Spatial and temporal patterns of *Pseudo-nitzschia* genetic diversity in the North Pacific Ocean from the Continuous Plankton Recorder survey

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ABSTRACT: Several species of the marine diatom *Pseudo-nitzschia* can produce the neurotoxin domoic acid that is responsible for the seafood-borne illness amnesic shellfish poisoning in humans, marine wildlife mortalities and prolonged closures of fisheries resulting in economic losses to coastal communities. Since the year 2000, *Pseudo-nitzschia* species have been monitored in the Pacific Ocean with the Continuous Plankton Recorder (CPR). We used a combination of scanning electron microscopy with high-throughput and Sanger sequencing of CPR survey samples to compare the diversity of phytoplankton, including *Pseudo-nitzschia* species, from the north-eastern Pacific Ocean over 3 climatically different years: 2002, 2005 and 2008. A *Pseudo-nitzschia*-specific primer set targeting a partial region of the large subunit ribosomal DNA (rDNA) revealed spatially separated communities of *Pseudo-nitzschia*. The coastal region was dominated by a diverse array of *P. fraudulenta* unique sequences (operational taxonomic units), whereas the offshore region was rich in *P. multiseries* and contained a wide range of other *Pseudo-nitzschia* taxa, many not previously observed in this region. In 2008, exceptionally cold sea surface temperatures were recorded, influenced by a strong negative Pacific Decadal Oscillation signal. In that year, a more diverse assemblage of species was present in a spring open water sample, whereas *P. fraudulenta* was unusually rare from a coastal autumn sample. This is the first application of high-throughput genetic methods to uncover patterns of *Pseudo-nitzschia* genetic diversity from archival CPR samples, demonstrating the value of using CPR for plankton community analysis in rarely sampled regions of the oceans.

KEY WORDS: *Pseudo-nitzschia* · Continuous Plankton Recorder · CPR · Pacific Ocean · High-throughput sequencing · Large subunit ribosomal DNA · Genetic diversity · Harmful algae

INTRODUCTION

Marine diatoms in the genus *Pseudo-nitzschia* are closely monitored in the eastern Pacific Ocean due to their capacity to produce the potent neurotoxin domoic acid (DA). DA can accumulate in filter-feed-ing fish and shellfish and be transferred through foodwebs to poison humans, marine mammals and seabirds (Work et al. 1993, Scholin et al. 2000). Symptoms of this poisoning in humans, called amnesic shellfish poisoning (ASP), include gastrointestinal distress, seizures, coma and permanent short-term

memory loss, with severe intoxications resulting in death (Perl et al. 1990). Monitoring programmes exist worldwide to protect human health from the effects of ASP. For example, in Washington State, USA, regular beach monitoring is conducted to look for cells of *Pseudo-nitzschia* in coastal waters (Trainer & Suddleson 2005), and shellfish are regularly tested for DA by the Washington State Department of Health. Shellfish harvesting closures are implemented when concentrations of DA exceed the regulatory limit for human consumption of 20 ppm in shellfish meat tissue. The first closure of recreational and commercial shellfish harvesting due to DA on the Washington State coast occurred in 1991 and resulted in a US$15–20 million revenue loss to local fishing communities (Horner & Postel 1993, Anderson 1995). The total estimated economic impact associated with a coastwide, year-long closure of the razor clam fishery, such as those that occurred in 1991–1992, 1998–1999 and 2002–2003, has been estimated at US$21.9 million (Dyson & Huppert 2010).

The Pacific Decadal Oscillation (PDO) is a pattern of ocean-climate variability that gives rise to very different climate regimes with implications for environmental parameters that influence *Pseudo-nitzschia* growth and toxicity. The PDO index is the first mode of monthly ocean sea surface temperature (SST) variability in the North Pacific Ocean poleward of 20° N (Mantua et al. 1997). When the PDO index is positive (negative), the coastal ocean in the Pacific Northwest is typically warmer (cooler) and the central north Pacific Ocean is cooler (warmer) (Mantua et al. 1997). The regional climate is also influenced by the PDO, with winter-time air temperature and precipitation in the US Pacific Northwest typically below normal during warm phases of the PDO. Historically, the warm and cool phases of the PDO have persisted for 20–30 yr, but in recent years, the PDO has been switching phases approximately every 5 yr and has closely tracked the El Niño/Southern Oscillation (ENSO). The mechanisms that give rise to the PDO are not fully understood; nevertheless, major changes in marine ecosystems and the distribution and ratios of nutrients in the Pacific Ocean have been documented to occur when the PDO changes phase (Botsford et al. 1997, Mantua et al. 1997). In general, biological productivity is enhanced off the coast of Alaska and inhibited off the coast of the contiguous USA during warm phases of the PDO, while the reverse is true during cold phases (Hare et al. 1999). Phytoplankton communities, including *Pseudo-nitzschia* species, may be affected by changing temperature, salinity and nutrient distributions that may

co-occur with PDO phase changes. Previous studies have observed different phytoplankton communities, including different *Pseudo-nitzschia* species, in offshore eastern Pacific regions versus coastal Pacific regions (Ribalet et al. 2010 and Table 1). In fact, recent work suggests that warm phases of the PDO (and ENSO) are directly related to US west coast toxic *Pseudo-nitzschia* bloom events (McCabe et al. 2016).

The Continuous Plankton Recorder (CPR) is an instrument designed to be towed from ships of opportunity on their normal sailings and provides opportunities for sampling plankton communities in rarely sampled regions of the open oceans. It works by filtering plankton on a moving band of silk mesh over long distances. The CPR survey was originally designed to collect zooplankton and higher abundances of larger phytoplankton. As such, the silk gauze that collects plankton has a mesh size of ~270 µm. Collection of phytoplankton by the CPR survey would be considered suboptimal, yet phytoplankton as small as 5 µm (coccolithophorids) have been retained (Richardson et al. 2006). This is because the large volume of water filtered (3 m^3) deposits large amounts of plankton that clog the mesh, effectively reducing the aperture size and retaining smaller plankton (Batten et al. 2003b). Additionally, phytoplankton can be trapped on the silk collecting gauze; the silk material is thicker and stickier than nylon used in plankton nets, with micro-threads that extend into the aperture. The CPR measurement of the phytoplankton colour index, a proxy for total phytoplankton abundance, correlates well with fluorometric and satellite-measured chlorophyll *a*, although it is seasonally variable (Batten et al. 2003a, Raitsos et al. 2005). *Pseudo-nitzschia* species are typically between 40 and 175 µm long, smaller than the mesh size, but can occur in long chains and so may be retained more readily than other smaller or non-chain forming phytoplankton.

The CPR survey monitors phytoplankton on 2 routes in the North Pacific (Batten et al. 2006). One is a 3000 km trans-Pacific route from Vancouver, Canada, to Hokkaido, Japan, through subpolar waters. This route has been sampled seasonally since 2000 during both warm and cool phases of the PDO. CPR samples are immediately preserved in formalin and archived, and offer an opportunity to examine the spatial distribution of *Pseudo-nitzschia* species over different temperature and ocean-climate regimes. At present, *Pseudo-nitzschia* retained on the mesh are examined microscopically and classified into 2 cell-width morphotypes, *P. seriata* (>3 µm) complex and *P. delicatissima* (<3 µm) complex (hereafter referred to as *P. seriata*- and *P. delicatissima*-sized cells, respec-

Table 1. Coastal and open-ocean *Pseudo-nitzschia* species reported from the Pacific Ocean in the literature with their approximate dimensions. WA: Washington, OR: Oregon, CA: California, HNLC: high-nutrient, low-chlorophyll, LM: light microscopy, TEM: transmission electron microscopy, SEM: scanning electron microscopy. Shaded cells indicate species that overlap both coastal and open-ocean niches. Question mark indicates uncertain identification in citation

Pseudo-nitzschia sp.	Niche	Pacific Ocean region	Width (µm)	Length (µm)	Identification method	Reference
P. pungens	Coastal	USA (WA, OR, CA); Peru; Mexico; SE Pacific	2.4–5.3	74–174	Genetic; LM; TEM	Fryxell et al. (1997), Hubbard et al. (2008), Stonik et al. (2011), Trainer et al. (2012)
P. multiseries	Coastal	USA (WA, CA); Peru; SE Pacific	3.4–6.0	68–140	Genetic; LM; TEM	Fryxell et al. (1997), Hubbard et al. (2008), Stonik et al. (2011), Trainer et al. (2012)
P. seriata	Coastal	USA (WA, CA); Peru; SE Pacific	5.5–8.0	75–160	Genetic; LM; TEM	Gómez et al. (2007), Hubbard et al. (2008), Stonik et al. (2011)
P. australis	Coastal	USA (WA, OR, CA)	6.5–8.0	75–144	Genetic, SEM	Fryxell et al. (1997), Hubbard et al. (2008), Gar ia-Mendoza et al. (2009), Trainer et al. (2012)
P. subpacifica	Coastal	USA (WA, CA)	5–7	33–70	Genetic, LM	Fryxell et al. (1997), Hubbard et al. (2008)
P. cuspidata	Coastal	USA (CA, WA)	~3	30–80	Genetic; LM	Fryxell et al. (1997), Auro (2007), Lundholm et al. (2012), Trainer et al. (2012)
P. calliantha	Coastal	USA (WA); Peru; Western Pacific	4–6	30–72	LM; TEM	Marchetti et al. (2006), Stonik et al. (2011)
P. multistriata	Coastal	Peru; SE Pacific	2.5–3.8	38–65	LM; TEM	Gómez et al. (2007), Stonik et al. (2011)
P. obtusa	Coastal	Peru; SE Pacific	4.5–5.5	61–100	LM; TEM	Gómez et al. (2007), Stonik et al. (2011)
P. cf. caciantha	Coastal	Peru; SE Pacific	3.5–5	53–75	LM; TEM	Gómez et al. (2007), Stonik et al. (2011)
P. americana	Coastal	Peru	~3	16–40	LM	Gómez et al. (2007)
P. subfraudulenta	Coastal	Mexico, USA (CA)	3.7–7.0	65–133	LM	Fryxell et al. (1997), Zamudio-Resendiz et al. (2014)
P. subpacifica	Coastal	Peru	3.8–5.8	36–68	SEM	Tenorio et al. (2016)
P. hasleana	Coastal	USA (WA)	1.5–2.8	37–79	Genetic, SEM	Lundholm et al. (2012)
P. australis	Coastal	USA (WA, OR, CA)	6.5–8.0	75–144	Genetic, SEM	Fryxell et al. (1997), Hubbard et al. (2008), Gar ia-Mendoza et al. (2009), Trainer et al. (2012)
	Open	NE Pacific	6.5–8.0	75–144	SEM	Trainer et al. (2012)
P. fraudulenta	Coastal	USA (WA); Peru; SE Pacific	4.5–10.0	50–119	Genetic; LM; SEM; TEM	Horner & Postel (1993), Fryxell et al. (1997), Hubbard et al. (2008), Stonik et al. (2011)
	Open	NE Subarctic Pacific (Station AL)	4.5–10.0	50–119	LM, SEM, TEM	Silver et al. (2010)
P. pseudodelicatissima	Coastal	USA (WA); Mexico	1.3–2.5	59–140	LM; SEM	Fryxell et al. (1997), Trainer et al. (2002), Zamudio-Resendiz et al. (2014)
	Open	NE Subarctic Pacific (Station AL)	1.3–2.5	59–140	LM; SEM; TEM	Silver et al. (2010)
P. delicatissima	Coastal	USA (WA); SE Pacific	1–2	40–76	Genetic; LM; TEM	Fryxell et al. (1997), Hubbard et al. (2008), Stonik et al. (2011), Trainer et al. (2012)
	Open	SE Pacific HNLC	1–2	40–76	LM	Gómez et al. (2007)

