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GENETIC POPULATION STRUCTURE OF *PSEUDO-NITZSCHIA PUNGENS* (BACILLARIOPHYCEAE) FROM THE PACIFIC NORTHWEST AND THE NORTH SEA[1]

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Several species of the diatom *Pseudo-nitzschia* produce the neurotoxin domoic acid (DA). Consumption of fish and shellfish that have accumulated this potent excitotoxin has resulted in severe illness and even death in humans, marine mammals, and seabirds. *Pseudo-nitzschia pungens* (Grunow ex Cleve) Hasle is a cosmopolitan diatom commonly occurring in the waters of the Pacific Northwest (PNW) and the eastern North Atlantic, including the North Sea. However, genetic and physiological relationships among populations throughout this large geographic distribution have not been assessed. Population genetic parameters (e.g., Hardy–Weinberg equilibrium, linkage equilibrium, F_{ST}) calculated for *P. pungens* collected from the Juan de Fuca eddy region in the PNW indicated the presence of two distinct groups that were more divergent from each other than either was from a *P. pungens* sample from the North Sea. Geographic heterogeneity was also detected within each of the two PNW groups. These results suggested that the populations of *P. pungens* recently mixed in the Juan de Fuca eddy region (a seasonally retentive feature off the coasts of Washington State, USA, and Vancouver Island, Canada) but did not exchange genetic material by sexual reproduction. Alternatively, these two groups may be cryptic (morphologically identical, but reproductively isolated) species. Identifying cryptic diversity in *Pseudo-nitzschia* is important for bloom prediction and aiding the identification of molecular markers that can be used for rapid detection assay development.

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

Abbreviations: **DA, domoic acid; HWE, Hardy–Weinberg equilibrium; PNW, Pacific Northwest; WC, whole cell**

Diatoms are distributed in almost all aquatic habitats and form important components of marine and freshwater food webs. Species of only three diatom genera (*Amphora*, *Nitzschia*, and *Pseudo-nitzschia*) have been shown to produce the neurotoxin domoic acid (DA) (Bates 2000). DA-producing diatoms can have adverse effects on ecosystems, including commercially and recreationally exploited shellfish, resulting in considerable ecological, human health, and economic impacts (HARRNESS 2005). Although tens of thousands of diatom species are already described, Mann (1999) suggested that species boundaries may have been drawn too broadly and many species likely contain several reproductively isolated entities worthy of recognition at the species level. Such cryptic species may be of particular interest in the study of the genus *Pseudo-nitzschia,* in which at least 12 of the ~30 described species produce measurable amounts of the neurotoxin DA (Hasle and Syvertsen 1997, Bates 2000, Fryxell and Hasle 2003, Lundholm et al. 2003, Trainer et al. 2008).

Molecular analyses have revealed genetically distinct groupings within several morphologically defined diatom species (Gallagher 1980, Lundholm et al. 2003, Montresor et al. 2003, Rynearson and Armbrust 2004, Sarno et al. 2005), though studies using microsatellite markers for population genetic analysis in diatoms are still relatively limited. Microsatellites have become a commonly used marker in population genetics due to their codominant inheritance, ubiquity in the genome, and the advantage of rapid and cost-efficient screening (Balloux and Goudet 2002). Furthermore, microsatellites reveal much higher genetic diversity per locus than other markers, such as allozymes, providing higher statistical power in population identification and the distinction between sexual and clonal reproduction (Halkett et al. 2005). For example, *Ditylum brightwellii* (Rynearson and Armbrust 2000, 2004) in the Strait of Juan de Fuca and Puget Sound showed very clear population structure, probably caused by adaptation of cells to the water properties of their respective

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environments (Rynearson and Armbrust 2004). In contrast, little temporal and spatial genetic differentiation was found in *P. pungens* in the North Sea, suggesting extensive dispersal and genetic panmixia (Evans et al. 2005). Additionally, Evans et al. (2004) demonstrated a high degree of variation among isolates of *Pseudo-nitzschia multiseries* (Hasle) Hasle from Canada, Europe, and Russia. Such population analyses provide particular insights when they can be combined with pertinent features of the oceanic environment like currents, eddies, and fronts that could contribute to or limit the exchange of cells between areas.

In the Pacific Northwest (PNW), the Juan de Fuca eddy (Fig. 1a) has been identified as a retentive site for phytoplankton and is a confirmed initiation site for toxic *Pseudo-nitzschia* blooms that can impact areas on the outer coast of Washington State, USA (Trainer et al. 2009). *P. pungens*, specifically, is frequently observed in the PNW where it produces low levels of DA (Trainer et al. 1998) and therefore serves as a model to help gain insight into the population structure of other potentially toxic *Pseudo-nitzschia* species. The Juan de Fuca eddy is situated in a region where oceanographic processes work to mix water masses, as well as any associated toxic cells, from sources such as waters from depth (Trainer et al. 1998, Rines et al. 2002), outflow from the Strait of Juan de Fuca (Freeland and Denman 1982), and the California undercurrent (Hickey and Banas 2003).

P. pungens commonly occurs in both PNW (Horner et al. 2000) and North Sea (Casteleyn et al. 2008) waters, making these two sites ideal candidates for comparing genetic population structure in this diatom species. This study had two main objectives. First, we investigated differences in the population structure of *P. pungens* from the two different ocean basins (the PNW, Pacific, and the North Sea, Atlantic). Second, we examined the genetic population structure of the PNW *P. pungens* to explore the possibility of identifying the sources of toxic cells that impact human and ecosystem health in the PNW region.

