003 from Helgoland waters (48% of the allelic tests and 53% of the F_{ST} values; Supplemental Table 4, http://www.blackwellpublishing.com/products/journals/suppmat/JPY/JPY04149/JPY04149sm.htm). It is difficult to account for the genetic distinctiveness of this group. Because these isolates were collected at a weekend, they originated from harbor waters rather than from offshore. Harbor waters are less mixed, and so it is possible that their partial isolation led to the observed genetic differentiation. However, other isolates collected from the same harbor but at different times were not similarly differentiated. It is also possible that this result was due to the small sample size involved and that if more isolates collected on this day had been genotyped, such a pattern may not have emerged.

Within the three sampling periods there was also limited evidence of weak genetic differentiation (12% of the allelic tests and 6% of the F_{ST} values were significant; Supplemental Table 5, http://www.blackwellpublishing.com/products/journals/suppmat/JPY/JPY04149/JPY04149sm.htm). Within the spring 2003 sampling period, the 15 isolates collected on 4 May were again involved in a large proportion of the significant tests (five of the six allelic tests and both of the F_{ST} values; Supplemental Table 5). The observed temporal patterns within sampling periods were not straightforward; for example, isolates collected from consecutive days during a sampling period were sometimes significantly differentiated (e.g. 28 and 29 September 2003; Supplemental Table 5), whereas those collected on days further apart were not.

The generally low levels of genetic differentiation together with the high levels of genetic diversity suggest that there are no major barriers to gene flow between *P. pungens* isolates in the German North Sea. It is therefore likely that the 464 isolates originated from one single large and generally well-mixed population. Similarly, low levels of genetic differentiation have been reported in other planktonic organisms; for example, Shankle et al. (2004) recorded just one significant Φ_{ST} value of 0.043 between groups of *P. micans* isolates sampled over a period of 2 years. Rynearson and Armbrust (2004), in contrast, reported F_{ST} values of up to 0.245 between populations of *D. brightwellii*; this degree of genetic differentiation was associated with barriers to dispersal, such as partial isolation in different estuaries or water bodies and/or adaptation to local conditions.

To determine the scale at which marked genetic differentiation can be detected within *P. pungens*, populations would have to be sampled over longer time periods and over larger spatial scales. The only *P. pungens* isolates collected from outside the North Sea in the current study were three isolated from eastern Canadian waters in 2002. Despite the extensive physical separation of the collection sites, these three isolates only introduced two new alleles. This contrasts markedly with a study of *P. multiseries*, where a single Pacific isolate introduced 11 new alleles at six microsatellite loci in a study predominantly based on Canadian Atlantic isolates (Evans et al. 2004). The introduction of fewer alleles by the Canadian isolates in the current study could be because there are fewer obvious barriers to the flow of water from the Atlantic to the North Sea than from the Pacific Ocean to the Canadian east coast. Alternatively, it is possible that *P. pungens* is a truly cosmopolitan diatom species, being able to tolerate a wide range of environmental conditions with few barriers impeding its dispersal.

Our results, in combination with those obtained by Rynearson and Armbrust (2004), demonstrate that diatom population genetics and population dynamics are complex, suggesting that many different factors are involved. Much more research is needed if we are to understand the true distribution of species and the dynamics of their populations over both local and biogeographical scales, a particularly important goal for toxic species such as *P. multiseries*. Such research will also provide necessary empirical tests of the recently resurfaced hypothesis that most microorganisms are cosmopolitan in their distributions (Finlay 2002). Because of problems of cryptic speciation, however, the use of microsatellite markers alone is not sufficient; instead an integrative approach is required.

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