Table 1 (continued)

Pseudo-nitzschia sp.	Niche	Pacific Ocean region	Width (µm)	Length (µm)	Identification method	Reference
P. heimii/ P. cf. heimii	Coastal	USA (WA); Peru; SE Pacific	4–6	67–120	LM; TEM	Fryxell et al. (1997), Gómez et al. (2007), Stonik et al. (2011)
	Open	NE Pacific (Ocean Station PAPA); NE Subarctic Pacific (Station AL)	4–6	67–120	LM; SEM; TEM	Marchetti et al. (2006), Silver et al. (2010)
P. lineola	Coastal		1.8–2.7	56–112	Genetic; LM; SEM	Fryxell et al. (1997), Hernández-Becerril (1998, 2007), Gar ia-Mendoza et al. (2009)
	Open	NE Subarctic Pacific (Station AL)	1.8–2.7	56–112	LM; SEM; TEM	Silver et al. (2010)
P. turgidula/ P. cf. turgidula	Coastal	California	1.3–2.5	30–80	LM	Fryxell et al. (1997)?
	Open	NE Pacific (Ocean Station PAPA); NE Subarctic Pacific (Station AL)	1.3–2.5	30–80	LM; SEM; TEM	Silver et al. (2010), Trick et al. (2010)
P. grannii, P. cf. grannii	Open	NE Pacific (Ocean Station PAPA); NE Subarctic Pacific (Station AL)	1.5–2.5	25–79	LM; SEM; TEM	Silver et al. (2010), Trick et al. (2010)
P. dolorosa	Open	NE Pacific (Ocean Station PAPA)	2–3.2	30–59	LM; TEM	Marchetti et al. (2006)
P. inflatula	Coastal	USA (CA)	1.5–2.5	6–100	LM	Fryxell et al. (1997)?
	Open	NE Subarctic Pacific (Station AL)	1.5–2.5	6–100	LM; SEM; TEM	Silver et al. (2010)

tively). Identification to lower taxonomic levels is not possible due to the limitation of light microscopy in identifying the minute morphological differences between species (Hasle 1993). Because of the cryptic and pseudo-cryptic morphological diversity of *Pseudo-nitzschia* species, morphological and genetic taxonomic approaches are now often used in tandem (Lundholm et al. 2006). Most studies use all or part of the ribosomal internal transcribed spacer (ITS) for identification, which has been found to distinguish species and even intraspecific populations within species (Lundholm et al. 2003, Orsini et al. 2004, Amato et al. 2007, Hubbard et al. 2008, Andree et al. 2011, Lim et al. 2012, Penna et al. 2013). The large subunit (LSU) ribosomal DNA (rDNA) has also been used successfully, although with a lesser degree of resolution in identifying species or species groups (Lundholm et al. 2002, McDonald et al. 2007).

The use of genetic taxonomic approaches to identify *Pseudo-nitzschia* species from archived samples can be limited by how the samples are preserved. Despite the use of buffered formalin to reduce hydrolytic fragmentation of DNA molecules, formalin-preservation still causes methylation as well as methylol modification of nucleobases and cross-linking between nucleotides or together with proteins (Paireder et al. 2013, Karmakar et al. 2015). Therefore, genetic analysis of formalin-preserved CPR samples presents challenges. Nevertheless, recent successes in genetic identification of species from CPR samples dating as far back as 1961 include the coccolithophore *Emiliania huxleyi* (Ripley et al. 2008), various microbial eukaryotes as small as 1 µm in size (McQuatters-Gollop et al. 2015), the harmful alga *Karenia mikimotoi* (Al-Kandari 2012) and the bacterium *Vibrio cholerae* (Vezzulli et al. 2012, 2016). The use of 454 GS FLX+ high-throughput sequencing technology (HTS), or similar HTS technology such as MiSeq (Illumina), is suitable for environmental barcoding of samples, as it uses small (150–300 bp) amplicon sizes and provides 500–1000 Mb per run (Scholz et al. 2012). In this study, we examined *Pseudo-nitzschia* species assemblages in the eastern North Pacific Ocean region in oligotrophic open waters compared to coastal waters off Vancouver Island, Canada, during both warm (2002 and 2005) and cool (2008) phases of the PDO. We used rDNA LSU primers designed for the genus *Pseudo-nitzschia* (McDonald et al. 2007) to determine species distributions in 30 CPR samples. Eleven of these

samples were able to generate PCR products for HTS and Sanger sequencing of clone libraries of PCR products (clone library sequencing, CLS), providing a species-level comparison of *Pseudo-nitzschia* diversity in coastal and open Pacific waters.

MATERIALS AND METHODS

CPR samples

The CPR is deployed on the trans-Pacific route between Vancouver, Canada, and Hokkaido, Japan, every 3 mo. CPR transects along the route were divided into 2 regions: (1) the Eastern region, including the shelf of North America to −134° E plus 1 sample at −136° E, and (2) the Central region, including the open-ocean region from −134° to −148° E (Fig. 1). Thirty samples out of a total of 159 were initially selected for genetic analysis to represent 3 seasons (spring, summer and autumn) during 2002, 2005 and

2008 (Fig. 1, Table 2). Eleven of the 30 samples successfully generated genetic results (see 'Materials and methods – PCR amplification and sequencing'). Samples were chosen on the basis of high *Pseudo-nitzschia* abundance determined from light microscopy. To compare diatom abundance and communities at different PDO phases, mean abundances of total diatoms and *Pseudo-nitzschia* species from all 159 samples were calculated for each season, year and region. The mean abundance of diatoms and *Pseudo-nitzschia* cells were calculated for Central or Eastern Pacific regions per season per year (termed seasonal means) from standard cell counts of all CPR samples (total 159) from 2002, 2005 and 2008 so that there were 2–4 CPR samples per seasonal mean (see Fig. 7).

Phytoplankton community analysis

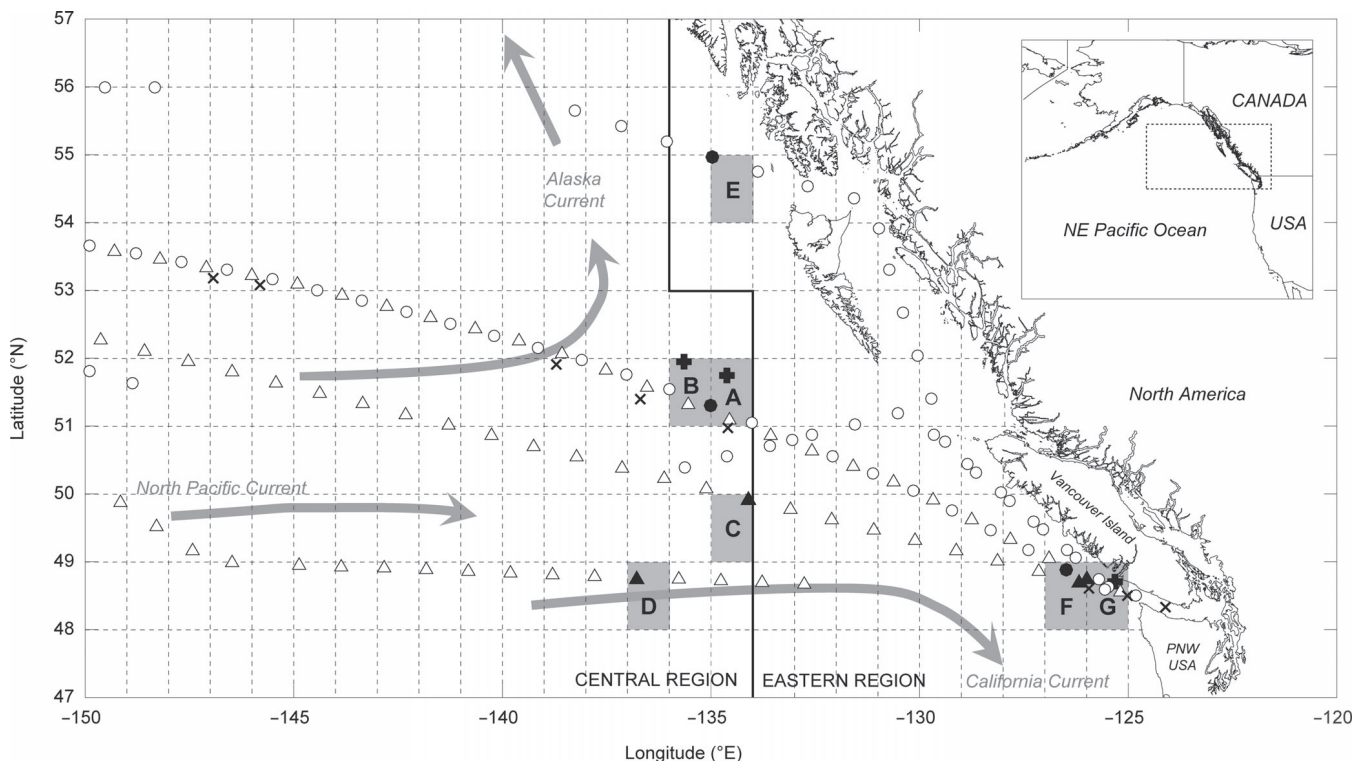Phytoplankton taxa were identified and counted from CPR samples as described by Batten et al.

