J Appl Phycol (2012) 24:1465–1475
DOI 10.1007/s10811-012-9803-7

Genetic diversity of *Pseudo-nitzschia brasiliana* (Bacillariophyceae) from Malaysia

Hong-Chang Lim · Po-Teen Lim · Suriyanti Nyun-Pau Su · Sing-Tung Teng · Chui-Pin Leaw

Received: 23 August 2011 / Revised and accepted: 24 January 2012 / Published online: 22 February 2012

Abstract To clarify the genetic diversity of a potentially toxic pennate diatom, *Pseudo-nitzschia brasiliana* found in Malaysian waters, 30 strains of *P. brasiliana* were established into clonal culture since May 2008. The ultrastructure of these strains was examined for confirmation of species identification. The genetic marker, internal transcribed spacer (ITS) of nuclear-encoded ribosomal DNA was used to examine the genetic diversity of *P. brasiliana* isolated from different geographical localities. The ITS sequences of *P. brasiliana* were highly conserved in their secondary structures, with five helices in the first internal transcribe spacer (ITS1) and four universal helices in the second internal transcribe spacer (ITS2) with a pseudo-helix. No compensatory base change was observed among the strains examined. Genetic divergences among the Malaysian strains ranged from 0.07 to 0.54%. The present study revealed a high genetic homogeneity of Malaysian *P. brasiliana* strains.

Keywords *Pseudo-nitzschia brasiliana* · Genetic diversity · ITS rDNA · Secondary structure · Minimum spanning network

Introduction

Pseudo-nitzschia is a genus of chain-forming pennate diatoms widely distributed in brackish to marine environments, from polar to temperate and equatorial waters (Hasle 2002). Blooms of some *Pseudo-nitzschia* species have raised issues of human toxicity due to contamination of the neurotoxin, domoic acid, in shellfish (e.g., Bates et al. 1989). While most *Pseudo-nitzschia* species are cosmopolitan, some have restricted distribution known to date. The distribution of *Pseudo-nitzschia seriata* is confined to temperate waters in the Northern Hemisphere (Hasle 2002), e.g., European waters (Cusack et al. 2004). *Pseudo-nitzschia turgiduloides* is only found in the cold Antarctic waters of the Southern Hemisphere (Scott and Thomas 2005). *Pseudo-nitzschia lineola* and *Pseudo-nitzschia turgidula* have been reported in both Northern and Southern Hemispheres, but not in the warm tropical waters (Almandoz et al. 2007, 2008).

In recent years, studies of *Pseudo-nitzschia* population genetics have been undertaken through various genetic approaches. These include the application of microsatellites markers (Evans et al. 2005; Adams et al. 2009; Casteleyn et al. 2009, 2010), inference of secondary structure in the internal transcribed spacer (ITS) rDNA region (Casteleyn et al. 2008), and the development of automated ribosomal intergenic spacer analysis (Hubbard et al. 2008). To date, the population structure of *Pseudo-nitzschia* on a global scale was only examined in *Pseudo-nitzschia pungens* due to its worldwide distribution (Hasle 2002; Casteleyn et al. 2008, 2009, 2010). This was the first worldwide population genetic study with microsatellites in *Pseudo-nitzschia* (Evans et al. 2005). On a local scale, the population genetics of *Pseudo-nitzschia multistriata* (D'Alelio et al. 2009a) and the Malaysian *P. pungens* (Lim et al. 2011a) were inferred from the ITS region of nuclear-encoded ribosomal RNA (rRNA) gene.

Pseudo-nitzschia brasiliana was first described in Brazil (Lundholm et al. 2002). Subsequently, this species was reported to be distributed worldwide, mainly in Brazil (Lundholm et al. 2002), Spain (Quijano-Scheggia et al. 2008, 2009a), and China (Li et al. 2010).

H.-C. Lim · P.-T. Lim · S. N.-P. Su
Faculty of Resource Science and Technology,
Universiti Malaysia Sarawak,
Kota Samarahan 94300 Sarawak, Malaysia

S.-T. Teng · C.-P. Leaw (✉)
Institute of Biodiversity and Environmental Conservation,
Universiti Malaysia Sarawak,
Kota Samarahan 94300 Sarawak, Malaysia
e-mail: cpleaw@ibec.unimas.my

Little is known about the distribution of *Pseudo-nitzschia* in the South China Sea, particularly in the Malaysian waters (Lim et al. 2011b). This study aims to investigate the intraspecific genetic variation and geographic distribution patterns of Malaysian *P. brasiliana*. In this study, clonal cultures of *P. brasiliana* were established from different locations in Malaysian waters. The genetic diversity of the species was examined using the ITS region of rRNA gene with the inference of its secondary structure.

Materials and methods

Sampling, culture and morphological observation

Samples were collected from the coastal waters of Sabah (Pulau Mamutik and Kota Kinabalu), Sarawak (Samariang Batu and Santubong), Malaysian Borneo, and Negeri Sembilan (Port Dickson), the west coast of Peninsular Malaysia (Fig. 1). Phytoplankton samples were collected by vertical haul using a 20-μm mesh plankton net during high tide. Clonal cultures were established by isolating single chains using a finely drawn micropipette under an Olympus IX51 inverted light microscope.

Cultures were grown and maintained in sterile SWII medium (Iwasaki 1961) with pH of 7.8–7.9. The medium was enriched with 500 μM of sodium metasilicate. Natural filtered seawater with 30 psu was used as the medium base. Cultures were maintained at 25 ± 0.5°C under a 12:12-h light/dark photoperiod with light intensity of 200 μmol photons m^{-2} s^{-1} in a temperature-controlled cold white fluorescent incubator (Shelab, USA). A total of 30 clones of *P. brasiliana* were used in this study.

For species identification, organic material in the cell was cleaned by acid wash treatment as described in Bargu et al. (2002). Both field and cultured samples were treated with saturated $KMnO_4$, followed by the addition of 1 mL concentrated HCl. The mixtures were rinsed several times with distilled water to remove remaining salt and acid.

For transmission electron microscopy (TEM), the acid-cleaned materials were mounted on a square mesh Veco copper grid, dried, and examined under a JEOL JEM-1230 transmission electron microscope, while for scanning electron microscopic (SEM) observation, the acid-washed materials were mounted onto a track-etched polycarbonate membrane filter (pore size, 0.2 μm), attached to a stub with carbon tape, and sputter-coated with gold–palladium using a JEOL JFC-1600 magnetron sputter coating instrument. Specimens were examined under a JEOL JSM-6390LA SEM. Detailed morphological characteristics were examined and morphometric measurements performed on at least 30 acid-cleaned cells (Table 1).