MATERIALS AND METHODS

Sample collection. Surface water plankton samples were collected using a net (25 cm diameter x 60 cm length; 20 μm Nitex® mesh; Research Nets Inc., Bothell, WA, USA) deployed by hand over the side of the *R/V Atlantis* in September 2004 and September 2005 as part of the Ecology and Oceanography of Harmful Algal Blooms in the Pacific Northwest (ECOHAB-PNW) effort. *P. pungens* cells were isolated from single plankton net tows collected at one site within Barkley Sound (BS) in 2004 and at four sites in offshore waters at the edge of the Juan de Fuca eddy region in 2005 (E1, E2, E3, E4) (Fig. 1a). DNA from 14 *P. pungens* isolates from the North Sea waters of the German island of Helgoland and one isolate from the German island of Sylt was provided by Katharine Evans of the University of Bristol. Frozen aliquots of *P. pungens* culture were obtained from Griet Casteleyn of Ghent University for 10 isolates from the waters of the southern North Sea (Fig. 1b).

Cell isolation, identification, culture maintenance, and harvesting. The whole cell (WC) hybridization assay, which relies on a set

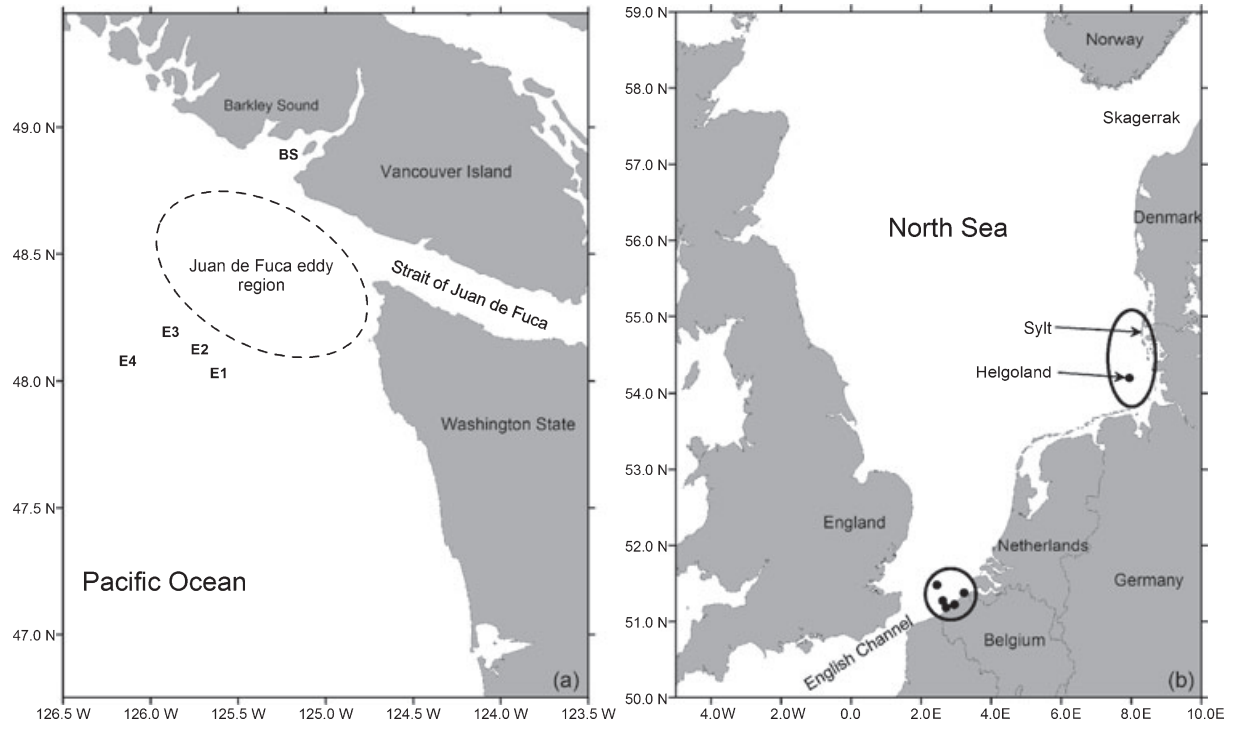

FIG. 1. (a) Pacific Northwest sample locations. *Pseudo-nitzschia pungens* was collected in 2004 (BS) and 2005 (E1, E2, E3, E4). The approximate location of the Juan de Fuca eddy is noted by the dotted ellipse. (b) North Sea sample locations. Ten *P. pungens* isolates were collected from the southern North Sea near Belgium. Fifteen isolates were from the waters off the islands of Helgoland and Sylt.