Fig. 1. Continuous Plankton Recorder (CPR) samples collected in 2002 (+), 2005 (O), 2008 (△) and 2014 (×; for scanning electron microscopy only) from the Eastern and Central Pacific ocean region. Samples were selected from 1–2 transects conducted during different seasons. Grid cells indicate the 1° latitude by 1° longitude spatial resolution of satellite-derived sea surface temperature (SST) used in this study. Gray-shaded grid cells (labelled A–G) contain the 11 CPR samples used for molecular analysis represented by shaded symbols +, ● and ▲. These correspond to the time series of SST anomalies shown in Fig. S3 in the Supplement. Details of samples subjected to molecular analysis are listed in Tables 2 & 4. Central and Eastern regions are bisected by the −134° E longitude line (−136° E for the northern transect) for the community composition analyses. Inset: Pacific Northwest region studied

Table 2. Summary of Continuous Plankton Recorder (CPR) samples used in this study and seasonal mean values of the Pacific Decadal Oscillation (PDO) index (sPDO) for the sampling season. Sample codes denote season (autumn, summer, spring), year (2002, 2005, 2008) and region (Eastern or Central) of origin and are listed following their longitudinal position. sPDO values for autumn ('Au') are the mean of September, October, and November; summer ('Su') is the mean of June, July, and August; and spring ('Sp') is the mean of March, April, May. Samples 21VJ5 (Au02E(2)) and 21VJ45 (Au02C(2)) are regional duplicates of 21VJ1 and 21VJ41

CPR sample	Month	Year	Latitude (° N)	Longitude (° E)	Location	Code	sPDO
21VJ1	10	2002	48.71	−125.42	Eastern	Au02E	0.79
21VJ5	10	2002	48.71	−125.42	Eastern	Au02E(2)	0.79
139VJ1	7	2008	48.76	−125.99	Eastern	Su08E	−1.57
146VJ5	9	2008	48.7	−126.17	Eastern	Au08E	−1.52
83VJ5	5	2005	48.88	−126.48	Eastern	Sp05E	0.69
77VJ7	4	2005	54.97	−134.97	Eastern	Sp05NE	1.42
21VJ41	10	2002	51.75	−134.61	Central	Au02C	0.79
132VJ17	5	2008	48.76	−136.79	Central	Sp08C	−1.20
146VJ37	9	2008	49.92	−134.11	Central	Au08C	−1.52
83VJ41	5	2005	51.3	−135.02	Central	Sp05C	0.69
21VJ45	10	2002	51.95	−135.64	Central	Au02C(2)	0.79

(2003a). Hard-shelled phytoplankton were counted under a light microscope by viewing 20 fields of view (diameter 295 µm) across each sample under high magnification (450×) and recording the presence of all the taxa in each field (presence in 20 fields is assumed to reflect a more abundant organism). These 20 fields amount to 1/10 000 of the area of the filtering silk. Cell abundances per field (H) were then calculated for each taxon (Robinson & Hiby 1978):

$$H = -\ln(k/20) \qquad (1)$$

where k is the number of empty microscope fields (out of 20) observed. Multiplication by the proportion of the sample examined gave cell abundances in each sample. A category system was used to calculate the average abundance sample^{-1}, ranging from 0–750 000 sample^{-1} (for a full explanation of the sampling technique, see Richardson et al. 2006). The 2 main groups of *Pseudo-nitzschia* that were routinely recorded in CPR samples were distinguished by their width in valve view, with the *P. delicatissima*-sized cells being smaller than 3 µm in width and the *P. seriata*-sized cells having a width exceeding 3 µm. Inconclusive species were recorded as *Nitzschia* spp. The mean sample taxonomic abundances for each year/region/season unit were transformed using $\log_{10}(x + 1)$, where x is abundance, for all 159 CPR samples.

DNA extraction

Each CPR sample represents a collection over 10 nautical miles (n miles) and is equivalent to filtering ~3 m^3 of water (Richardson et al. 2006). A quarter piece of a CPR sample was cut so that it represented the entire 10 n miles but only a quarter of the volume of filtered plankton (0.75 m^3). The CPR silk piece was cut into 1 cm^2 square pieces and placed into 30 ml of TE buffer. The procedure for extracting DNA is described in detail elsewhere (Ripley et al. 2008) and is only briefly outlined here. The CPR silk piece was washed and agitated in TE buffer for 24 h, the plankton was recovered by centrifugation, resuspended into 1 ml fresh TE buffer and divided into 2 duplicate 500 µl samples. Both duplicates were treated with Proteinase K and sodium dodecyl sulfate (SDS) for 48 h, followed by a phenol:chloroform:isoamyl alcohol (25: 24:1) extraction. The upper aqueous layer from the phenol:chloroform step was further extracted by chloroform:isoamyl alcohol (24:1). DNA was precipitated with ammonium chloride and ethanol extraction and resuspended in 30 µl of TE buffer.

PCR amplification and sequencing

PCR amplification of 30 CPR samples and genomic DNA from 2 non-preserved cultures of *Pseudo-nitzschia multiseries* (culture lost) and *Pseudo-nitzschia fraudulenta* (CCAP1061/6) from the Culture Collection of Algae and Protozoa (SAMS, Scotland) was attempted using a 600−800 bp LSU marker (Scholin et al. 1994) and ITS markers (White et al. 1990, Hubbard et al. 2008, Andree et al. 2011). The ITS marker amplifications yielded no amplicons except for very faint products for samples 139VJ5, 139VJ37 and 146VJ5 and genomic *P. fraudulenta*

DNA. Amplification of diluted genomic DNA (1:10, 1:100, 1:1000) in a subset of samples also failed. A number of nested PCR strategies were used for ITS amplification with no success. With most amplification reactions (except for these 3 samples) resulting in failure using the ITS marker, it was eliminated from this study (see Table S1 in the Supplement at www.int-res.com/articles/suppl/m606p007_supp.pdf). However, a nested PCR amplification approach using LSU markers was successful in yielding products in CPR samples and the cultures. General eukaryotic LSU primers D1R and D2C (Scholin et al. 1994) resulted in 22/30 amplicons from CPR samples (size 600−800 bp). Amplifications were carried out with the Promega PuReTaq kit using 2 µl of genomic DNA (ranging from 25−1073 ng µl^{-1}, mean 288 ng µl^{-1}) which were then diluted by 1:100 in a reaction volume of 25 µl containing 3 mM MgCl$_2$, 0.2 mM dNTPs, 0.4 µM each of primers and 1 unit of *Taq* polymerase. PCR conditions were 95°C for 5 min, then 35 cycles of denaturation at 95°C for 30 s, annealing at 45°C for 45 s and extension at 72°C for 45 s, and a final extension step of 72°C for 10 min. The *Pseudo-nitzschia*-specific LSU nested primer set D1-186F and D1-548R (McDonald et al. 2007) was then used on first-round PCR products (D1R-D2C) to amplify a 362 bp product that was successful in 11/22 first-round amplicons. PCR reaction conditions were as above except 1 µl of first-round amplification product was used for a template. PCR cycling conditions were the same as for D1R/D2C, except the annealing temperature was 50°C and the final 72°C extension lasted 5 min.

CLS analysis

To confirm that only *Pseudo-nitzschia* were amplified using the LSU nested primer set, a clone-library sequencing study was performed on 6 nested PCR products that successfully generated positive clones and subsequently sequences (see Table 4). Cloning was carried out using the TOPO TA® cloning kit (Life Technologies) using 25 µl of One Shot® INVαF′ competent cells for 5 µl of PCR product with primer dimers removed using ExosapIT (Affymetrix). A total of 162 transformed colonies from the remaining samples was prepared for sequencing according to the manufacturer's instructions, except that DNA from colonies was prepared by dissolving a colony into 10 µl of sterile water and heat-denatured at 95°C for 2 min. Sequencing reactions were performed in a 20 µl reaction volume with 1 µl of BigDye v.3.1 and 5× buffer

(supplied by Applied Biosystems), 1 µl of 3.2 µM primer (either M13F or M13R) and 20−50 ng of PCR product. The amplicons were sequenced using capillary electrophoresis by Source Bioscience (Nottingham, UK). The CLS dataset was trimmed using BioEdit v. 1999-2013 software (Hall 1999) to remove cloning sites, checked using BLAST (Altschul et al. 1990) for initial identification and added to the HTS dataset (see 'Phylogenetic analysis of sequences'). Repeat sequencing of Au08E was carried out using primer set PfrauLSUF (5′ GTT TGG GAT TGC AGC TCT AAT TTG GTG G 3′) and PfrauLSUR (5′ GAG CTT GCG TGG GTT TGG GTT GGT TGA TC 3′), performed using a Bioline SensiFAST probe no-ROX kit using 10 µl of SensiFAST reagent, 0.8 µl of each primer and 4 µl of DNA in a 20 µl reaction volume, in a Rotor Gene 6000 (Qiagen) real time PCR cycler under the following conditions: 95°C for 3 min, followed by 45 cycles at 95°C for 5 s, 67°C for 10 s, and 72°C for 30 s. The sequencing was carried out by Source Bioscience using PfrauLSUF primer.

HTS analysis and operational taxonomic unit generation

To obtain better diversity representation, HTS was conducted on the LSU products of 9 samples (see Table 4) that contained sufficiently concentrated DNA. Despite a wide range of genomic DNA concentrations, the difference in PCR product concentrations from the *Pseudo-nitzschia*-specific nested reaction was no more than 9 ng µl^{-1} between samples. All PCR products were diluted to 50 ng µl^{-1} and sent to MrDNA Molecular Research Laboratory (Shallowater, Texas, USA) for a custom assay with primers D1-186F and D1-548R, using a single-step, 30-cycle PCR using HotStarTaq Plus Master Mix Kit (Qiagen) and PCR conditions as described earlier for this primer set. Following the PCR step, amplicon products from all samples were mixed in equal concentrations and purified using Agencourt Ampure beads (Agencourt Bioscience). Samples were sequenced using Roche 454 FLX titanium instruments and reagents and following the manufacturer's guidelines.

Various bioinformatics pipelines incorporated into the Bio-Linux (Field et al. 2006) operating software based on Ubuntu 10.4 were tailored toward the analysis of eukaryotic LSU amplicons. The Python-based QIIME software (Caporaso et al. 2010) script split_libraries.py was used to quality-check reads using default settings and to trim primers and tags. In total, 39 731 sequence reads were retrieved from 9 samples

from HTS sequencing ranging from 2632–6506 reads sample^{-1}. Additional filtering criteria were applied with a sliding window quality score of 50 to remove poor-quality sequences and to include reads greater than 300 bp (a primer mismatch of 1), and manual chimera-checking was performed on aligned sequences (denoise step). Operational taxonomic unit (OTU) picking steps were performed on denoised sequence data by clustering sequences at 90 and 99% using UCLUST to allow for abundance pre-sorting (Edgar 2010) in order to obtain a range of representative taxa. Each OTU was a unique sequence that was at least 1% different from other OTUs. These sequences were exported into BioEdit (Hall 1999) for more precise analysis of OTU identities. Additional quality checks were carried out by BLAST analysis to ensure that no chimeras or low-quality sequences were retained. All sequences were deposited in Gen-Bank (see Table S2 in the Supplement).

Phylogenetic analysis of sequences

An initial BLAST search of the D1-186F and D1-548R *Pseudo-nitzschia*-specific LSU fragment (McDonald et al. 2007) was carried out to check that all HTS and CLS datasets belonged to *Pseudo-nitzschia* and no chimeras were present. Non-redundant hits to our sequences that contained species information were used for phylogenetic analysis. We also used the search term '*Pseudo-nitzschia* large ribosomal' to capture 394 *Pseudo-nitzschia* sequences. An additional 30 other pennate and centric diatom species were added as an outgroup. All reference sequences were downloaded in May 2016, September 2017 and May 2018. These were combined with environmental HTS and CLS and automatically aligned and trimmed using CLUSTALW in BioEDIT (Hall 1999) to approximately 320 bp. The alignment was 538 bp long including gaps and contained 785 sequences in total (see Table S3 in the Supplement). The alignment was exported into MEGA 6.0 (Tamura et al. 2013) for phylogenetic analysis with the maximum likelihood (ML) method using the general time reversible model and 4 gamma distribution categories to model evolutionary rate differences among sites (4 categories +*G*, parameter = 1.6667). A partial deletion option was selected in which all positions with less than 95% site coverage were eliminated, resulting in 325 positions analysed in the final dataset. ML bootstrap analyses were carried out with 1000 pseudo-replicates. Initial tree(s) for the heuristic search were obtained by applying the neighbour-joining method

to a matrix of pairwise distances (PWDs) estimated using the maximum composite likelihood approach. The tree with the highest log likelihood (−11161.8314) was selected, and the percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree was drawn to scale, with branch lengths measured in the number of substitutions site^{-1}. Newick files were exported into the Interactive Tree of Life (Letunic & Bork 2016), where clades were compressed for clarity. Annotation and further compression was carried out on clades containing identical or highly similar sequences, using Adobe Illustrator software, to allow visualisation of environmental sequences. An additional ML phylogeny of longer (381 bp) CLS reads of the D1-186F, D1-548R LSU fragment from 6 CPR samples was performed for better identification of environmental sequences (Fig. S1 in the Supplement). The alignment was 430 bp long including gaps, with 352 environmental and public sequences, and the phylogeny was built using the same tree building methods described above (+*G*, parameter = 0.4770) with 280 positions analysed in the final dataset. Investigation of genetic PWDs was also carried out but did not reveal a clear distinction within and between species (see Fig. S2 in the Supplement). Hence PWD metrics were not used to evaluate species here.