Total genomic DNA extraction

Mid-exponential clonal cultures of *Pseudo-nitzschia* were harvested by centrifugation (10,000 rpm for 10 min) in microfuge tubes. Cells were lysed by adding 2× cetyltrimetyl ammonium bromide (CTAB) buffer consisted of 0.02 M EDTA, 0.06 M CTAB, 0.1 M Tris-Base, 1.4 M NaCl, and 1 mL of 2-β-mercaptoethanol, with an addition of 5 μL Proteinase K (20 mg mL^{-1}; Qiagen, Germany). The mixture was incubated at 60°C for 45 min to 1 h. This was followed by adding an equal volume of chloroform/isoamyl alcohol (C/I, 24:1), spun at 10,000 rpm for 10 min. Total genomic DNA was precipitated by adding an equal volume of absolute ethanol and 25 μL of 3 M NaCl. The sample was kept in −20°C for 3 to 4 h. The sample was then centrifuged at 13,000 rpm for 10 min and the DNA pellet rinsed with 70% ethanol, followed by another centrifugation. The DNA pellet was air-dried, dissolved in 50 μL of TE buffer (10 mM Tris–HCl, pH 7.4, and 1 mM EDTA, pH 8.0), and stored at −20°C until further analysis.

Gene amplification and sequencing

The ITS region (ITS1–5.8S–ITS2) was amplified using the universal primers ITS1, 5′-TCC GTA GGT GAA CCT GCG G-3′ and ITS4, 5′-TCC TCC GCT TAT TGA TAT GC-3′ (Vivantis Technologies, Malaysia; White et al. 1990). The region was amplified in a 25-μL reaction mixture which contained 1× PCR buffer (Promega, Madison, USA); 2.5 mM $MgCl_2$; 0.4 mM of each dATP, dTTP, dCTP, and dGTP (Qiagen); 0.02 μM of each primer; 2.5 U *Taq* polymerase (Promega); and approx. 80 μg μL^{-1} genomic DNA template. The PCR condition was as follows: initial denaturation at 94°C for 4 min, followed by 35 cycles (30 s at 94°C, 30 s at 51°C, and 60 s at 72°C), and final extension at 72°C for 7 min.

The PCR products were purified using Wizard® PCR Preps DNA Purification kit (Promega) according to manufacturer's instructions. Sequencing was carried out using an ABI 377 automated sequencer (PE Applied Biosystems, USA). Sequencing for each sample was carried out on both strands. The nucleotide sequences obtained were initially edited using the BioEdit Sequence Alignment Editor, version 7.0.9.0 (Hall 1999), followed by ClustalX 2.0 (Thompson et al. 1997). Complete sequence was obtained for further analyses.

Secondary structure prediction and sequence–structure alignment

The ITS1 region was predicted through homologous modeling where *P. pungens* sequence (AM778733) was used as the template (Casteleyn et al. 2008) in the ITS2 Database III (http://its2.bioapps.biozentrum.uni-wuerzburg.de/cgi-bin/

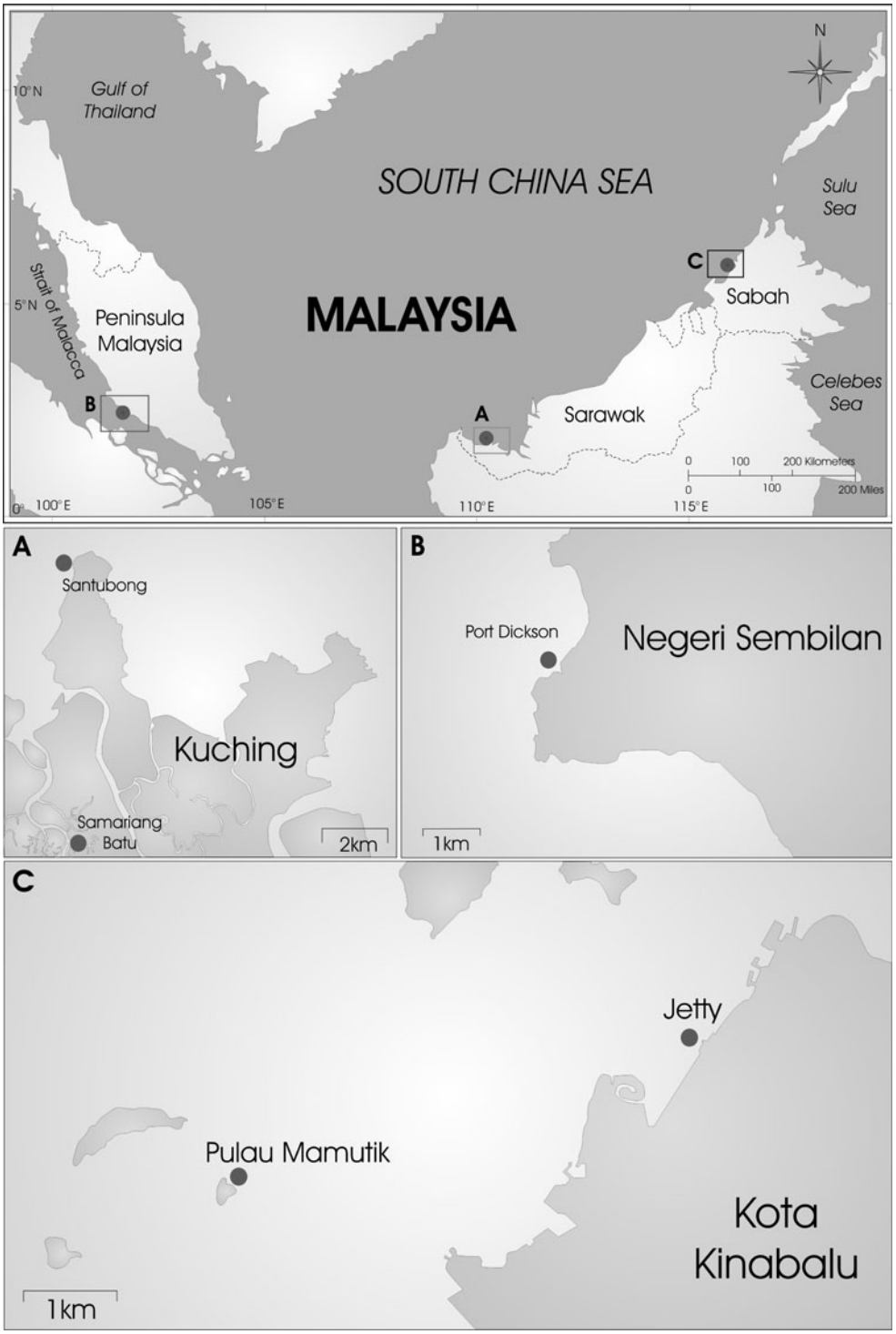

Fig. 1 Sampling locations in Malaysia where *P. brasiliana* strains were collected: Santubong and Samariang Batu in Kuching (**a**), Port Dickson in Negeri Sembilan (**b**), and Pulau Mamutik and Jetty in Kota Kinabalu (**c**)

index.pl?custom; Schultz et al. 2006; Selig et al. 2008; Koetschan et al. 2010). The ITS2PAM50 matrix was chosen and the percentage of transfer helices at 75% similarity was selected. The 5.8S region was constructed in the same manner by referring to the secondary structure model of Gottschling and Plotner (2004). The ITS2 region of *P. pungens*, strain PnMt45 (HQ111412), was used to construct the secondary structure by free energy minimization using RNAstructure 5.02 (Mathews et al. 2004). The predicted structure was then used as a template in homologous modeling for the rest of the sequences obtained in this study (Table 2). Available sequences of *P. brasiliana* were retrieved from GenBank for the secondary structure analyses (Table 2).