of *Pseudo-nitzschia* species-specific fluorescent probes that target LSU rRNA sequences, was performed to confirm the presence of *P. pungens* in the PNW plankton net samples (Miller and Scholin 1996, see below for details). Two to three drops of net tow sample were added to 0.75 mL f/2 growth medium (Guillard and Ryther 1962) in 48-well plates and maintained in either an on-deck flowing seawater incubator with ambient sunlight or in a temperature- and light-controlled incubator for up to 15 d prior to isolation in the laboratory. A single presumptive *P. pungens* cell or chain was isolated from each well of the 48-well plates with a drawn out capillary pipette, then rinsed three times in sterile f/2 medium and placed into an individual well of a new 48-well plate containing f/2 medium (0.75 mL). The plates were maintained in a temperature- and light-controlled incubator at 12°C on a 12:12 light:dark (L:D) cycle and at a photon flux density of ~85 μEinsteins $\cdot$ $m^{-2} \cdot s^{-1}$. The isolates were examined microscopically for contamination with other algal species every 2 d, and cultures were reisolated where necessary. Once the isolates were free of contamination and had grown to a high cell density, they were transferred to glass culture tubes (20 × 150 mm) containing f/2 medium (15 mL) and maintained under the same incubation conditions described above. When the cultures reached a high cell density (visibly colored growth medium), ~0.5 mL from each was transferred to fresh f/2 medium, while the remaining culture was harvested onto nitrocellulose filters (Millipore HA, 25 mm diameter, 0.45 μm; Millipore, Billerica, MA, USA) and stored at −80°C until DNA extraction. It should be noted that bias can be introduced in these population studies because the data are generated only from diatoms that grow in f/2 medium.

The WC assay was performed on each culture to confirm positive identification of *P. pungens* cells. Fixatives and buffers for the WC assay were made following Miller and Scholin (2000): 25X SET buffer (3.75 M NaCl, 25mM EDTA, 0.5 M Tris, pH 7.8); modified saline ethanol (73.5% high grade 95% ethanol, 16.5% RNase free water, 10% 25X SET buffer); hybridization buffer (78.8% RNase free water, 20% 25X SET buffer, 1% IGEPAL-CA630, 0.2% polyadenylic acid). LSU rRNA probes (puD1, uniC, uniR) were obtained following the sequences described in Miller and Scholin (2000). A 1.0 mL aliquot of live culture was filtered onto a 25 mm polycarbonate membrane filter and fixed with 5.0 mL modified saline ethanol for ~2 h. The filter was then rinsed with 1.0 mL of 45°C hybridization buffer and resuspended in 0.5 mL 45°C hybridization buffer to which was added 12 μL of the *P. pungens*–specific probe (puD1). Additionally, positive and negative controls (uniC and uniR probes, respectively) were prepared using *P. pungens* cultures along with each set of isolates analyzed by the WC assay using the above steps. The probes were then allowed to hybridize at 45°C for ~2 h. The unbound probe was washed away, and the filter was viewed with an epifluorescence microscope (Zeiss Axiostar plus; Carl Zeiss MicroImaging Inc., Thornwood, NY, USA) where isolates were identified as *P. pungens* if there was noticeable fluorescence compared to the negative control. A subset of the isolates was analyzed by SEM for morphological characteristics, for example, rows of poroids in the frustule, described in Hasle et al. (1996) to confirm the results of the WC assay.

DNA extraction and quantitation. DNA was extracted from the harvested PNW *P. pungens* cultures and the southern North Sea aliquots using Qiagen (Valencia, CA, USA) DNeasy Plant Mini kits with the following modifications. To prepare the material for the extraction procedure, 500 μL Qiagen lysis buffer, 5.0 μL RNase A (Qiagen, 100 mg $\cdot$ mL^{-1}) and 2.5 μL Proteinase K (Qiagen, 20 mg $\cdot$ mL^{-1}) were added directly to the tube containing the filter with the harvested culture. The tubes were then vortexed at the highest setting for 30 s and centrifuged (Eppendorf 5415 C; Eppendorf AG, Hamburg, Germany) for 5 min (5,223*g*) to pellet the cells. The filters were removed, and the tubes were vortexed for 2–3 s to resuspend the material. The thawed cultures from the southern North Sea were centrifuged, and the supernatant was removed prior to the addition of lysis buffer, RNase A, and Proteinase K. Following incubation at 65°C for 1 h, the Qiagen procedure was followed according to the manufacturer's instructions, with only one elution in 70 μL of Qiagen buffer AE. DNA was quantified by PicoGreen staining on an FLx800 plate reader (Biotek Instruments Inc., Winooski, VT, USA), and DNA concentration was normalized to 0.2 ng $\cdot$ μL^{-1}.

PCR and genotyping. PCR was used to amplify four microsatellite loci (PP2, PP3, PP5, and PP6) using labeled primers developed by Evans and Hayes (2004). PCR was carried out in 20 μL reaction volumes containing 2.0 ng DNA and 1.5 mM (for PP2), 2.0 mM (for PP3, and PP5) or 2.5 mM $MgCl_2$ (for PP6). Volumes and concentrations of all other PCR reaction components (primers, buffer, *Taq* polymerase, and dNTPs) followed Evans and Hayes (2004). After a 3 min initial denaturing step at 94°C, 35 cycles were performed: 1 min at 94°C (denaturing), 1 min at 56°C (annealing), and 30 s at 72°C (extension). This step was followed by a final extension step at 72°C for 20 min. Amplified fragments were separated on an ABI 3100 (Applied Biosystems, Foster City, CA, USA) genetic analyzer, and allele sizes were estimated using the ABI GeneScan and Genotyper software.

Analysis of microsatellite data. GENEPOP version 3.4 (Raymond and Rousset 1995) was used to calculate basic descriptive population genetic statistics including allele frequencies, numbers of alleles, observed heterozygosity (H_o), and expected heterozygosity (H_e) for each sample. Allelic richness, a measure of the number of alleles independent of sample size, was calculated per locus for each sample in FSTAT version 2.9.3.2 (Goudet 2001). The program MICRO-CHECKER (Van Oosterhout et al. 2004) was used to check for errors due to large allele dropout and stuttering.