Scanning electron microscopy

In order to confirm the morphological types of *Pseudo-nitzschia* captured by the CPR survey, 8 of the genetically analysed CPR samples from the trans-Pacific route from Vancouver, Canada, to Hokkaido, Japan, during 2002–2008 (Table 2) and an additional set from 2014 were analysed by scanning electron microscopy (SEM). *Pseudo-nitzschia* cells were identified to species level. Small subsamples of CPR mesh containing preserved phytoplankton material were cut to size, inserted into 15 ml centrifuge tubes and vortexed with 10 ml of MilliQ® water. Subsamples (1–2 ml) were removed and centrifuged in microcentrifuge tubes. Pellets were rinsed in MilliQ water 1–2 more times to remove any remaining preservative and then oxidized with 4–5 drops of saturated potassium permanganate solution, cleared with 3 rinses of concentrated hydrochloric acid (HCl) and washed in 3 rinses of MilliQ water to remove HCl. Finally, pellets were resuspended in approximately 0.5 ml MilliQ water and filtered onto 13 mm diameter, 0.2 µm pore size polycarbonate filters (Millipore). Filters were then glued to aluminum SEM stubs,

coated with gold-palladium and examined in a JEOL 6360LV SEM.

Satellite-derived SST and PDO

Satellite-derived SST values were obtained on a 1° × 1° grid in the Eastern (134° to 125° E, 49° to 56° N) and Central (148° to 134° E, 49° to 56° N) regions of the NE Pacific. Optimum interpolation SST V2 data are provided by the National Oceanic and Atmospheric Administration (NOAA), Office of Oceanic and Atmospheric Research (OAR), Earth System Research Laboratory (ESRL), Physical Sciences Division (PSD), Boulder, Colorado, USA, from their website at www.esrl.noaa.gov/psd/. Seasonal mean values of SST were interpolated for the Eastern and Central regions to determine spatial variability in the regions during seasons and years when CPR samples were collected. Temporal variability in monthly SST was determined by examining standardized anomalies for grid cells that encompassed locations where CPR samples were collected (grid cells A–H in Fig. 1) from 2000 through 2010. Standardized anomalies were calculated by dividing the anomalies by the climatological standard deviation, using the 11 yr baseline period from 2000 through 2010, such that the time series for each grid cell had a mean of 0 and a standard deviation of 1. Monthly values of the PDO index were obtained from the University of Washington Joint Institute for the Study of the Atmosphere and Ocean (JISAO 2014). Seasonal mean values of the PDO index (sPDO) were calculated for seasons and years when CPR samples were collected.

RESULTS

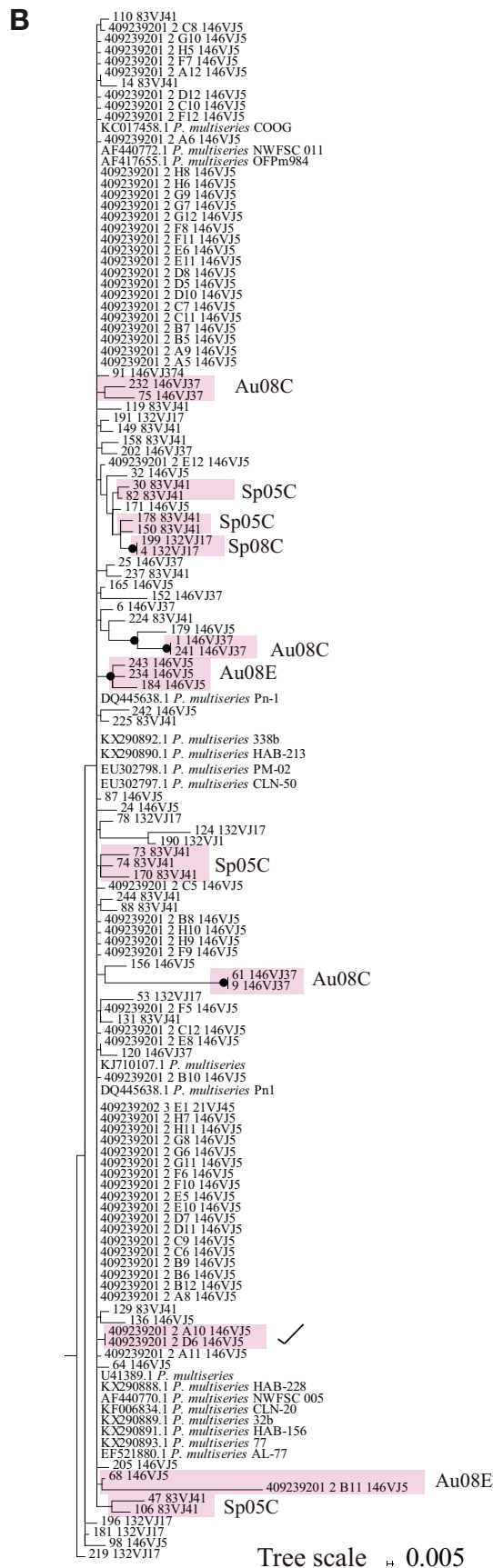### OTU identification of *Pseudo-nitzschia*

ML phylogenetic analysis of public and environmental sequences using the *Pseudo-nitzschia*-specific 320 bp LSU fragment (D1-186F, D1-548R, McDonald et al. 2007) on 11 CPR samples (Fig. 2) identified 36 terminal clades relating to 1 or more species, excluding the outgroup, most with low support, and 22 of these could be identified to single-species (excluding *P. pseudodelicatissima* and *P. delicatissima*) from strains characterised in previous studies (Table 3, Fig. 2). This includes *P. multiseries* and *P. fraudulenta* with intraspecific genetic distances of 0.3 and 0%, respectively. Although genetic distances were not clearly defined within and between species, within-species distances of *P. fraudulenta* and *P. multiseries* fell below distances between most species (≥1%, Fig. S2 in the Supplement). *P. fraudulenta* (Fig. 2) is contained in 1 large clade (see following subsection) with several other species clades. A large amount of structure appears in this clade, with public sequences appearing in 3 subclades. All CLS reads corresponding to *P. fraudulenta* in Fig. 2A were also identified as *P. fraudulenta* in a ML phylogeny with longer reads derived from the CLS dataset of the D1-186F, D1-548R LSU fragment (381 bp after trimming, Fig. S1 in the Supplement).

Four conspecific clades consisted of 2 species each (Fig. 2, Table 3). However, due to the lack of resolution of the small region used, these conspecific clades could not be resolved further. These were *P. pseudodelicatissima* and *P. mannii, P. subpacifica* and *P. heimii, P. dolorosa* and *P. micropora* and *P. delicatissima* and *P. arenysensis* (Fig. 2, Table 3). *P. delicatissima* and *P. arenysensis* are cryptic species only distinguishable by ITS (Amato et al. 2007, Quijano-Scheggia et al. 2009). *P. delicatissima* and *P. pseudodelicatissima* appeared in multiple clades, likely due to the lack of resolution of the smaller LSU fragment and because of distinct populations of these species (Orsini et al. 2004, Lundholm et al. 2006, Amato et al. 2007, McDonald et al. 2007, Moschandreou et al. 2012). A clade containing *P. pseudodelicatissima* (strains confirmed from 4 studies, Table 3, Fig. 2) also clustered with strains belonging to *P. pseudodelicatissima/cuspidata* group (Lundholm et al. 2003, Fernandes et al. 2014), *P. plurisecta, P. cuspidata* and *P. fukuyoi*, all related species that are morphologically similar to *P. pseudodelicatissima* (Lundholm et al. 2003, Lim et al. 2013, Orive et al. 2013) but could not be separated using this small marker. Confirmed strains of *P. pseudodelicatissima* also grouped with *P. mannii*, sister to *P. calliantha* as described by Amato & Montresor (2008). *P. turgidula,* a common and geographically distinct open-water Pacific species, appears twice, as a core group and as 1 strain grouped with 4 *P. delicatissima* strains; thus, the true diversity of *P. turgidula* is unclear. Only 1 representative of *P. turgiduloides,* a common open-water Pacific species (Silver et al. 2010), exists in public records but clustered with *Fragilariopsis* and *Neodenticula* (Fig. 2) likely due to the low resolving marker used here (less than 2% difference between the 3 species). An unknown *Pseudo-nitzschia* sp. genetic clade labelled MVR2015 related to *P. lineola* was also found. The remaining 22 other diatom species (excluding *Neodenticula* spp. and *Fragilariopsis* spp.) formed an outgroup that was separate and basal to other *Pseudo-nitzschia* species (Fig. 2).