The whole ITS sequences with structural information were unambiguously aligned using 4SALE (Seibel et al. 2006, 2008) through remote alignment. The aligned

Table 1 Detailed morphometric characteristics of *P. brasiliana* as observed in Malaysian strains compared with data from the literature

Strain name	Valves		Fibula		Striae (in 10 μm)	Poroid		Cell overlap
	Length (μm)	Width (μm)	Number in 10 μm	Central interspace		Number in 1 μm	Rows in 1 stria	
PnSm07 (n=30)	33.6–35.8	2.8–3.2	24	Absent	24–26			
PnSm18 (n=10)	35.8–37.1	2.9–3.4	21–23	Absent	22–25	7–9	2–3	
PnSm19 (n=30)	42.4–58.8	2.7–3.0	22–24	Absent	23–24	7–9	2–3	
PnSm20 (n=30)	31.9–36.8	2.6–3.0	21–25	Absent	22–24	9–10	2	1/10
PnSb34 (n=30)	51–55	2.3–3.6	24	Absent	25–27	9	2	
PnSb35 (n=30)	49–51	2.3–3.5		Absent				
PnSb39 (n=30)	53–56	2.6–3.7	20–22	Absent	19–23	8	2–3	
PnSb41 (n=30)	50–56	2.1–3.7	19–23	Absent	21–24	8	2–3	
PnSb42 (n=30)	50–64	2.2–3.9	24	Absent	23–24	9	2–3	
PnSb43 (n=30)	53–55	2.2–3.3	22	Absent	22–24	8–9	2–3	
PnSb46 (n=30)	51–56	2.2–3.9	20–21	Absent	22–23	9	2–3	
PnPm03 (n=30)	37–40	2.2–3.5	24	Absent	24–25	9	2–3	
PnPm04 (n=30)	36–42	2.0–3.2	21–22	Absent	21–22	9	2–3	
PnPm05 (n=30)	22–27	2.4–3.5		Absent				
PnPm06 (n=30)	24–27	2.0–3.3		Absent				
PnPm08 (n=30)	25.9–28.5	2.4–2.6		Absent				
Sepetiba Bay, Brazil[a] (n=97)	12–65	1.8–3.0	20–26	Absent	20–26	7–10	2–3	1/8–1/11
Vietnam[b] (n=20)	30.5–52	1.8–2.7	20–26	Absent	21–26	6–10	2–3	
Catalonia, Spain[c] (field materials, n=20)	34.9–38.1	2.5–2.9	23–26	Absent	26–27	8–10	2	
ICMB-172[d] (n=10)	31.4–32.6	2.9–3.3	22–24	Absent	23–25	8–9		
ICMB-175[d] (n=10)	33.3–34.7	2.8–3.2	22–25	Absent	23–25	7–8		
Cuyutlan Lagoon, Mexico[e] (field materials, n=300)	24–31.7	2.7–3.0	23–24	Absent	19–25	7–9	2	

n indicates the number of cells examined

[a] Lundholm et al. (2002)

[b] Skov et al. (2004)

[c] Quijano-Scheggia et al. (2008)

[d] Quijano-Scheggia et al. (2009a)

[e] Quijano-Scheggia et al. (2011)

Table 2 List of *P. brasiliana* strains used in the present study

Strain	Locality	Date of collection	GenBank accession	Isolator
PnSm07	Samariang Batu, Sarawak	28 May 2008	HQ111404	H.C. Lim
PnSm19	Samariang Batu, Sarawak	7 May 2009	HQ111405	H.C. Lim
PnSm20	Samariang Batu, Sarawak	7 May 2009	HQ111406	H.C. Lim
PnSb34	Santubong, Sarawak	30 June 2009	HQ111397	H.C. Lim
PnSb35	Santubong, Sarawak	30 June 2009	HQ111398	S.N.P. Su
PnSb39	Santubong, Sarawak	30 June 2009	HQ111399	S.N.P. Su
PnSb41	Santubong, Sarawak	30 June 2009	HQ111400	S.N.P. Su
PnSb42	Santubong, Sarawak	30 June 2009	HQ111401	S.N.P. Su
PnSb43	Santubong, Sarawak	30 June 2009	HQ111402	S.N.P. Su
PnSb46	Santubong, Sarawak	30 June 2009	HQ111403	S.N.P. Su
PnPm03	Pulau Mamutik, Sabah	25 June 2009	HQ111407	S.N.P. Su
PnPm04	Pulau Mamutik, Sabah	25 June 2009	HQ111408	S.N.P. Su
PnPm05	Pulau Mamutik, Sabah	25 June 2009	HQ111409	S.N.P. Su
PnPm06	Pulau Mamutik, Sabah	25 June 2009	HQ111410	S.N.P. Su
PnPm08	Pulau Mamutik, Sabah	25 June 2009	HQ111411	H.C. Lim
PnSm23	Samariang Batu, Sarawak	22 September 2010	JN252410	H.C. Lim
PnSm24	Samariang Batu, Sarawak	22 September 2010	JN252411	H.C. Lim
PnSm27	Samariang Batu, Sarawak	22 September 2010	JN252412	H.C. Lim
PnSm31	Samariang Batu, Sarawak	5 October 2010	JN252413	H.C. Lim
PnSm33	Samariang Batu, Sarawak	5 October 2010	JN252414	H.C. Lim
PnSm34	Samariang Batu, Sarawak	5 October 2010	JN252415	H.C. Lim
PnSm35	Samariang Batu, Sarawak	5 October 2010	JN252416	H.C. Lim
PnSm36	Samariang Batu, Sarawak	5 October 2010	JN252417	H.C. Lim
PnSm39	Samariang Batu, Sarawak	5 October 2010	JN252418	H.C. Lim
PnSm40	Samariang Batu, Sarawak	5 October 2010	JN252419	H.C. Lim
PnKk15	Kota Kinabalu, Sabah	2 October 2010	JN252423	H.C. Lim
PnKk28	Kota Kinabalu, Sabah	2 October 2010	JN252427	H.C. Lim
PnKk31	Kota Kinabalu, Sabah	2 October 2010	JN252429	H.C. Lim
PnPd03	Port Dickson, Negeri Sembilan	December 2010	JQ627613	S.T. Teng
PnPd18	Port Dickson, Negeri Sembilan	December 2010	JQ627615	S.T. Teng
Xt3C	Vietnam	10 May 2005	DQ062662	J. Skov
MMDL50479	China	16 May 2010	HM236161	
MMDL50485	China	16 May 2010	HM236162	
MMDL50486	China	16 May 2010	HM236163	
ICMB-172	Olympic Harbor, NW Mediterranean Sea	3 December 2007	EU327364	S. Quijano-Scheggia
ICMB-175	Olympic Harbor, NW Mediterranean Sea	3 December 2007	EU327365	S. Quijano-Scheggia

sequences with secondary structure information were saved in ".xfasta" extension for analysis in ProfDistS 0.9.8 (Wolf et al. 2008).