Concordance of genotypic distributions to Hardy–Weinberg expectations was determined by exact tests (Guo and Thompson 1992) in GENEPOP, with Markov chain parameters of 1,000 for dememorization number, 300 batches, and 1,000 iterations per batch. Statistical significance is reported both before and after nonsequential Bonferroni correction (Rice 1989). The F_{IS} statistic, a measure of deviation from Hardy–Weinberg equilibrium (HWE, Wright 1951) was estimated following Weir and Cockerham (1984) in FSTAT. The program MULTILOCUS version 1.2 (Agapow and Burt 2001) was used to calculate $\bar{r}_d$ a measure of linkage disequilibrium (the nonrandom association of alleles at different loci). One thousand randomizations of the data for each sample were performed to test whether the observed $\bar{r}_d$ values were significantly larger than expected if alleles assorted at random. F_{ST} (Wright 1951), an estimator of genetic population differentiation, was estimated following Weir and Cockerham (1984), and exact tests for pair-wise differentiation were performed in ARLEQUIN 3.1 (Excoffier et al. 2005). Significance of the pair-wise F_{ST} tests is reported before and after nonsequential Bonferroni corrections. Pair-wise R_{ST} (Slatkin 1995), a measure of population differentiation similar to F_{ST} that also takes microsatellite allele size into account, was calculated in GENEPOP.

The software STRUCTURE version 2.1 (Pritchard et al. 2000) was used to examine the substructure in the PNW samples without a priori identification of populations. STRUCTURE employs a Bayesian model-based clustering method using multilocus genotype data to infer population structure and assign individuals to populations that best conform to HWE and linkage equilibrium. STRUCTURE was run using the ancestry with admixture model, 100,000 burn-in iterations, and a run length of 100,000 iterations assuming one to 10 distinct clusters (K). Vähä and Primmer (2006) suggest that a *q*-value, the proportion of the genotype of an individual that is derived

from a given cluster, of 0.10 be used as the most efficient threshold to separate between purebred and hybrid individuals. Therefore, only isolates with proportions ≥0.9 for a specific cluster were retained in that population for subsequent analyses. Microsatellite data from the populations identified by STRUCTURE were analyzed for HWE, linkage disequilibrium, and population differentiation as described above. Additionally, a standardized genetic differentiation measure allowing for differences in within population variability, G'_{ST}, was calculated according to Hedrick (2005). Within-population structure was analyzed after isolates were sorted according to their sample of origin (BS, E1, E2, E3, and E4). Pair-wise F_{ST} values were then calculated, and an analysis of molecular variance (AMOVA) performed in ARLEQUIN.

RESULTS

Identification and genotyping. A total of 175 *P. pungens* isolates collected from five locations (Fig. 1a) were identified and genotyped for this study. SEM was performed on five of these isolates and confirmed the *P. pungens* identifications made using the WC (Miller and Scholin 1996) assay. Of these isolates, 172 were successfully genotyped at all four loci, revealing 147 unique multilocus genotypes.

Microsatellite data analysis. Analysis with MICROCHECKER indicated that for both the pooled PNW sample and the North Sea sample, there was no evidence for scoring errors due to large allele dropout or stutter at any locus. General descriptive statistics, including allele size ranges, number of alleles, allelic richness, observed number of genotypes, observed heterozygosity, and expected heterozygosity, for all samples are given in Table S1 (in the supplementary material). The pooled PNW sample deviated from HWE and had a significant deficit of heterozygotes at all loci, while there were no significant deviations in the North Sea sample, both before and after nonsequential Bonferroni correction (Table 1). The pooled and individual PNW samples showed higher multilocus linkage ($\bar{r}_d$) than would be expected in ideal sexually reproducing populations (Table 1). Conversely, the $\bar{r}_d$ value for the North Sea sample was not significantly larger than that of the randomized data, which suggested random assortment of alleles in this sample (Table 1).

Significant genetic differentiation (F_{ST}) was detected between the pooled PNW and the North Sea samples (0.242, $P < 0.05$) as well as between the individual PNW samples and the North Sea sample ($P < 0.05$, Table 2). R_{ST} was also high between the pooled PNW and North Sea samples (0.472) as well as between the individual PNW samples and the North Sea sample (Table 2). R_{ST} was always greater than F_{ST}, reflecting the difference in allele sizes among samples. Comparisons made between the PNW and North Sea *P. pungens* should be interpreted with caution due to the small size of the North Sea sample and the broad geographic extent from which the isolates were collected.

PNW population structure analysis. Data analysis using the program STRUCTURE indicated that the PNW sample set was likely made up of a mixture of two populations (Fig. S1 in the supplementary

TABLE 1. HWE (Hardy–Weinberg Equilibrium) probability *P*-values, F_{IS} (measure of deviation from HWE), and linkage equilibrium for the individual Pacific Northwest (PNW) samples, pooled PNW, North Sea, PNW1[a], and PNW2[a].