Fig. 2 (this and the next page). (A) Large subunit (LSU) maximum likelihood (ML) phylogeny from a 538 bp alignment of partial LSU fragments (D1-186F, D2-548R) from public reference sequences and environmental *Pseudo-nitzschia* sequences from 11 Continuous Plankton Recorder (CPR) samples. Some clades have been collapsed for clarity number of collapsed taxa are shown in parentheses, whilst those containing environmental sequences are shown in colour (blue: *P. fraudulenta*; green: *P. hasleana*; red: *P. multiseries*; orange: environmental groups). Grey collapsed clades are outgroups. Environmental sequence identities are listed in Table S2 in the Supplement. Bootstrap values over 70 are shown by a circle at tree nodes. Symbols next to tree leaf labels refer to strains identified in other studies (see key top right), detailed in Table 3. Pink shaded boxes indicate clades belonging to a specific location and time. The checkmark indicates CLS clades also recovered in Fig. S1 in the Supplement. Genetic distance is shown at bottom right

B

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110 83VJ41
409239201 2 C8 146VJ5
409239201 2 G10 146VJ5
409239201 2 H5 146VJ5
409239201 2 F7 146VJ5
409239201 2 A12 146VJ5
14 83VJ41
409239201 2 D12 146VJ5
409239201 2 C10 146VJ5
409239201 2 F12 146VJ5
KC017458.1 P. multiseries COOG
409239201 2 A6 146VJ5
AF440772.1 P. multiseries NWFSC 011
AF417655.1 P. multiseries OFPm984
409239201 2 H8 146VJ5
409239201 2 H6 146VJ5
409239201 2 G9 146VJ5
409239201 2 G7 146VJ5
409239201 2 G12 146VJ5
409239201 2 F8 146VJ5
409239201 2 F11 146VJ5
409239201 2 E6 146VJ5
409239201 2 E11 146VJ5
409239201 2 D8 146VJ5
409239201 2 D5 146VJ5
409239201 2 D10 146VJ5
409239201 2 C7 146VJ5
409239201 2 C11 146VJ5
409239201 2 B7 146VJ5
409239201 2 B5 146VJ5
409239201 2 A9 146VJ5
409239201 2 A5 146VJ5
91 146VJ374
232 146VJ37        Au08C
75 146VJ37
119 83VJ41
191 132VJ17
149 83VJ41
158 83VJ41
202 146VJ37
409239201 2 E12 146VJ5
32 146VJ5
30 83VJ41        Sp05C
82 83VJ41
171 146VJ5
178 83VJ41       Sp05C
150 83VJ41
199 132VJ17      Sp08C
4 132VJ17
25 146VJ37
237 83VJ41
165 146VJ5
152 146VJ37
6 146VJ37
224 83VJ41
179 146VJ5
1 146VJ37        Au08C
241 146VJ37
243 146VJ5
234 146VJ5       Au08E
184 146VJ5
DQ445638.1 P. multiseries Pn-1
242 146VJ5
225 83VJ41
KX290892.1 P. multiseries 338b
KX290890.1 P. multiseries HAB-213
EU302798.1 P. multiseries PM-02
EU302797.1 P. multiseries CLN-50
87 146VJ5
24 146VJ37
78 132VJ17
124 132VJ17
190 132VJ1
73 83VJ41
74 83VJ41        Sp05C
170 83VJ41
409239201 2 C5 146VJ5
244 83VJ41
88 83VJ41
409239201 2 B8 146VJ5
409239201 2 H10 146VJ5
409239201 2 H9 146VJ5
409239201 2 F9 146VJ5
156 146VJ5
61 146VJ37
9 146VJ37        Au08C
53 132VJ17
409239201 2 F5 146VJ5
131 83VJ41
409239201 2 C12 146VJ5
409239201 2 E8 146VJ5
120 146VJ37
KJ710107.1 P. multiseries
409239201 2 B10 146VJ5
DQ445638.1 P. multiseries Pn1
409239202 3 E1 21VJ45
409239201 2 H7 146VJ5
409239201 2 H11 146VJ5
409239201 2 G8 146VJ5
409239201 2 G6 146VJ5
409239201 2 G11 146VJ5
409239201 2 F6 146VJ5
409239201 2 F10 146VJ5
409239201 2 E5 146VJ5
409239201 2 E10 146VJ5
409239201 2 D7 146VJ5
409239201 2 D11 146VJ5
409239201 2 C9 146VJ5
409239201 2 C6 146VJ5
409239201 2 B9 146VJ5
409239201 2 B6 146VJ5
409239201 2 B12 146VJ5
409239201 2 A8 146VJ5
129 83VJ41
136 146VJ5
409239201 2 A10 146VJ5    ✓
409239201 2 D6 146VJ5
409239201 2 A11 146VJ5
64 146VJ5
U41389.1 P. multiseries
KX290888.1 P. multiseries HAB-228
AF440770.1 P. multiseries NWFSC 005
KF006834.1 P. multiseries CLN-20
KX290889.1 P. multiseries 32b
KX290891.1 P. multiseries HAB-156
KX290893.1 P. multiseries 77
EF521880.1 P. multiseries AL-77
205 146VJ5
68 146VJ5
409239201 2 B11 146VJ5   Au08E
47 83VJ41        Sp05C
106 83VJ41
196 132VJ17
181 132VJ17
98 146VJ5
219 132VJ17
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Tree scale ⊢ 0.005

ML phylogeny of longer reads derived from the CLS dataset derived from the D1-186F, D1-548R LSU fragment (381 bp after trimming) also confirmed the presence of *P. multiseries* in these samples (Fig. S1 in the Supplement). This tree was more robust, with *P. multiseries* and *P. pungens* as sister species adjacent to clades containing *P. brasiliana*, *P. americana*, *P. multistriata*, *P. seriata* and *P. australis* that could be separated into their respective species, as also recovered by Lim et al. (2013).

Environmental species distribution: genetic and SEM identification

ML phylogeny of environmental sequences (Fig. 2, Table 3) of the trimmed LSU fragment (D1-186F, D1-548R, McDonald et al. 2007) from 11 CPR samples generated a total of 359 sequences: 163 CLS (many identical) and the 196 OTUs from the HTS dataset. All sequences are identified in Table S2 in the Supplement. CLS reads from 6 CPR samples were identified either as *P. multiseries*, *P. fraudulenta* or *P. pungens* (Table 4, Fig. S1 in the Supplement). The *P. fraudulenta* clade contained 212 HTS and CLS environmental sequences (Fig. 2B), and 117 HTS and CLS environmental sequences were identified as *P. multiseries* (Fig. 2B). As *P. multiseries* was an unusual finding in open-water samples, we confirmed its presence in the Au08E sample using a different primer (see 'Materials and methods', GenBank accession no. M820151). A minority of OTUs were related to other species (Table 3, see Fig. 2A): 17 OTUs clustered with *P. hasleana* and a clade of 10 OTUs (Environmental Group 2) formed a sister clade to *P. turgiduloides*, *Neodenticula* spp. and *Fragilariopsis* spp. The identity of Environmental Group 2 could not be determined; genetic distances of these OTUs to this sister clade ranged from 2–9%. BLAST identities of OTU 235, 56, 89 and 109 from Environmental Group 2 showed 98, 99, 99 and 99%, respectively to *P. subpacifica*, while BLAST identity hits of OTU 126 and 166 revealed 98% similarity to *P. subpacifica* and to *P. hasleana*. Three other OTUs could not be identified (Environmental Group 1) within *Pseudo-nitzschia* species. OTUs 92, 219 and 247 were sister to *P. multiseries*. These OTUs showed 99%

Fig. 2. (B) The expanded subtree shows microdiversity of *Pseudo-nitzschia multiseries* clade from Panel A. Clade label abbreviations are shown in the key bottom left of panel A. Genetic distance is shown at bottom right

Table 3. *Pseudo-nitzschia* species groups identified in this study from maximum likelihood (ML) phylogeny (Fig. 2) and related to previous studies showing the number of environmental sequences from this study that corresponds to each group

MP clade group name	Species in clade	Strain correspondence with previous studies	No. of environmental sequences
P. abrensis	*P. abrensis*	None	0
P. dolorosa, *P. micropora*	*P. dolorosa, P. micropora, P. delicatissima*	Orsini et al. (2004)-*P. delicatissima*, Lim et al. (2012), Ajani et al. (2013)	0
P. mannii, *P. pseudodelicatissima*	*P. mannii,* *P. pseudodelicatissima*	Orsini et al. (2002), Lim et al. (2012), Ajani et al. (2013)	0
P. kodamae	*P. kodamae*		0
P. hasleana	*P. hasleana*	Lundholm et al. (2012), Ajani et al. (2013)	17
P. calliantha	*P. calliantha*	Lim et al. (2012), Lundholm et al. (2012), Ajani et al. (2013)	0
P. cf. *delicatissima*	*P.* cf. *delicatissima,* *Pseudo-nitzschia* spp.		0
P. delicatissima, *P. arenysensis* 1 and 2	*P. delicatissima,* *P. arenysensis,* *Pseudo-nitzschia* sp. *P. pseudodelicatissima*	Lundholm et al. (2012), Orsini et al. (2002), Lim et al. (2012)	0
P. delicatissima, *P. turgidula,* *P. pseudodelicatissima*	*P. delicatissima,* *P. turgidula,* *P. pseudodelicatissima*		0
P. delicatissima	*P. delicatissima,*	Amato et al. (2007), McDonald et al. (2007)	0
P. multistriata	*P. multistriata*	Lim et al. (2012, 2013), Ajani et al. (2013)	0
P. australis	*P. australis*	Lim et al. (2012, 2013), Ajani et al. (2013)	0
P. seriata	*P. seriata*	Lim et al. (2012, 2013), Ajani et al. (2013)	0
P. brasiliana	*P. brasiliana*	Lim et al. (2012, 2013), Ajani et al. (2013)	0
P. linea	*P. linea*	None	0
P. americana	*P. americana*	Ajani et al. (2013)	0
P. pungens	*P. pungens, P. pungens* var. *aveirensis*	Lim et al. (2012, 2013), Ajani et al. (2013)	0
P. multiseries	*P. multiseries*	Lim et al. (2012, 2013), Ajani et al. (2013)	117
P. subfraudulenta	*P. subfraudulenta*	Subclade of *P. fraudulenta* (Lim et al. 2012, 2013, Ajani et al. 2013)	0
P. lundholmiae	*P. lundholmiae*	Lim et al. (2013)	0
P. lineola	*P. lineola*	Lundholm et al. (2012), Ajani et al. (2013)	0
MVR2015	*Pseudo-nitzschia* sp. MVR2015 cl. 22-23, Bacillariophyceae MVR2015 cl. 21	None	0
P. inflatula	*P. inflatula,* *P. pseudodelicatissima*	Lim et al. (2012), Lundholm et al. (2012)	0
P. subpacifica, P. heimii	*P. subpacifica, P.heimii*	Lim et al. (2012), Ajani et al. (2013)	0
P. fraudulenta	*P. fraudulenta*	Lim et al. (2012, 2013), Ajani et al. (2013)	212
P. fryxeliana	*P. fryxelliana,* *P. pseudodelicatissima*	Lim et al. (2012, 2013) Lundholm et al. (2012), Ajani et al. (2013)	0
P. circumpora	*P. circumpora*	Lim et al. (2012)	0
P. turgidula	*P. turgidula,* *P.* cf. *turgidula*	Lundholm et al. (2012)	0
P. caciantha	*P. caciantha*	None	0
P. arctica (single sequence)	*P. arctica*	Percopo et al. (2016)	

Table 3 (continued)

ML clade group name	Species in clade	Strain correspondence with previous studies	No. of environmental sequences
P. granii (single sequence)	*P. granii*	Record not released yet	0
P. galaxiae	*P. galaxiae*	McDonald et al. (2007), Lim et al. (2012, 2013)	0
P. sabit	*P. sabit*	Teng et al. (2015)	0
P. turgiduloides, *Neodenticula*, *Fragilariopsis*	*Neodenticula seminae*, *Fragilariopsis cylindricus*, *F. curta*, *F. rhombica*, *F. vanheurkii*, *F. kerguelensis*	None	0
P. pseudodelicatissima, *P. cuspidata*, *P. plurisecta*, *P. fukuyoi*	*P. pseudodelicatissima*, *P. cuspidata*, *P. plurisecta*, *P. fukuyoi*	Ajani et al. (2013), Fernandez et al. (2014), Lundholm et al. (2012), Lim et al. (2012, 2013)	0
Environmental Group 1	OTU 92, 219, 247, sister to *P. multiseries*	None	3
Environmental Group 2	OTU 235, 56, 126, 118, 89, 109, 166, 3, 221, 140	None	10