Phylogenetic analysis based on ITS structural information

The ITS phylogenetic tree with structural information was reconstructed using the program ProfDistS 0.9.8 (Qt version; Müller et al. 2004; Friedrich et al. 2005; Rahmann et al. 2006; Wolf et al. 2008). RNA/DNA structure Profile Neighbor Joining (PNJ) was selected from the analysis with the parameters set as: bootstrap=1,000, distance correction model=General Time Reversible, Ratematrix Q=Q_ITS2, followed by bootstrap RNA structure of 1,000 replicates. The tree was rooted, with *P. pungens* (HQ111412) and *P. multistriata* (EF636677) as outgroups.

Haplotype determination

Genetic divergence estimation was performed using DnaSP v5 (DNA sequence polymorphism; Libraso and Rozas 2009). The aligned sequence–structural data was used. Sites

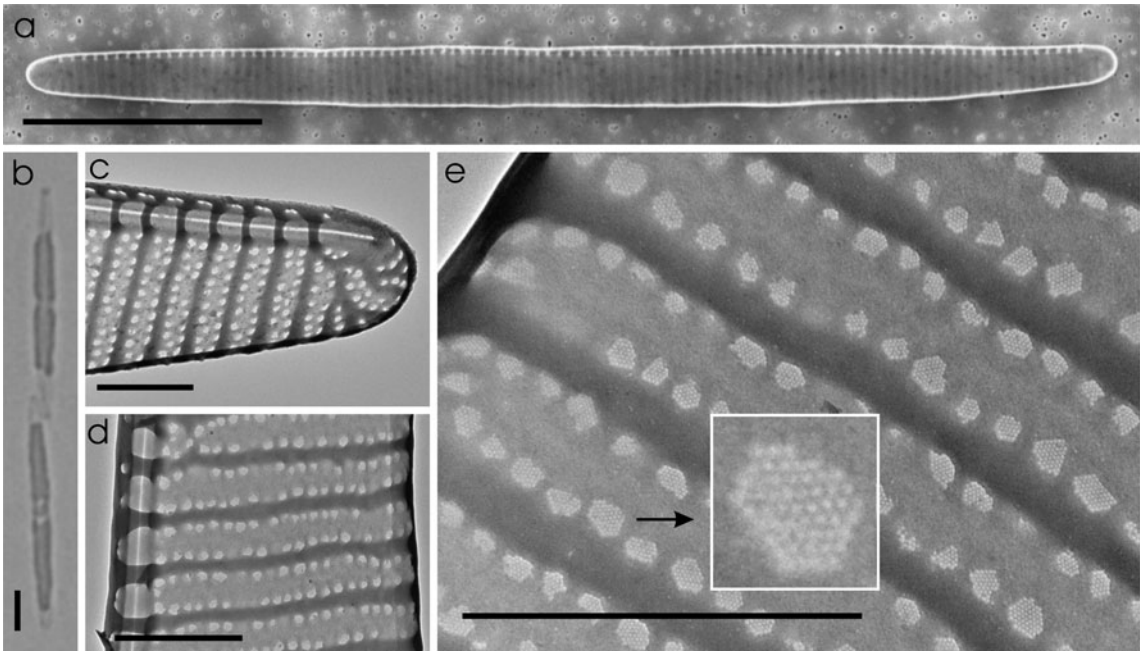

Fig. 2 *P. brasiliana*. **a** SEM. Valve view of frustule. **b** LM. 1/10 cells overlap. *Scale bar*, 10 μm. **c**–**e** TEM. **c** Broad round end apices. **d** Two rows of poroids in each striae. **e** Simple poroid, hexagonal arrangement (*inset*). *Scale bar*, 1 μm

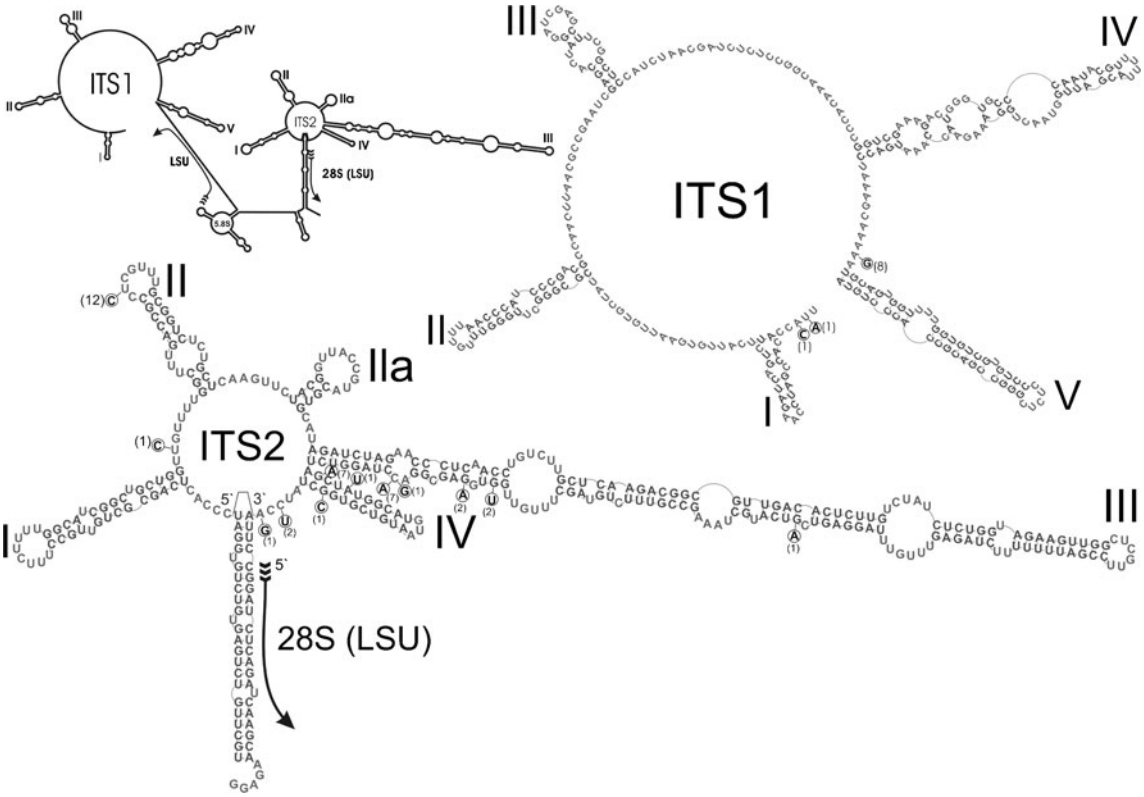

Fig. 3 Secondary structure of ITS1–5.8S–ITS2 rDNA of *P. brasiliana* strain PnSm07. Major helices were labeled as *I–V* in the ITS1 transcript and *I–IV* and *IIa* in the ITS2 transcript. *Numbers in brackets next to circles* indicate the base changes occurring among sequences of *P. brasiliana* in Malaysian waters