	Pacific Northwest sites								
Locus	BS	E1	E2	E3	E4	All PNW	PNW1[a]	PNW2[a]	North Sea
PP2									
HWE	**0.0014**[b]	**0.0000**[b]	0.0425[b]	0.0000[b]	0.0239[b]	**0.0000**[b]	0.6115	0.3646	0.8545
F_{IS} (W&C)	0.052	0.117[c]	0.138	0.016[c]	0.095	**0.064**[c]	−0.030	−0.090	−0.103
PP3									
HWE	0.0812	0.2283	0.0121[b]	0.0049[b]	0.2434	**0.0000**[b]	0.4039	0.5853	0.8229
F_{IS} (W&C)	0.120	0.107	0.180[c]	0.017[c]	0.147	**0.082**[c]	0.099	0.028	−0.076
PP5									
HWE	**0.0000**[b]	0.0950	**0.0001**[b]	**0.0000**[b]	0.0309[b]	**0.0000**[b]	**0.0001**[b]	0.2768	0.3445
F_{IS} (W&C)	**0.572**[c]	0.016	**0.187**[c]	**0.203**[c]	0.139[c]	**0.221**[c]	0.328[c]	0.076	0.074
PP6									
HWE	**0.0000**[b]	0.0079[b]	**0.0001**[b]	**0.0000**[b]	**0.0002**[b]	0.0000[b]	0.0619	n.d.	0.9085
F_{IS} (W&C)	0.516[c]	**0.447**[c]	**0.718**[c]	0.494[c]	**0.660**[c]	**0.557**[c]	0.114	n.d.	−0.114
Overall									
HWE	**H.S.**[b]	0.0000[b]	**0.0000**[b]	**H.S.**[b]	**0.0000**[b]	**H.S.**[b]	**0.0009**[b]	0.4626	0.9326
F_{IS} (W&C)	0.281	0.132	0.268	0.119	0.212	0.179	0.126	0.005	−0.060
$\bar{r}_d$	0.6273[d]	0.4172[d]	0.3939[d]	0.4043[d]	0.4971[d]	0.4496[d]	0.0446	−0.0113	0.0282

[a]PNW1 and PNW2 are the two populations identified through analysis using the STRUCTURE program.

[b]Significant deviation from HWE ($P < 0.05$); bold HWE values indicate significant deviation from HWE after nonsequential Bonferroni correction. H.S., highly significant. F_{IS} (W&C): measure of deviation from HWE calculated according to Weir and Cockerham (1984).

[c]Significant deviation from HWE and significant deficit of heterozygotes ($P < 0.05$); bold F_{IS} values indicate significant deficit of heterozygotes after nonsequential Bonferroni correction.

[d]Larger than expected value based on 1,000 randomizations of the data ($P < 0.001$). n.d., not determined. Overall F_{IS} was calculated over all loci.

TABLE 2. Values for pair-wise population differentiation estimators F_{ST} (lower half-matrix) and R_{ST} (upper half-matrix) for the individual Pacific Northwest samples and the North Sea.

	North Sea	Pacific Northwest Sites				
		BS	E1	E2	E3	E4
North Sea		0.343	0.370	0.401	0.441	0.310
BS	**0.237**[a]		−0.019	−0.011	0.008	−0.024
E1	**0.263**[a]	0.002		0.011	−0.009	−0.018
E2	**0.211**[a]	0.020	0.009		0.065	−0.006
E3	**0.260**[a]	0.006	−0.001	0.015		0.003
E4	**0.194**[a]	0.025	0.017	−0.002	0.013	

[a]Significant pair-wise test for differentiation ($P < 0.05$). F_{ST} is an estimator of population differentiation using allele frequency data, while R_{ST} is an estimator of population differentiation that uses allele size as well as allele frequency. F_{ST} values in bold are significant after nonsequential Bonferroni correction. Negative F_{ST} values can be caused by corrections for unequal sample size in the method of Weir and Cockerham (Feder et al. 1990). Negative R_{ST} values indicate that within-population variance in allele size is greater than the between-population variance (Goodman 1997).

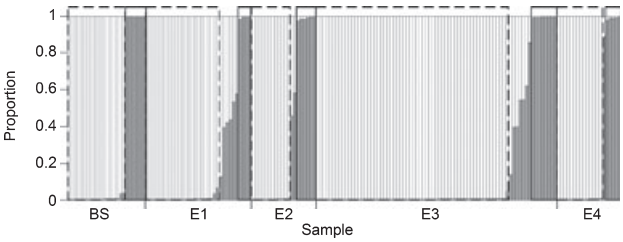

FIG. 2. Population assignments of Pacific Northwest (PNW) samples as determined by STRUCTURE. Each vertical bar represents an individual *Pseudo-nitzschia pungens* isolate. The isolates are grouped according to their original sample designations (i.e., BS, E1, E2, E3, E4), which are shown on the *x*-axis. For instance, sample E3 contained more isolates than sample E4, so the area occupied by E3 in this figure is greater than that of E4. The *y*-axis is the proportion of alleles derived from each population. Each vertical bar is partitioned into two shaded segments (gray and white). Each shaded segment represents the estimated membership fraction of that isolate in each of the populations. Predominantly gray bars (surrounded by solid rectangle) represent PNW1, while the predominantly white bars (surrounded by dashed rectangle) indicate PNW2. Isolates with intermediate proportions are potentially interspecific hybrids and were not assigned to either population.

material). Most isolates belonged to one of these two populations, PNW1 and PNW2 (Fig. 2), and the proportion of alleles originating from each of the two populations in each isolate followed a trimodal distribution ($q = 0$, 0.5, and 1, Fig. S2 in the supplementary material). Those isolates with intermediate proportions (≥0.1 and <0.9) represent potential hybridization between the two populations. Isolates with a proportion of alleles ≥0.90 stemming from one population were assigned to that population because this approach excluded the potential hybrids.