Table 4. *Pseudo-nitzschia* species identified by scanning electron microscopy (SEM) from a subset of the genetically analysed sample set. Sample ID relates to the Continuous Plankton Recorder (CPR) sample. The sequence analysis method indicates whether the samples were analysed using high-throughput sequencing (HTS) technology or clone library (CL) sequencing technology. The numbers of raw reads generated from HTS analysis are indicated where applicable. NA: not applicable

Sample ID	Method	HTS reads	Code	*Pseudo-nitzschia* species found by SEM	*Pseudo-nitzschia* species found by HTS	*Pseudo-nitzschia* species found by CL sequencing
21VJ1	454	3202	Au02E	*P. heimii* plus small undetermined species	*P. fraudulenta*	NA
21VJ5	CL		Au02E(2)	NA	NA	*P. fraudulenta*
139VJ1	454, CL	5038	Su08E	None (*Thalassiosira* spp. abundant)	*P. fraudulenta*	*P. fraudulenta*
146VJ5	454, CL	5962	Au08E	NA	*P. multiseries*, *P. fraudulenta*, Environmental Group 2	*P. multiseries*, *P. fraudulenta*, *P. pungens*
83VJ5	454	4548	Sp05E	NA	*P. fraudulenta*	NA
77VJ7	454	5154	Sp05NE	None	*P. fraudulenta*, Environmental Group 1	NA
21VJ41	454	3764	Au02C	*P. cuspidata*, *P. turgidula*, *P. heimii*	*P. fraudulenta*, Environmental Group 2	NA
21VJ45	CL		Au02C(2)	*P. cuspidata*, *P. turgidula*, *P. australis*	NA	*P. fraudulenta*
132VJ17	454	2925	Sp08C	*P. turgidula*	*P. multiseries*, *P. hasleana*, *P. fraudulenta*, Environmental Groups 1, 2	*P. multiseries* (checked using different sequencing primers)
146VJ37	454, CL	2632	Au08C	*P. turgidula*, *P. inflatula*, *P. fraudulenta*	*P. multiseries*	*P. fraudulenta*
83VJ41	454, CL	6506	Sp05C	*P. australis*	*P. multiseries*	*P. fraudulenta*

Table 5. List of *Pseudo-nitzschia* species identified by scanning electron microscopy (SEM) in 2014. Sample ID relates to the Continuous Plankton Recorder (CPR) sample; locations are shown in Fig. 1. Samples are listed following their longitudinal position

Sample ID	Environment	Sample latitude (° N)	Sample longitude (° E)	Month	Year	*Pseudo-nitzschia* species found by SEM
272VJ-1	Eastern, coastal	48.348	−124.135	8	2014	*P. fraudulenta, P. pungens, P. seriata, P. multiseries*
272VJ-5	Eastern, coastal	48.517	−125.077	8	2014	*P. fraudulenta, P. pungens, P. seriata, P. multiseries*
272VJ-9	Eastern, coastal	48.73	−126.02	1	2014	*P. fraudulenta, P. pungens, P. seriata, P. multiseries*
273VJ-3	Central, open	51.393	−136.688	2	2014	*P. inflatula, P. pseudodelicatissima, P. turgidula*
273VJ-11	Central, open	51.848	−138.707	3	2014	*P. inflatula, P. pseudodelicatissima, P. turgidula*
272VJ-45	Central, open	50.962	−134.648	9	2014	*P. inflatula, P. pseudodelicatissima, P. turgidula*
273VJ-39	Central, open	53.067	−145.865	4	2014	*P. inflatula, P. pseudodelicatissima, P. turgidula*
273VJ-43	Central, open	53.187	−146.96	4	2014	*P. inflatula, P. pseudodelicatissima, P. turgidula*

BLAST identity to *P. fraudulenta*. Overall BLAST identities were found to be inaccurate and were not in agreement with phylogenetic analysis. In comparison to the CLS dataset, HTS generated more diversity, and both datasets were mostly congruent (Fig. 2, see Table 4) in samples where both methods were used, showing consistency in detection. Even in the 2 cases where alternative samples were used, i.e. Au02E and Au02E(2), Au02C and Au02C(2), *P. fraudulenta* was identified in both samples either by CLS or HTS. The only inconsistencies were in samples Au08C and Sp05C, where *P. multiseries* was detected by HTS while CLS detected *P. fraudulenta*. Only CLS identified *P. pungens* in Au08E, which was absent in HTS-analysed samples. However, only a few CLS sequences were produced in these samples, so bias is likely here.

SEM identification was applied to a subset of the 2002–2008 samples used for genetic analysis (Table 4). This revealed typical coastal and open-water species composition also found in earlier studies of this region (Table 1). Since no *Pseudo-nitzschia* cells were found in 2 Eastern (coastal) samples, additional SEM analysis of samples from 2014 (Table 5, Fig. 3) were carried out from the same area to determine the extent that SEM can uncover species diversity from CPR samples. Both 2002–2008 (Table 4) and 2014

(Table 5) samples showed typical coastal and open-water species compositions compared to earlier Pacific studies (Table 1), confirming that CPR sampling is representative for *Pseudo-nitzschia* spp. SEM
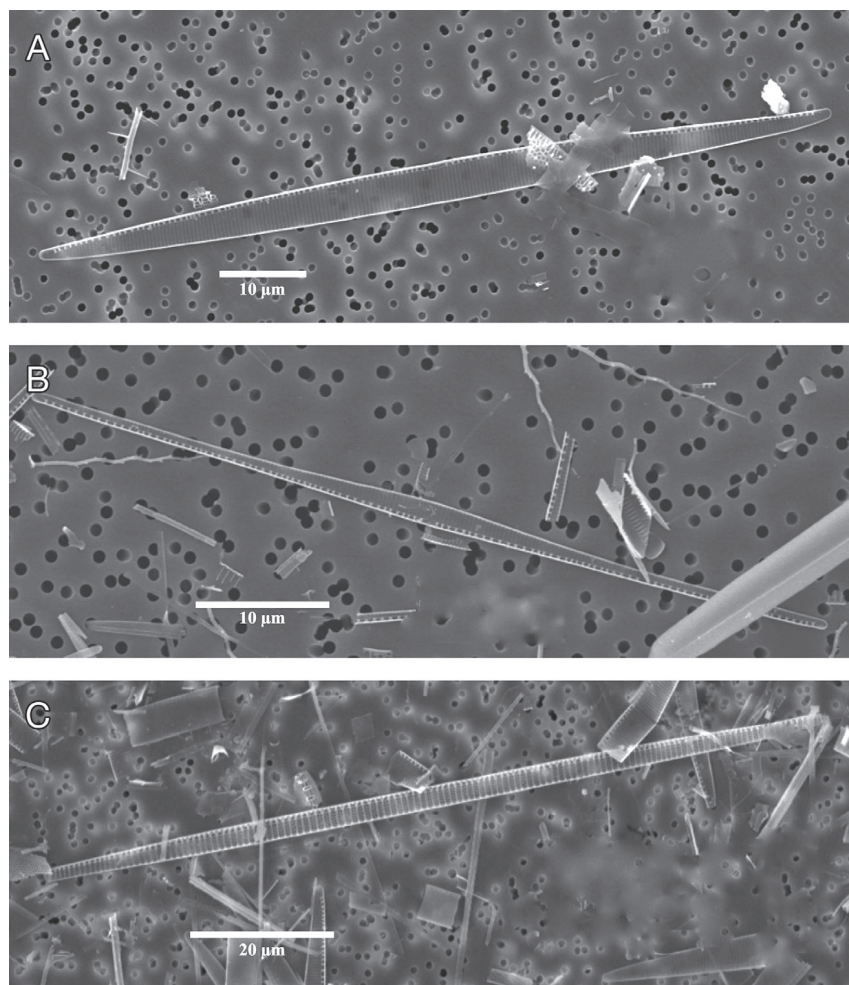

Fig. 3. Scanning electron microscopy images of (A) *Pseudo-nitzschia fraudulenta*, (B) *P. inflatula* and (C) *P. pungens* from the Continuous Plankton Recorder 2014 samples. See Table 5 for sampling locations

results from Central (open water) samples from 2002–2008 could be compared with those of 2014 and revealed different communities in which only *P. turgidula* was common to both sets. *P. heimii* and unidentified species were the only taxa identified from 2002–2008 in Eastern samples but were not present in 2014 Eastern samples. SEM-identification showed little correspondence with genetic results (Table 4). *P. fraudulenta*, *P. multiseries* and *P. pungens* were observable using both SEM and genetics, but only 1 sample (Au08C) showed direct correspondence by genetics and SEM and only for *P. fraudulenta*. Little seasonal variation was observed from both sets of SEM results in contrast to genetic results. *P. turgidula* and *P. inflatula* were found to be exclusively open-water species in previous studies but were not found genetically in any sample. Particularly striking was that only 1 species, *P. turgidula*, was found by SEM in the Sp08C sample, yet genetic results showed that this sample was the most diverse with 5 different genetic taxa. *P. multiseries* has not previously been observed in central samples, and *P. hasleana* has not yet been reported in central Pacific waters.

Ocean conditions

The last 'full' PDO cycle consisted of a cool phase from 1947 through 1976 followed by a warm phase from 1977 through (at least) the mid-1990s (Mantua et al. 1997, Zhang et al. 1997). In late 1998, the PDO entered a cold phase that lasted 4 yr, followed by a warm phase that lasted 3 yr (2002 through 2005), neutral until August 2007, and then a 6 yr cold phase through 2013 (interrupted briefly by the moderate El Niño in fall/winter of 2009/10). Monthly values of the PDO index from 2000 through 2010 are shown in Fig. 4. sPDO values were weakly positive during Autumn 2002 (Au02) and Spring 2005 (Sp05), and strongly negative during Spring 2008 (Sp08), Summer 2008 (Su08) and Autumn 2008 (Au08; Table 2). Note that even though the sPDO value was weakly positive during Au02, the PDO had just reversed polarity from cool to warm phase and conditions may have been more representative of transitional periods.

Temporal patterns of monthly SST anomalies for grid cells that encompassed locations where CPR samples were collected closely followed the PDO index in both the Central and Eastern regions (Fig. 4; and see Fig. S3 in the Supplement). No strong differences in local SST variability was apparent between the 2 regions, except that the cool PDO phase from
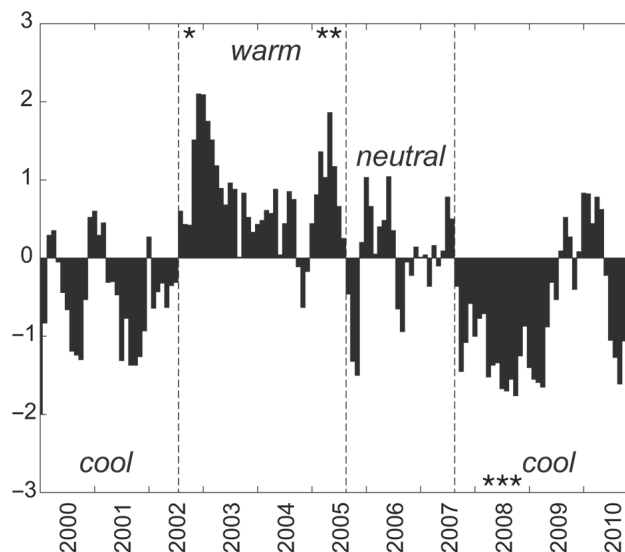

Fig. 4. Time series of monthly values of the Pacific Decadal Oscillation (PDO) index from 2000 through 2010 indicating warm, cool and neutral phases. Asterisks indicate months when CPR samples used in the molecular analyses were collected

late 1998 through 2001 was less pronounced in the Eastern region compared to the Central region. Within a region (Central or Eastern), temporal patterns of local SST variability for grid cells that encompassed locations where CPR samples were collected were very similar to one another and responded similarly to warm and cool phases of the PDO (Fig. 4; Fig. S3). Synoptic snapshots of SST in the NE Pacific Ocean during months when CPR samples were collected are shown in Fig. 5. These plots show the spatial patterns in the monthly average SST values across the regions during the cool (2002 and 2008) and warm (2005) PDO years and for months in the spring, summer and autumn. The synoptic snapshots of the regions during May 2005 and May 2008 allow a direct comparison of a warm and cool PDO year, respectively, with the average SST across both regions being ~2.9°C cooler in 2008 (Fig. 5C,D). During all months, the Central region was always cooler than the Eastern region, and southern waters were generally warmer compared to northern waters within the study area (Fig. 5). A strong seasonal pattern was also evident, whereby SST was cooler in the spring compared to the summer and autumn (Fig. 5).