Table 3 Nucleotide composition in the ITS region (in base pairs) in *P. brasiliana* from different locations obtained in this study

Nucleotide no.	2	3	142	221	495	497	514	667	699	700	702	703	704	710	720	723	725	727	742	744	749	750	752
h1	T	A	A	A	T	T	C	G	G	T	–	G	A	C	G	A	–	–	–	G	–	C	A
h2	.	.	.	.	.	.	T	.	.	.	–	.	.	.	.	.	–	–	–	.	–	.	.
h3	.	.	.	.	C	.	.	.	.	.	–	.	.	.	.	.	–	–	–	.	–	.	.
h4	.	.	.	G	.	.	.	.	.	.	–	.	.	.	.	.	–	–	–	.	–	.	.
h5	.	.	G	.	.	A	.	.	.	.	–	.	.	.	.	.	–	–	–	.	–	.	.
h6	.	.	.	.	.	.	T	.	.	.	–	.	.	.	.	.	G	–	–	.	–	.	.
h7	.	.	.	.	.	.	T	.	.	.	G	.	.	.	A	.	–	–	–	.	–	.	.
h8	.	.	.	.	.	.	.	.	.	.	–	.	.	T	.	.	–	–	–	.	–	.	.
h9	.	.	.	.	.	.	.	.	.	.	–	.	.	.	A	.	–	G	–	.	–	.	.
h10	.	.	.	.	.	.	T	.	.	.	–	.	.	T	.	.	–	–	G	.	–	.	.
h11	.	.	.	G	.	.	.	.	.	–	–	.	.	.	.	.	–	–	–	.	–	.	.
h12	.	.	.	G	.	.	.	.	T	–	–	A	–	.	.	.	–	–	–	.	–	.	.
h13	.	.	.	.	.	.	T	.	.	.	–	.	.	T	A	.	–	–	–	.	–	T	.
h14	A	C	.	.	.	.	T	.	.	.	–	.	.	T	A	.	–	–	–	C	–	T	G
h15	.	.	.	.	.	.	T	.	.	.	–	.	.	T	A	.	–	–	–	.	–	.	.
h16	.	.	.	.	.	.	.	.	.	–	–	.	.	.	.	.	–	–	–	.	T	.	.
h17	.	.	.	.	.	.	.	A	.	.	–	.	.	.	A	.	–	–	–	.	–	.	.
h18	.	.	.	.	.	.	T	.	.	.	–	.	.	G	A	.	G	–	–	.	–	.	.
h19	.	.	.	.	.	.	T	.	.	.	–	.	.	T	.	.	–	–	–	.	–	.	.
h20	.	.	.	.	.	.	.	.	.	.	–	.	.	T	.	T	–	–	–	.	–	.	.

Variations were in comparison to haplotype h1. Sites with identical nucleotide to the reference haplotype are indicated with ".". The numbering of nucleotides starts from ITS1 until the ITS2 region of strain PnSm19 (HQ111405)

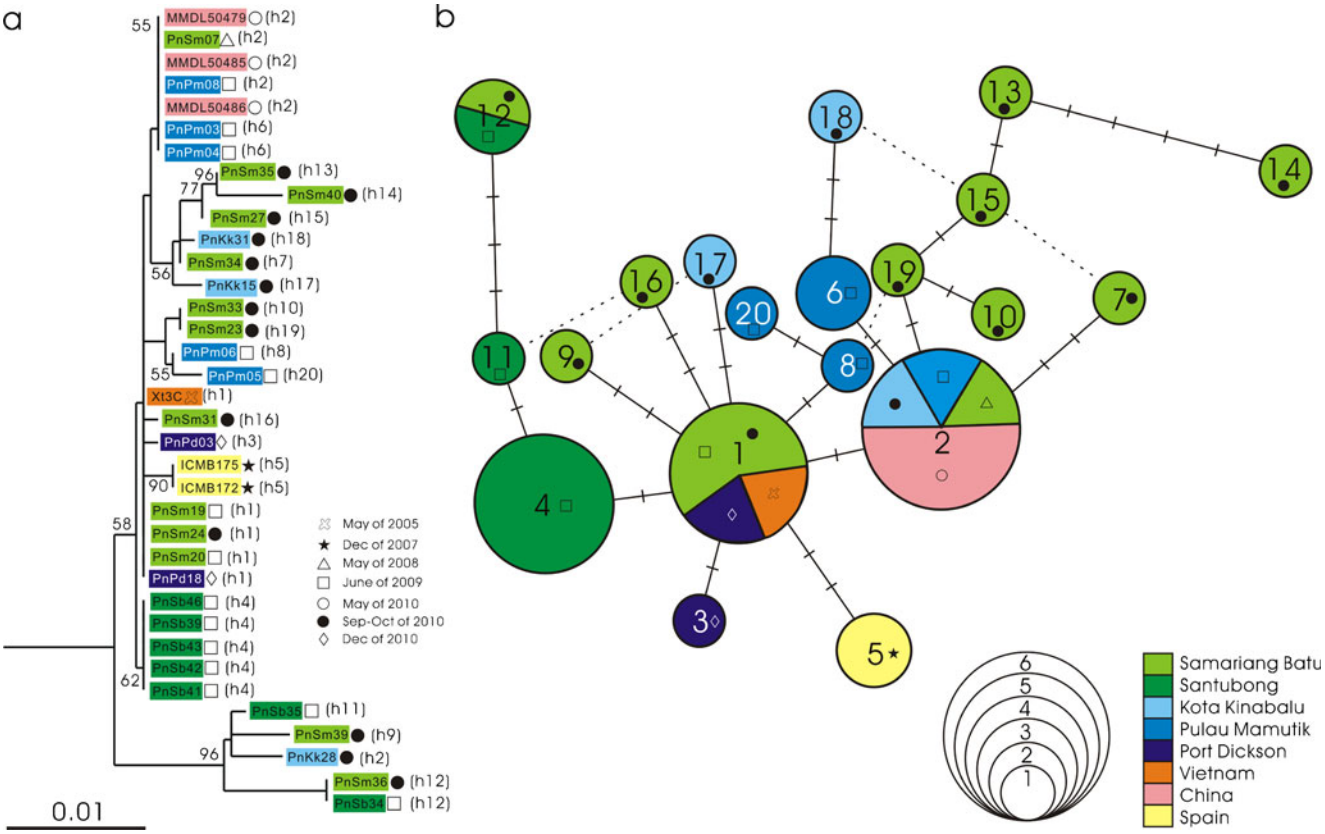

Fig. 4 **a** Profile neighbor joining tree of *P. brasiliana* derived from ITS nucleotide sequences with structural information. The tree was rooted by *P. pungens* (HQ111412) and *P. multistriata* (EF636677), with outgroup not shown. Values on the nodes correspond to posterior probability; only values >50% are shown. **b** Minimum spanning tree of the 20 haplotypes found among 36 strains of *P. brasiliana*. Each *circle* represents a haplotype and scaled with the number of strain as the frequency

with alignment gaps were considered in the analysis followed by defining sequence sets to respective locations accordingly. Invariable sites were included and haplotype data files were generated in both NEXUS and Arlequin (.arp) formats.