Allele frequency data for PNW1 and PNW2 (Fig. 3), plotted with the pooled PNW and North Sea samples, showed some overlap in allele size classes for loci PP2, PP3, and PP5, but there was no overlap at locus PP6 for PNW1 and PNW2. PNW1 did not deviate from HWE at loci PP2, PP3, or PP6 (Table 1), although there was a significant deficit of heterozygotes at locus PP5. PNW2 did not deviate from HWE at the three loci where variability was detected (PP2, PP3, PP5, Table 1). Over all loci, PNW1 deviated from HWE while PNW2 did not. The $\bar{r}_d$ values for PNW1 and PNW2 were similar to those in the North Sea (Table 1), indicating a random association of alleles within these two populations. Pair-wise tests of differentiation (F_{ST} and R_{ST}) for PNW1, PNW2, and the North Sea showed a high level of differentiation between all population pairs (Table 3), and R_{ST} values were always higher than F_{ST} values. Standardized genetic differentiation (G'_{ST}) indicated that when comparisons were made between population pairs, the variation due to differences between the pairs was 96% for the North Sea/PNW1 pair and also 96% for the PNW1/PNW2 pair, while 70% of variation was due to differences between the North Sea /PNW2 pair.

Subpopulation structure within PNW1 and PNW2 is shown in Table 4, although given the small sample sizes, the results should be interpreted with caution. All of the PNW1 components were significantly different from PNW2 components, and AMOVA results indicated that 41% of the variation was due to differentiation between the two groups, 2% was due to differentiation between sites within groups, and 57% was due to variation within sites. In PNW1, the BS sample was significantly differentiated from all the other samples ($P < 0.05$), and pair-wise F_{ST} values were relatively high, though after Bonferroni correction, only one pair-wise comparison was significant (Table 4). Similarly, E4 was differentiated in PNW2, though F_{ST} values were lower and mostly not significant after Bonferroni correction.

DISCUSSION

Our microsatellite data demonstrated the existence of two populations of *P. pungens* in the PNW, which were genetically as divergent from each other as each of them was from conspecifics collected in a different ocean basin, the North Sea. Although the taxonomic status of these groups is presently unclear, our results support the notion of high cryptic diversity within marine diatoms (Mann 1999). Our results also indicate geographic subpopulation structure within each of the PNW populations and at least some sexual reproduction within individual populations.

Sexual reproduction and population genetic parameters. Population genetic equilibria, such as HWE and linkage equilibrium, depend on assumptions of an ideal population, including sexual reproduction. Like all diatoms, *Pseudo-nitzschia* mean cell size is reduced through vegetative (clonal) reproduction

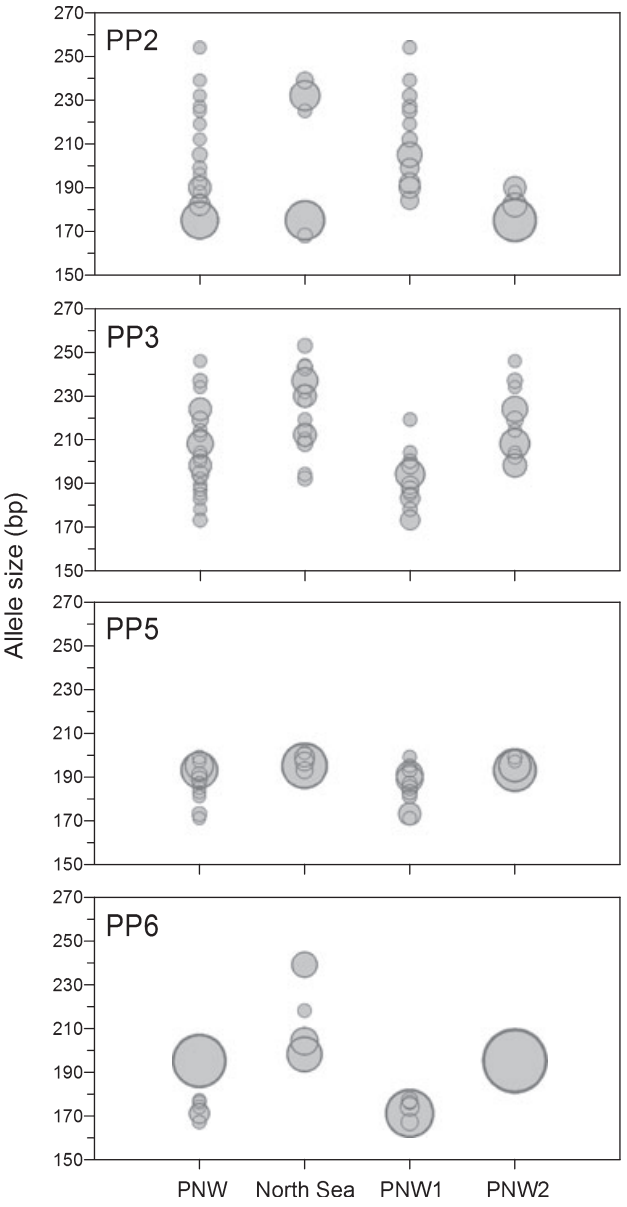

FIG. 3. Allele frequencies for the Pacific Northwest (PNW), North Sea, PNW1, and PNW2. Individual circles are centered at a specific allele size. The area of a particular circle is proportional to the frequency of that allele.

until cell size is restored via auxospore formation, usually through sexual reproduction (which can occur on the order of once every 3 years for *Pseudo-nitzschia*) after cells reach a certain minimum size (Davidovich and Bates 1998). However, computer models demonstrate clearly that both equilibria are relatively robust against violation of the assumption of sexual reproduction, and only at very high rates of clonal reproduction will deviations of genotypic proportions from expectations become significant (Balloux et al. 2003).