CPR diatom community analysis

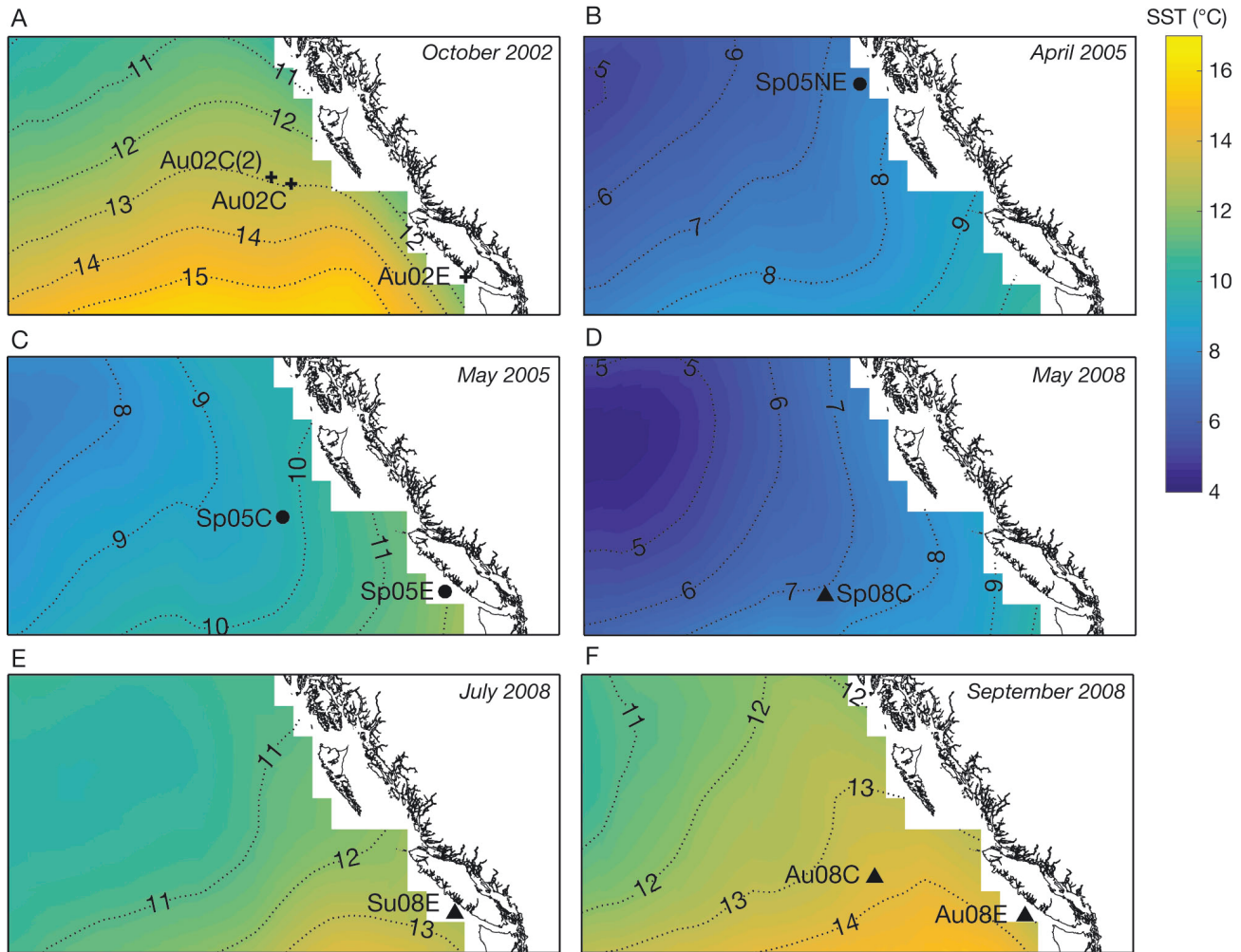Comparing diversity of HTS-generated OTUs between samples (Fig. 6) revealed that Eastern samples

Fig. 5. Spatial variability in monthly averages of satellite-derived sea surface temperature (SST) in the NE Pacific during months when the Continuous Plankton Recorder (CPR) samples used in the molecular analyses were collected. Maps show contoured SST during (A) October 2002 (Au02E, Au02C and Au02C(2)); (B) April 2005 (Sp05NE); (C) May 2005 (Sp05E and Sp05C); (D) May 2008 (Sp08C); (E) July 2008 (Su08E); and (F) September 2008 (Au08E and Au08C)

were dominated by *P. fraudulenta*, whereas Central samples were more variable. *P. fraudulenta* diversity was present in 8 of the 9 HTS samples and was common in all coastal (Eastern Pacific) samples, except for Au08E. A large proportion of *P. fraudulenta* OTUs was observed in Au02C. By contrast, *P. multiseries* OTU diversity was generally dominant when *P. fraudulenta* was rare. *P. multiseries* was common in spring and autumn samples. Three samples contained a large proportion of *P. multiseries* OTUs (Sp05C, Au08C, Au08E). A small proportion of *P. multiseries* OTUs were present in Au02C, Sp08C, Sp05E and Su08E. Endemic novel diversity was observed within *P. fraudulenta* and *P. multiseries* (Fig. 2A,B, respectively). A substantial amount of substructure was present in both species that points to the existence of local populations. Many clades only contained envi-

ronmental *P. fraudulenta* (clustering with CLS reads confirmed as *P. fraudulenta*, Fig. S1 in the Supplement). Those that clustered with public *P. fraudulenta* were mostly from coastal samples. Seven *P. fraudulenta* environmental OTU clades originated only from single samples: Au02E (1 clade), Su08E (4 clades), Sp05NE (1 clade) and Sp05E (1 clade). Two of these clades were also recovered using longer CLS phylogeny (Fig. S1). By contrast, 10 novel clades of *P. multiseries* environmental OTUs belonged to Au08E (2 clades), Sp05C (4 clades), Au08C (3 clades) and Sp08C (1 clade). One clade of CLS environmental sequences was also recovered in *P. multiseries* longer CLS phylogeny (Fig. S1). Many of the *P. multiseries* clades also corresponded with public sequences of strains (KC710107, EF521880, AF417655, KC017458). These public sequences were related to each other,
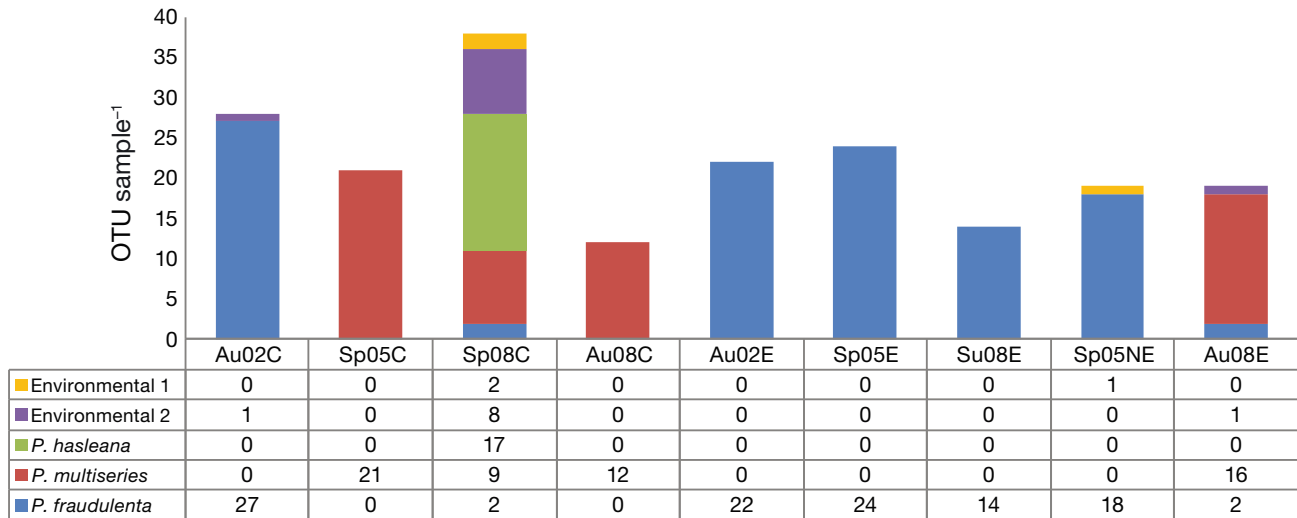
	Au02C	Sp05C	Sp08C	Au08C	Au02E	Sp05E	Su08E	Sp05NE	Au08E
Environmental 1	0	0	2	0	0	0	0	1	0
Environmental 2	1	0	8	0	0	0	0	0	1
P. hasleana	0	0	17	0	0	0	0	0	0
P. multiseries	0	21	9	12	0	0	0	0	16
P. fraudulenta	27	0	2	0	22	24	14	18	2