Haplotype diversity was calculated. Pairwise difference was performed using AMOVA significant test, and minimum spanning tree (Rohlf 1973) was performed to infer the haplotype relationship using Arlequin 3.5.1.2 (Excoffier and Lischer 2010).

Table 4 Genetic distances in the ITS region of *P. brasiliana*

P. brasiliana	Sm1	Sm2	Sm3	Sm4	Sb	Kk	Pm	Pd	Vietnam	China	Spain
Sm1 (*n*=1)	0										
Sm2 (*n*=2)	0.13	0									
Sm3 (*n*=3)	0.18	0.22	0.27								
Sm4 (*n*=7)	0.49	0.51	0.51	0.27							
Sb (*n*=7)	0.36	0.23	0.45	0.51	0.18						
Kk(*n*=3)	0.19	0.21	0.26	0.45	0.44	0.29					
Pm (*n*=5)	0.27	0.31	0.33	0.26	0.54	0.36	0.44				
Pd (*n*=2)	0.20	0.07	0.29	0.33	0.29	0.28	0.38	0.13			
Vietnam (*n*=1)	0.13	0	0.22	0.29	0.23	0.21	0.31	0.07	0		
China (*n*=3)	0	0.13	0.18	0.22	0.36	0.19	0.27	0.20	0.13	0	
Spain (*n*=2)	0.40	0.27	0.49	0.18	0.49	0.48	0.58	0.33	0.27	0.40	0

Diagonal elements represent the average number of pairwise differences within population (P_iX, 100%); lower diagonal elements represent the percentage of the average number of pairwise differences between populations (P_iXY, 100%)

Sm Samariang Batu [1 (May 2008), 2 (May 2009), 3 (September 2010), 4 (October 2010)], *Sb* Santubong, *Kk* Kota Kinabalu, *Pm* Pulau Mamutik, *Pd* Port Dickson

Results

Morphology

Morphological characterizations by electron microscopy analyses (SEM and TEM) confirmed the identity of the 30 strains of *P. brasiliana*: 13 strains from Samariang Batu, 7 from Santubong, 5 from Pulau Mamutik, 3 from Kota Kinabalu, and 2 from Port Dickson. The detailed morphological characteristics of *P. brasiliana* obtained in this study fitted well to the original description of Lundholm et al. (2002) (Fig. 2 and Table 1).

Secondary structures of the ITS region

Nucleotide sequences of the ITS region generated in this study for *P. brasiliana* were deposited in GenBank with the accessions shown in Table 2. In this study, the transcript folding pattern of the ITS transcripts in *P. brasiliana* (PnSm07) was proposed (Fig. 3). In the ITS1 transcript, a multi-branch loop with five helices (I–V) was identified in all the sequences. Helix V was the longest helix with more than 17 paired bases; the positions of the helices were well conserved. The secondary structure of the 5.8S rRNA possessed three helices: a basal pairing forming a loop with two helices, a single helix, and the 5′ end of 5.8S rRNA complimented with the 3′ end of 28S LSU rRNA. The divergence of 5.8S rRNA was low, and the secondary structure of 5.8S rRNA was highly conserved with similar structure observed among the *P. brasiliana* strains. In the ITS2 transcript, the loop was closed and formed a proximal stem, the 5.8S–28S LSU interaction. The secondary structure of the ITS2 transcript consisted of four helices (I–IV) and one pseudo-Helix, IIa. Helix III was the longest among the helices.

No compensatory base change and hemi-compensatory base change were found in the ITS2 transcript comparisons among the 36 strains examined.

Genetic diversity of *P. brasiliana*

The ITS region was highly variable with 23 differing nucleotide sites defining 20 haplotypes. The haplotype sequences were aligned readily with seven insertions and deletions (indels; Table 3). See Table 3 for the list of haplotypes found among the strains from different locations collected at different temporal seasons. However, PNJ analysis did not show any trend in the clustering of *P. brasiliana* strains from different locations, nor different sampling periods. No geographic and temporal structuring was observed in the PNJ tree (Fig. 4a).

A haplogroup of *P. brasiliana* from Malaysia comprised 19 haplotypes, h1–h20 (except h5). Two haplotypes (h1 and h2) were common and shared between different geographic locations. Haplotype h1 was present in strains from Samariang Batu, Port Dickson, and Vietnam. Haplotype h2, the most common haplotype, was found in strains from Samariang Batu, Pulau Mamutik, Kota Kinabalu, and China, but absent in strains from the other locations analyzed. Haplotype h5 was confined to the population from Spain. Generally, strains of *P. brasiliana* grouped according to the haplotypes and not the geographic locale.

Haplotypes h1 and h2 appeared at one extreme of the network, giving rise to the rest of the haplotypes (Fig. 4b). Strains of *P. brasiliana* isolated from Samariang Batu showed an intriguing haplotype variation, with 11 haplotypes (h1, h2, h7, h9, h10, h12–h16, and h19) identified from the location. This is also the location with the most isolates. Strains isolated from Pulau Mamutik of Sabah, PnPm05 (h20) and PnPm06 (h8), PnKk15 (h17) from Kota Kinabalu, PnPd03 (h3) from Port Dickson, and Spanish strains (h5, ICMB172 and ICMB175) were interconnected with haplotype h1.

Among the strains of *P. brasiliana*, pairwise genetic differences were relatively low, with the exception of the strains between Pulau Mamutik (Pm) and Santubong (Sb), with a genetic divergence of 0.54%. The highest pairwise difference of *P. brasiliana* within populations in Bornean waters was observed in Pulau Mamutik population (0.44%; Table 4).

Discussion

Electron microscopic observations of all *P. brasiliana* strains obtained in this study confirmed the species identity. Strains of *P. brasiliana* isolated from Santubong, Samariang Batu, Pulau Mamutik, and Port Dickson were morphologically identical (Fig. 2). The latter two locations represent new records of *P. brasiliana* found in Malaysian waters. The species was previously reported from Kuching waters (Lim et al. 2011b). Most recently, *P. brasiliana* was confirmed as a domoic acid producer and toxin was detected from strains of S-9 isolated from Bizerte Lagoon, Tunisia (Sahraoui et al. 2011). However, none of the strains from Malaysia tested thus far showed any detectable toxins (Lim et al. 2010).

Secondary structures of the ITS region of rDNA of *P. brasiliana* were relatively conserved between the different ITS types (Fig. 3). The inter-strain diversity found in *P. brasiliana* was low even, though with high numbers of haplotypes found in this study. Strains from Kuching (Samariang Batu and Santubong) were the most diverse with 13 haplotypes found, followed by strains from Sabah (Pulau mamutik and Kota Kinabalu). Strains from Port Dickson in the Straits of Malacca, although geographically isolated from the strains from Kuching and Sabah in the South China Sea, showed no significant genetic difference and shared a single haplogroup.