The assumption of Hardy–Weinberg and linkage equilibrium is the basis of the STRUCTURE analyses, which attempt to find groups of individuals within a sample that fulfill these assumptions (Pritchard et al. 2000, Falush et al. 2003). After separation of the two populations in the PNW, most loci did not deviate from HWE or linkage equilibrium (Table 1), and $\bar{r}_d$values calculated for PNW1 and PNW2 were not significant, indicating that there was a random association of alleles in these groups (Table 3). Furthermore, the North Sea sample showed no significant deviations from HWE or linkage equilibrium, indicating at least occasional sexual reproduction and demonstrating the conformance of population parameters to STRUCTURE assumptions.

TABLE 3. Values for pair-wise population differentiation estimators F_{ST} (lower half-matrix) and R_{ST} (upper half-matrix) values for the Pacific Northwest (PNW1 and PNW2) and the North Sea.

	PNW1[a]	PNW2[a]	North Sea
PNW1[a]		0.804	0.636
PNW2[a]	**0.418**[b]		0.540
North Sea	**0.274**[b]	**0.352**[b]	

[a]PNW1 and PNW2 are the two populations identified through analysis using the STRUCTURE program.

[b]Significant pair-wise test for differentiation ($P < 0.05$). F_{ST} is an estimator of population differentiation using allele frequency data, while R_{ST} is an estimator of population differentiation that uses allele size as well as allele frequency. Bold F_{ST} values indicate significance after nonsequential Bonferroni correction.

Cryptic P. pungens *species.* The results of the STRUCTURE analysis suggested that there were two distinct presumably randomly mating populations or, alternatively, two cryptic species present in the PNW sample. The taxonomic status of these groups is uncertain and depends on specific species concepts, but the magnitude of differentiation warrants further investigations of species identity. The SEM analysis of five isolates showed that they all possessed the morphological characteristics of *P. pungens*; however, one isolate was from PNW1, three were from PNW2, and one was an intermediate.

The R_{ST} value of >0.80 for the PNW1/PNW2 pair may indicate the presence of two morphologically similar species represented by the PNW1 and PNW2 populations. Along with the relatively high F_{ST} values, G'_{ST} values (Hedrick 2005) support the high differentiation between North Sea and PNW pairs, although it appears that PNW2 is more closely related to the North Sea sample than PNW1. There is precedent for cryptic forms of *Pseudo-nitzschia* species as *Pseudo-nitzschia pseudodelicatissima* was separated into distinct species groups based on TEM analysis (Lundholm et al. 2003), and reproductive isolation was demonstrated among genetically distinct groups of *Pseudo-nitzschia delicatissima* (Amato et al. 2007). Additionally, the two internal transcribed spacer (ITS) clades reported by Casteleyn et al. (2008) may correspond to the two populations identified in the present study.

TABLE 4. Pair-wise F_{ST} values for the Pacific Northwest (PNW) after population assignment using STRUCTURE.

	PNW1 BS (6)	PNW1 E1 (4)	PNW1 E2 (6)	PNW1 E3 (8)	PNW1 E4 (5)	PNW2 BS (18)	PNW2 E1 (23)	PNW2 E2 (12)	PNW2 E3 (60)
PNW1 E1 (4)	0.149[a]								
PNW1 E2 (6)	0.127[a]	0.045							
PNW1 E3 (8)	0.089[a]	0.019	−0.015						
PNW1 E4 (5)	**0.162**[a]	0.063	−0.033	0.020					
PNW2 BS (18)	**0.462**[a]	**0.495**[a]	**0.434**[a]	**0.462**[a]	**0.441**[a]				
PNW2 E1 (23)	**0.447**[a]	**0.475**[a]	**0.418**[a]	**0.449**[a]	**0.422**[a]	−0.006			
PNW2 E2 (12)	**0.424**[a]	**0.462**[a]	**0.393**[a]	**0.428**[a]	**0.409**[a]	0.022	0.002		
PNW2 E3 (60)	**0.451**[a]	**0.472**[a]	**0.421**[a]	**0.453**[a]	**0.425**[a]	0.002	−0.004	−0.006	
PNW2 E4 (14)	**0.427**[a]	**0.466**[a]	**0.395**[a]	**0.431**[a]	**0.419**[a]	0.054[a]	**0.052**[a]	0.017	0.023[a]

[a]Significant pair-wise F_{ST} ($P < 0.05$); bold F_{ST} values indicate significance after nonsequential Bonferroni correction. Negative F_{ST} values can be caused by corrections for unequal sample size in the method of Weir and Cockerham (Feder et al. 1990). Individuals were compared within populations based on the original sample from which they came. Column and row headers indicate the population determined by structure analysis (i.e., PNW1 or PNW2) followed by the original sample (i.e., BS, E1, E2, E3, or E4) and, in parentheses, the number of individuals in that sample. For example, there were six individuals from original sample BS that were assigned to population PNW1.