Fig. 6. Diversity of Environmental *Pseudo-nitzschia* operational taxonomic unit (OTU) diversity found in samples analysed from high-throughput sequencing environmental reads, clustered at 99 and 90% identity. Sample codes as in Fig. 5
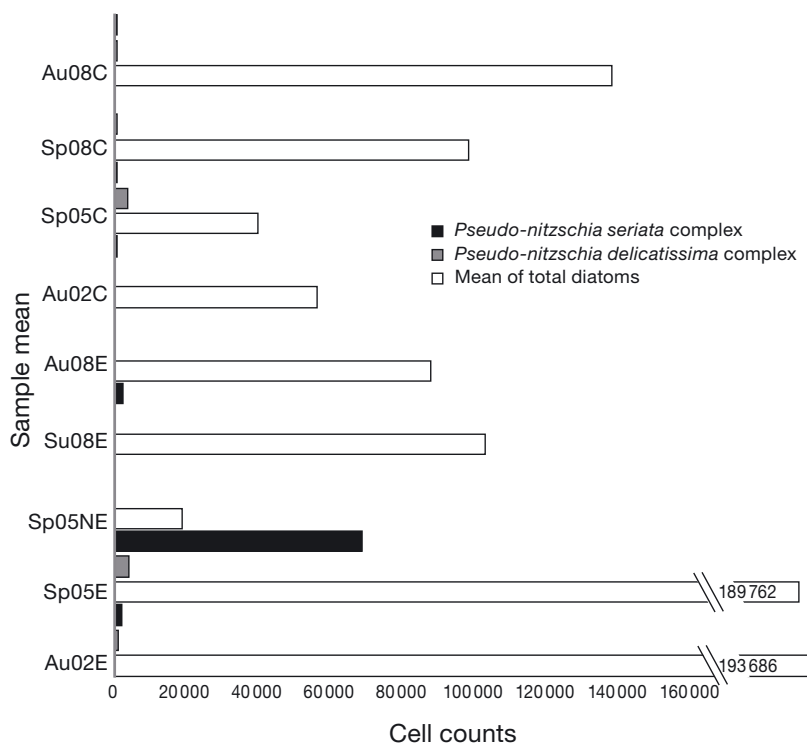
Fig. 7. Average cell counts of all diatoms (open bars) compared to *Pseudo-nitzschia seriata*-sized cells and *Pseudo-nitzschia delicatissima*-sized cells in each region. Sample codes as in Fig. 5

but globally distributed (Thessen et al. 2009, Ajani et al. 2013). SEM results did not reveal any seasonality in the 2002−2008 or 2014 samples (Tables 4 & 5). Community composition by SEM analysis was very homogenous within each region. Within this small

sample set, no clear trend was observed between genetically detected species or population patterns and PDO phase, with species and novel clades found equally in positive and negative PDO phases. However, taxa composition in Sp08C stood out as unusually diverse compared to all other samples (Fig. 6) which was collected when SSTs were exceptionally cold. Furthermore, the dominance of *P. multiseries* in A08E was different from genetic community composition of other Eastern samples. It is noteworthy that no pattern emerged between sample age and species richness that might indicate degradation-related alterations, nor were any patterns related to genomic DNA concentration.

Fig. 7 compares the seasonal mean (mean per region over a season for a given year) abundance of total diatoms versus the larger *P. seriata*-sized cells (>3 µm width), and smaller *P. delicatissima*-sized cells (<3 µm width). No correspondence was found between the seasonal means (Fig. 7) or average cell counts of *Pseudo-nitzschia* spp. (data not shown) in samples used for genetic analysis and the number of LSU sequences (Table 4). The abundance of *Pseudo-nitzschia* was not related to the genetic diversity of species found in samples or to SEM detection. The Sp05E sample contained the highest number of *Pseudo-nitzschia* (~7000 cells),

mostly consisting of *P. seriata*-sized cells. In comparison, other samples contained fewer than 2000 *Pseudo-nitzschia* cells. In total, *P. seriata*-sized cells were present in 9 out of 16 seasonal means. Four seasonal means recorded *P. delicatissima*-sized cells, with only 1 of those not recording *P. seriata*-sized cells. More *Pseudo-nitzschia* were recorded in the warmer year of 2005 compared to 2002 and 2008. *Pseudo-nitzschia* spp. ranged from 1.5–33% (seasonal mean) of the total diatoms. Total diatoms were generally more abundant in Eastern versus Central regions, except for autumn 2005 and 2008, but no geographic pattern was discernible for *Pseudo-nitzschia* seasonal means.

DISCUSSION

High-throughput genetic analysis is becoming cheaper and can complement microscopic counts to delineate *Pseudo-nitzschia* in more detail. Our study reveals that HTS can be used on formalin-preserved samples and that these genetic studies are an important addition to microscopic diversity studies in the Pacific, uncovering novel diversity of species and their distributions. This phylogeny is not as resolved as those using the longer D1-D3 LSU reads (Lim et al. 2013), but this phylogeny could separate many species. Four taxa were uncovered, 2 potentially novel and 2 species showing new distributions. Species diversity identified from SEM in these samples differed from that generated by genetics, but was similar in composition to previous studies listed in Table 1, mostly based on microscopic identification. *P. multiseries* was the second most dominant species group found in this study, and an unexpected finding, as it has not previously been reported in open waters of the Pacific. The finding of potentially harmful species in open Pacific waters has implications for monitoring harmful species in Pacific waters and modelling their distribution.

Both genetic and SEM diversity revealed contrasting species communities in coastal Pacific waters, which are generally iron-rich, nitrate-poor with high phytoplankton productivity compared to open-water communities, which have lower phytoplankton productivity, because these waters are iron-poor but nitrate-rich, so called 'high-nutrient, low-chlorophyll' [HNLC] regions (Harrison et al. 1999, Ribalet et al. 2010). Studies in NE Pacific waters revealed that phytoplankton and *Pseudo-nitzschia* spp. communities were structured by a nutrient gradient from coastal transitional to open-water zones, revealing different communities in coastal, transitional and open-water zones (Ribalet et al. 2010). Some of the sampling sites from Ribalet et al. (2010) were near to our CPR sampling stations where we found extraordinary intra-species diversity in *P. fraudulenta* and *P. multiseries* by HTS. Much of this intra-specific diversity was restricted to localised areas specific to 1 year in many cases. This leads us to hypothesize that this may be a species complex with local isolated populations adapted to different regions. Further confirmation of biogeographical population structure in *P. fraudulenta* has been shown in the Mediterranean region (Moschandreou et al. 2012). On the other hand, we found environmental variants of a globally distributed *P. multiseries* population that was also described by Ajani et al. (2013) and Thessen et al. (2009). This may indicate local adaptation in a cosmopolitan population. This study supports the physiological findings of Thessen et al. (2009), revealing that *Pseudo-nitzschia* can adapt to multiple environments, perhaps due to its high genetic variability that allows multiple ecotypes to succeed each other. DA-producing strains of *P. multiseries* (Pn-1) and two identical *P. fraudulenta* strains (Pn-9, Pn-12) studied by Thessen et al. (2009) were highly similar to environmental sequences uncovered in this study. However, identical strains showed different growth response and DA production when exposed to different nutrient regimes (Thessen et al. 2009), revealing a more complex control mechanism of DA toxin production in *Pseudo-nitzschia*.

Pseudo-nitzschia abundances determined from microscopic counts of CPR samples were greater in 2005, when SST was highest, compared to 2002 and 2008 (with lower SST), indicating greater growth of potentially toxic species with warmer waters and thus potentially a link with PDO. The small sample size prohibits the identification of any conclusive patterns with the PDO or seasonality, but our results suggest that temperature may influence species composition. Both Central and Eastern autumn samples in 2002 and 2008 were similar in SST and were similar in species composition, despite different nutrient regimes in these regions. Spring samples were the most diverse and harboured all novel diversity, particularly Sp08C when SST was at its lowest. *P. multiseries* appears to prefer cooler waters, as its diversity was highest in central regions where SST was lower.

P. multiseries is a large cosmopolitan species (Hasle 2002) that has been reported from coastal locations (Forbes & Denman 1991, Horner & Postel 1993, Hasle 2002, Trainer et al. 2012). Our finding that genetically identified *P. multiseries* dominated *Pseudo-nitzschia* species assemblages in 2 of the 4

Central region samples is therefore unusual and was not supported by SEM results from partially destroyed samples. *P. multiseries* was observed in 3 Eastern samples from 2014, indicating that it is captured by the CPR. One possible explanation is that *P. multiseries* environmental DNA (e-DNA) was disseminated to central open waters from coastal regions which would be undetectable by microscopy. However, studies have shown that e-DNA has a rapid degradation rate in seawater, and even small fragments of 100 bp can only last days (Thomsen & Willerslev 2015). Thus it would be difficult to conceive of e-DNA surviving the approximately 800 km (430 n miles) from coastal to open-water communities. There is a possibility of sample contamination from DNA of broken cells taken from Eastern samples leaking through on the CPR sample roll or from the formalin tank to contaminate Central samples. CPR samples are collected on a roll of silk with another layer of silk sandwiched over the plankton layer. Several layers of silk separate samples collected from open and coastal regions (Richardson et al. 2006). Central and Eastern samples are separated by approximately 430 n miles. The longest CPR tow route is 500 n miles, requiring 5 m of silk, so these samples are farthest away from each other, separated by no less than 4 m of silk on a roll (Richardson et al. 2006). Therefore, the possibility of contamination remains but is remote. The diversity of OTUs in these 4 independent Central samples should be equivalent or more in their Eastern counterparts if contamination from the latter was the source of *P. multiseries.* However, this is not the case, and furthermore, OTUs specific only to Central samples were not present in Eastern samples from the same tow, making contamination an unlikely option. The alternative explanation is that local populations of *P. multiseries* are supported by mesoscale-level Haida eddies containing nutrients transported up to 1000 km from their point of origin to HNLC regions of the NE Pacific (Whitney & Robert 2002). These currents could also bring and support local coastal and cosmopolitan populations for extended periods that may create new hybrid forms, thus resulting in a mixture of localised and global populations.

A good match was found between HTS and CLS genetic approaches in terms of species detected except for 2 central samples likely due to low recovery of clone-library sequences from these samples. The diversity within species from CLS was severely depleted compared to HTS, and thus this approach is not recommended for diversity studies. There could

be several reasons for the lack of congruity between genetic and SEM results. Sampling and processing differences using the 2 methods are likely a main contributor. Diatoms are one of a few examples where genetics and SEM correspond with adequately resolved genetic markers (Malviya et al. 2016). However, SEM is not a high-throughput method, which may mean diversity is lost, especially in this case where part of the sample was destroyed for genetic analysis. Likewise, there are bioinformatic biases. Many reads were shorter than the 300 bp required for a good identification whilst the use of traditional pairwise alignment sequence dissimilarity (PSD, Nguyen et al. 2016) clustering methods similar to the method used in this paper, has been shown to create poor OTU clusters, reducing diversity in the final dataset. Better clustering methods using curated and representative sequence databases to identify OTUs within the bioinformatic pipeline would also improve the OTU retrieval process.

Other reasons for bias are sample condition, DNA extraction, genetic assays and the lack of resolution of this marker. These variables combined with population structure within several species common to Pacific Eastern waters, such as *P. pseudodelicatissima* will reduce sequence quality and diversity. The use of new technology such as MinION sequencing (Oxford Nanopores), that directly sequences long-reads of genomic DNA without an amplification step, would reduce bias brought about by PCR analysis of mixed templates (Suzuki 1996, Kalle et al. 2014) and allow better delineation of species with longer reads. The lack of public reference sequences especially for Pacific open-water species such as *P. turgidula* and *P. inflatula* could be an additional factor hindering their detection. Increased database representation would improve the phylogeny delineating species or populations within species, improving the identification of unknown environmental sequences.

It is clear from this study that more comparative work with both genetics and SEM would benefit characterisation of *Pseudo-nitzschia* in this region. Nevertheless, new species to this region have been uncovered using the HTS approach on archival formalin-preserved samples. A novel group of sequences has been found, and the distribution of *P. hasleana* has been extended to open Pacific waters which demonstrates that diversity in the NE Pacific is under-characterised. Open-water species deserve further study to capture and culture representatives to determine their environmental preferences. Their response to nutrients and temperature make them valuable indicators of ocean health.

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