Homothallic species shows a more advantageous life strategy in reproduction compared with heterothallic species.

Several species of *Pseudo-nitzschia* are known to possess heterothallic auxosporulated mating behavior, e.g., *Pseudo-nitzschia arenysensis* (Quijano-Scheggia et al. 2009b), *Pseudo-nitzschia delicatissima* (Amato et al. 2005), *Pseudo-nitzschia mannii* (Amato and Montresor 2008), *Pseudo-nitzschia multiseries* (Davidovich and Bates 1998; Hiltz et al. 2000), *P. multistriata* (D'Alelio et al. 2009b), *P. pungens* (Chepurnov et al. 2005) and *Pseudo-nitzschia pseudodelicatissima* (Davidovich and Bates 1998). Among the many species of *Pseudo-nitzschia*, *P. brasiliana* is one of the species that possessed homothallic auxosporulation (Quijano-Scheggia et al. 2009a). This may explain why more haplotypes were retained in the populations, as observed in the Samariang Batu strains.

In this study, haplotype h1 was observed in several sampling occasions in June 2009 and September–October 2010 from the same locale. Although no sexual reproduction experiment was carried out for Malaysian *P. brasiliana*, successful interbreeding had been reported in several *Pseudo-nitzschia* species, with a success rate of 0.6% in *P. multistriata* (D'Alelio et al. 2009a) and 0–0.7% in *P. pungens* (successful breeding within clades 1 and 2, but no breeding between clades; Casteleyn et al. 2008). The Malaysian *P. brasiliana* may have high possibility to be sexually compatible based on ITS2 transcript conservation.

From the present results, we showed that *P. brasiliana* has no geographic structuring pattern. Surface current circulation patterns found in the South China Sea may explain the broad-scale homogeneity where the currents move northward during the southwest monsoon in a clockwise direction and reversed during the northeast monsoon period in an anticlockwise direction (Idris et al. 2007). Therefore, various potential sources of *P. brasiliana* populations may be homogenized by the oceanographic properties of the South China Sea. The potential exchange of distant populations through ballast water remains a problem to be investigated in the future.

Our result showed high genetic homogeneity among *P. brasiliana* from Malaysia, and it was well supported by the low sequence divergence as shown in Table 4. It can be envisaged that no speciation occurs, although they are geographically distant. Our data showed that the dispersal by geographical distance do not lead to a genetic differentiation within the marine planktonic diatom *P. brasiliana*. These oppose the assumption that geographically separated populations can lead to high diversity in planktonic organisms or diverge into cryptic species.

Acknowledgements We are grateful to the two anonymous reviewers for their comments and suggestions. H.C. Lim was supported by a PhD scholarship from the Ministry of Higher Education (MoHE). This project was financially supported by the MoHE Fundamental Research Grant Scheme (FRGS) 06(12)/677/2008(10) to P.T. Lim, eScienceFund 02-01-09-SF0054, and International Foundation of Science (IFS) E4745-1 to C. P. Leaw.

References

Adams NG, Trainer VL, Rocap G, Herwig RP, Hauser L (2009) Genetic population structure of *Pseudo-nitzschia pungens* (Bacillariophyceae) from the Pacific Northwest and the North Sea. J Phycol 45:1037–1045

Almandoz GO, Ferrario ME, Ferreyra GA, Schloss IR, Esteves JL, Paparazzo FE (2007) The genus *Pseudo-nitzschia* (Bacillariophyceae) in continental shelf waters of Argentina (Southwestern Atlantic Ocean, 38–55°S). Harmful Algae 6:93–103

Almandoz G, Ferreyra G, Schloss I, Dogliotti A, Rupolo V, Paparazzo F, Esteves J, Ferrario M (2008) Distribution and ecology of *Pseudo-nitzschia* species (Bacillariophyceae) in surface waters of the Weddell Sea (Antarctica). Polar Biol 31:429–442

Amato A, Montresor M (2008) Morphology, phylogeny, and sexual cycle of *Pseudo-nitzschia mannii* sp. nov. (Bacillariophyceae): a pseudo-cryptic species within the *P. pseudodelicatissima* complex. Phycologia 47:487–497

Amato A, Orsini L, D'Alelio D, Montresor M (2005) Life cycle, size reduction patterns, and ultrastructure of the pennate planktonic diatom *Pseudo-nitzschia delicatissima* (Bacillariophyceae). J Phycol 41:542–556

Bargu S, Koray T, Lundholm N (2002) First report of *Pseudo-nitzschia calliantha* Lundholm, Moestrup and Hasle 2003, a new potentially toxic species from Turkish Coast. J Fish Aquat Sci 19:479–483

Bates SS, Bird CJ, Freitas ASW, Foxall R, Gilgan M, Hanic LA, Jhonson GR, McCulloch AW, Odense P, Pocklington R, Quilliam MA, Sim PG, Smith JC, Rao DVS, Todd ECD, Walter JA, Wright JLC (1989) Pennate diatom *Nitzschia pungens* as the primary source of domoic acid, a toxin in shellfish from eastern Prince Edward Island, Canada. Can J Fish Aquat Sci 46:1203–1215

Casteleyn G, Chepurnov VA, Leliaert F, Mann DG, Bates SS, Lundholm N, Rhodes L, Sabbe K, Vyverman W (2008) *Pseudo-nitzschia pungens* (Bacillariophyceae): a cosmopolitan diatom species? Harmful Algae 7:241–257

Casteleyn G, Evans K, Backeljau T, D'hondt S, Chepurnov V, Sabbe K, Vyverman W (2009) Lack of population genetic structuring in the marine planktonic diatom *Pseudo-nitzschia pungens* (Bacillariophyceae) in a heterogeneous area in the Southern Bight of the North Sea. Mar Biol 156:1149–1158

Casteleyn G, Leliaert F, Backeljau T, Debeer A-E, Kotaki Y, Rhodes L, Lundholm N, Sabbe K, Vyverman W (2010) Limits to gene flow in a cosmopolitan marine planktonic diatom. Proc Natl Acad Sci 107:12952–12957

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

Cusack C, Raine R, Patching J (2004) Occurrence of species from the genus *Pseudo-nitzschia* Peragallo in Irish waters. Biology & Environment: Proc Roy Irish Acad 104:55–74

D'Alelio D, Amato A, Kooistra WHCF, Procaccini G, Casotti R, Montresor M (2009a) Internal transcribed spacer polymorphism in *Pseudo-nitzschia multistriata* (Bacillariophyceae) in the Gulf of Naples: recent divergence or intraspecific hybridization? Protist 160:9–20

D'Alelio D, Amato A, Luedeking A, Montresor M (2009b) Sexual and vegetative phases in the planktonic diatom *Pseudo-nitzschia multistriata*. Harmful Algae 8:225–232

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

Evans KM, Kühn SF, Hayes PK (2005) High levels of genetic diversity and low levels of genetic differentiation in north sea *Pseudo-*

nitzschia pungens (Bacillariophyceae) populations. J Phycol 41:506–514

Excoffier L, Lischer HEL (2010) Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Mol Ecol Resourc 10:564–567