Our STRUCTURE analyses revealed intermediate isolates that could represent interspecific hybrids. The intermediate *q* scores in STRUCTURE (Fig. S2) suggest that these individuals are the product of fairly recent hybridization events. In total, 16 of the 172 isolates were suspected hybrids (~10%), a large proportion given that the high F_{ST} values suggested very little recent gene flow between the populations. A more exhaustive analysis of potential hybrid strains identified in the present study is presented by Casteleyn et al. (2009).

Structure within PNW populations. Subpopulation structure within PNW1 is suggested by the overall deviation from HWE (Table 1) and the results of pair-wise F_{ST} analysis (Table 4), though sample sizes are quite small. Interestingly, the genetic structure appears to be different in the two populations. The differentiation in PNW1 may suggest a stable population in isolated inshore areas of Barkley Sound, potentially maintained by geographic isolation and oceanographic fronts. There is less of a geographic pattern in the genetic differentiation among PNW2 samples, and the differentiation of E4 may be related to local upwelling events or eddies. Further studies incorporating a temporal aspect and explicit correlation to concurrent oceanographic features are required to resolve the structure within each of the two populations. Nevertheless, our data indicated that such population differentiation is likely to exist and may potentially be used to identify the source of harmful algal blooms.

Physical oceanography and genetic structure in Pseudo-nitzschia. It is possible that the PNW1 and PNW2 populations originated from different sources and oceanographic conditions in the Juan de Fuca eddy region were responsible for mixing them together. The Juan de Fuca eddy is a semipermanent feature that initiates in the spring and declines in the fall (Freeland and Denman 1982). *Pseudo-nitzschia* cells are known to persist at depth at very low light levels (Trainer et al. 1998, Rines et al. 2002), and these cells could be transported to surface waters via upwelling or vertical mixing during periods when the eddy is present. Additionally, the northward-flowing California undercurrent, the source of upwelled waters on the Washington coast and one of the main components of Juan de Fuca eddy water (MacFadyen et al. 2008), has been suggested as a means of transport for phytoplankton from the south (Hickey and Banas 2003). This undercurrent could therefore be another mechanism that introduces new populations of *P. pungens* to the Juan de Fuca eddy region. Populations of *P. pungens* associated with the outflowing Strait of Juan de Fuca water could also be mixed with those of oceanic origin in the Juan de Fuca eddy region. MacFadyen et al. (2005) showed that drifting buoys deployed in the Strait of Juan de Fuca will either exit the area via the northwestward flowing Vancouver Island coastal current or become trapped in the Juan de Fuca eddy. Therefore, various potential sources of *P. pungens* could be homogenized by oceanographic properties in the Juan de Fuca eddy region. To investigate this further, discrete samples from each of these sources need to be analyzed for *P. pungens* population structure.

Implications. The results of this study suggest that current morphological characteristics used to identify *Pseudo-nitzschia* species could be supported by incorporating molecular data. The detection of distinct *P. pungens* populations and the hypothesis that they could represent cryptic species also has implications for other *Pseudo-nitzschia* species in the PNW. Historical toxic events on the Washington State outer coast have been attributed to *Pseudo-nitzschia australis* Frenguelli (Taylor and Horner 1994) and *P. pseudodelicatissima* (Adams et al. 2000, Trainer et al. 2002). Additionally, *P. australis* has recently been responsible for the closure of shellfish harvesting in Puget Sound for the first time since testing for DA began in Washington State (Bill et al. 2006) where it was hypothesized that an oceanic population of *P. australis* had been advected into Puget Sound.

Additionally, cryptic variation within morphologically identified species may be related to toxin production. For example, multiple rDNA ITS types of *P. delicatissima* occurred sympatrically in the Gulf of Naples under normal conditions, whereas during blooms just one of these ITS types dominated (Orsini et al. 2004). The authors suggested that cryptic diversity could explain the existence of toxic and nontoxic strains of the same species and that blooms of particular strains may be triggered by specific environmental conditions. Further, of the two ITS clades identified by Casteleyn et al. (2008) within a group of presumably *Pseudo-nitzschia pungens* clones, one of these clades had morphological features that conformed to *P. pungens* var. *cingulata* as described by Villac and Fryxell (1998). With many *Pseudo-nitzschia* species capable of producing DA, including *P. pungens* (Trainer et al. 1998), errors in species identification could adversely impact management decisions, especially since isolate-specific differences in DA production have been observed (Mos 2001). The global distribution of many *Pseudo-nitzschia* species (Hasle 2002) suggests that such cryptic diversity may be common and highly relevant for the prediction of harmful algal blooms. Using microsatellite analysis to identify the sources of cells and understanding the environmental conditions in which certain strains may thrive will aid in mitigating impacts (e.g., economic and cultural) to shellfish harvesters in the PNW.

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Supplementary Material

The following supplementary material is available for this article:

Figure S1. Results of STRUCTURE analysis for 1–10 populations in the Pacific Northwest (PNW) sample.

Figure S2. Histogram showing a trimodal distribution for the proportion of alleles stemming from populations PNW1 and PNW2 (see Fig. 2) for all isolates after analysis by STRUCTURE.

Table S1. Descriptive statistics for Pacific Northwest (PNW) and North Sea samples.

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