Friedrich J, Dandekar T, Wolf M, Müller T (2005) ProfDist: a tool for the construction of large phylogenetic trees based on profile distances. Bioinformatics 21:2108–2109

Gottschling M, Plotner J (2004) Secondary structure models of the nuclear internal transcribed spacer regions and 5.8S rRNA in Calciodinelloideae (Peridiniaceae) and other dinoflagellates. Nucleic Acids Res 32:307–315

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Symp Ser 41:95–98

Hasle GR (2002) Are most of the domoic acid-producing species of the diatom genus *Pseudo-nitzschia* cosmopolites? Harmful Algae 1:137–146

Hiltz M, Bates SS, Kaczmarska I (2000) Effect of light:dark cycles and cell apical length on the sexual reproduction of the pennate diatom *Pseudo-nitzschia multiseries* (Bacillariophyceae) in culture. Phycologia 39:59–66

Hubbard KA, Rocap G, Armbrust EV (2008) Inter- and intraspecific community structure within the diatom genus *Pseudo-nitzschia* (Bacillariophyceae). J Phycol 44:637–649

Idris NH, Seeni M, Mohd I (2007) Sea surface current circulation pattern in the South China Sea derived from satellite altimetry. Asian Conference on Remote Sensing, Kuala Lumpur, Malaysia

Iwasaki H (1961) The life-cycle of *Porphyra tenera* in vitro. Biol Bull 121:173–187

Koetschan C, Förster F, Keller A, Schleicher T, Ruderisch B, Schwarz R, Müller T, Wolf M, Schultz J (2010) The ITS2 Database III—sequences and structures for phylogeny. Nucleic Acids Res 38:D275–279

Li Y, Ma YY, Lu SH (2010) Morphological characteristics of *Pseudo-nitzschia americana* complex in Daya Bay, China. Acta Hydrobiol Sin 34:851–855

Libraso P, Rozas J (2009) DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25:1451–1452

Lim HC, Su SNP, Hartina MA, Kotaki Y, Leaw CP, Lim PT (2010) Toxicity of diatom *Pseudo-nitzschia* (Bacillariophyceae) analyzed using high performance liquid chromatography (HPLC). J Sci Technol Tropics 6:S116–S119

Lim HC, Lim PT, Su SNP, Teng ST, Leaw CP (2011a) Genetic diversity of *Pseudo-nitzschia pungens* (Bacillariophyceae) in Borneo, Malaysia. Coast Mar Sci 35:58–63

Lim HC, Lim PT, Su SNP, Kotaki Y, Leaw CP (2011b) Morphological observation of two species of *Pseudo-nitzschia* (Bacillariophyceae). Coast Mar Sci 35:52–57

Lundholm N, Hasle GR, Fryxell GA, Hargraves PE (2002) Morphology, phylogeny and taxonomy of species within the *Pseudo-nitzschia americana* complex (Bacillariophyceae) with descriptions of two new species, *Pseudo-nitzschia brasiliana* and *Pseudo-nitzschia linea*. Phycologia 41:480–497

Mathews DH, Disney MD, Childs JL, Schroeder SJ, Zuker M, Turner DH (2004) Incorporating chemical modification constraints into a dynamic programming algorithm for prediction of RNA secondary structure. Proc Natl Acad Sci U S A 101:7287–7292

Müller T, Rahmann S, Dandekar T, Wolf M (2004) Accurate and robust phylogeny estimation based on profile distances: a study of the Chlorophyceae (Chlorophyta). BMC Evol Biol 4:20

Quijano-Scheggia S, Garcés E, Sampedro N, Lenning KV, Flo E, Andree K, Fortuno J-M, Camp J (2008) Identification and characterisation of the dominant *Pseudo-nitzschia* species (Bacillariophyceae) along the NE Spanish coast (Catalonia, NW Mediterranean). Sci Mar 72:343–359

Quijano-Scheggia S, Garcés E, Andree K, Fortuño JM, Camp J (2009a) Homothallic auxosporulation in *Pseudo-nitzschia brasiliana* (Bacillatiophyta). J Phycol 45:100–107

Quijano-Scheggia S, Garcés E, Lundholm N, Moestrup Ø, Andree K, Camp J (2009b) Morphology, physiology, molecular phylogeny and sexual compatibility of the cryptic *Pseudo-nitzschia delicatissima* complex (Bacillariophyta), including the description of *P. arenysensis* sp. nov. Phycologia 48:492–509

Quijano-Scheggia S, Olivos-Ortiz A, Gaviño-Rodríguez JH, Castro-Ochoa F, Rivera-Vilarelle M, Galicia-Pérez M, Patiño-Barragan M (2011) First report of *Pseudo-nitzschia brasiliana* and *P. micropora* (Bacillariophycea) found in Cuyutlan Lagoon, Mexico. Revista Bio Mar Ocean 46:189–197

Rahmann S, Müller T, Dandekar T, Wolf M (2006) Efficient and robust analysis of large phylogenetic datasets. In: Hsu HH (ed) Advanced data mining technologies in bioinformatics. Idea Group Publishing, Hershey, pp 104–117

Rohlf FJ (1973) Algorithm 76. Hierarchical clustering using the minimum spanning tree. Comput J 16:93–95

Sahraoui I, Bates SS, Bouchouicha D, Mabrouk HH, Hlaili AS (2011) Toxicity of *Pseudo-nitzschia* populations from Bizerte Lagoon, Tunisia, southwest Mediterranean, and first report of domoic acid production by *P. brasiliana*. Diatom Res 26:293–303

Schultz J, Müller T, Achtziger M, Seibel PN, Dandekar T, Wolf M (2006) The internal transcribed spacer 2 database—a web server for (not only) low level phylogenetic analyses. Nucleic Acids Res 34:W704–707

Scott FJ, Thomas DP (2005) Diatoms. In: Scott FJ, Marchant HJ (eds) Antartic marine protists. Canberra & Hobart: Australian Biological Resources Study; Australian Antarctic Division, pp 13–201

Seibel PN, Müller T, Dandekar T, Schultz J, Wolf M (2006) 4SALE—a tool for synchronous RNA sequence and secondary structure alignment and editing. BMC Bioinf 7:498

Seibel PN, Müller T, Dandekar T, Wolf M (2008) Synchronous visual analysis and editing of RNA sequence and secondary structure alignments using 4SALE. BMC Res Notes 1:91

Selig C, Wolf M, Müller T, Dandekar T, Schultz J (2008) The ITS2 Database II: homology modelling RNA structure for molecular systematic. Nucleic Acids Res 36:D377–380

Skov J, Ton TP, Do TBL (2004) Bacillariophyceae. In: Larsen J, Nguyen NL (eds) Potentially toxic microalgae of Vietnam. Opera Botanica 140, pp 23–51

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols. Academic, New York, pp 315–322

Wolf M, Ruderisch B, Dandekar T, Müller T (2008) ProfdistS: (Profile-) distance based phylogeny on sequence–structure alignments. Bioinformatics 24:2401–2402