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Genetic diversity of *Pseudo-nitzschia pungens* (Bacillariophyceae) in Borneo, Malaysia

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Abstract — *Pseudo-nitzschia pungens* is a chain forming cosmopolitan marine pennate diatom. It is known to produce the neurotoxin, domoic acid (DA) which is responsible for amnesic shellfish poisoning (ASP). In this study, clonal cultures of *P. pungens* were established from Sabah and Sarawak. To examine the genetic diversity of *P. pungens*, the ITS region of rDNA was amplified. Nucleotide sequences obtained were used in secondary structure modeling based on homologous modeling and free energy minimization. The ITS sequences of *P. pungens* showed common structure of ITS1 and ITS2 transcripts. Universal motifs were found in the ITS2 transcript with pyrimidine-pyrimidine (U–U) mismatch and AAA motif between Helix II-III and UGGU motif in the Helix III. Unambiguous orthologous structural sequence alignment was subsequently used in genetic population analyses. Seven haplotypes were found in the Borneon populations. Kuching haplotype is exclusive from the six haplotypes found in Kudat population. The Kuching and Kudat strains formed two distinctive groups inferred by the Profile Neigboring Joining (PNJ) tree. High F_{ST} value (>0.69) suggested no gene flow among the populations of *P. pungens* in Sabah and Sarawak.

Key words: *Pseudo-nitzschia pungens*, ITS, rDNA, population genetic, haplotype

Introduction

Pseudo-nitzschia is a group of chain forming pennate diatom. The diatom is widely distributed in brackish to marine environments in tropical and temperate regions. Blooms of some species of *Pseudo-nitzschia* have resulted in human intoxication after consuming shellfish mollusk contaminated with neurotoxin, domoic acid (DA). *Pseudo-nitzschia pungens* has been known by its vast distribution, mainly in the Atlantic and Pacific waters (Hasle et al. 1996, Stonik et al. 2001, Sterh et al. 2002, Cusack et al. 2004, Kaczmarska et al. 2005, Quijano-Scheggia et al. 2008, Klein et al. 2010, Moschandreou and Nikolaidis 2010), and a few reports from South and East China Sea (Eun et al. 2001, Skov et al. 2005, Ai et al. 2005).

Many studies of *Pseudo-nitzschia pungens* have been conducted to understand its population genetic structure. These included the application of microsatellites markers (Evans et al. 2005, Casteleyn et al. 2009, Nicolaus et al. 2009), inference of secondary structure in ITS rDNA region (Casteleyn et al. 2008) and the development of species specific primers known as automated ribosomal intergenic spacer analysis (ARISA) (Hubbard et al. 2008). However, little is known of the distribution of *Pseudo-nitzschia* in the South China Sea, particularly in waters of Malaysia. In our plankton survey along the northwest coasts of Sabah and Sarawak, we had found six species of *Pseudo-nitzschia*, namely *P. brasiliana*, *P. cuspidata*, *P. dolorosa*, *P. micropora*, *P. pungens* and *P. circumpora* where *P. pungens* and *P. brasiliana* are two common species found in the waters.

In this study, clonal cultures of *Pseudo-nitzschia pungens* were established from three locations of Sabah and Sarawak. The ITS region of the nuclear encoded ribosomal DNA (rDNA) was amplified and sequenced. Nucleotide sequences together with the secondary structure information were used to estimate the population structure of the isolates. Population structure of *P. pungens* was investigated, inter- and intrapopulation genetic diversity of this species was evaluated. We hypothesized that the *P. pungens* in Malaysian Borneo were not isolated based on different geographical region.

Materials and Methods

Sampling, culture establishment and species identification

Plankton samples were collected from Santubong and Muara Tebas (Sarawak) and Kudat (Sabah) (Fig. 1) by using a plankton net with 20 μm opening. Single chain of *Pseudo-*

nitzschia cells were isolated for clonal culture establishment. Cultures were grown in SWII medium (Iwasaki 1961) at 30 psu with pH adjusted to 7.8–7.9 and maintained at 25°C under irradiance of 100 μmol photons $m^{-2} s^{-1}$ at 12 : 12 h light : dark cycle.

Species identification of *Pseudo-nitzschia* was carried out under light microscopy, scanning and electron microscopy according to Hasle et al. (1996), Villac and Fryxell, (1998), Cusack et al. (2004), Almandoz et al. (2007), Moschandreou and Nikolaidis (2010).

Fig. 1. Map showing sampling locations, Kuching (Sarawak) and Kudat (Sabah).

Genomic DNA extraction and ITS region amplification

Late exponential cultures of *Pseudo-nitzschia* were used for genomic DNA extraction according to Vinod (2004). Region of internal transcribed spacer (ITS) was amplified using universal primers ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) (Orsini et al. 2004). The amplicons were purified using Wizard® PCR Preps DNA Purification kit (Promega, Madison, WI, USA). Sequencing was carried out using an ABI 377 automated sequencer (PE Applied Biosystems, Foster City, CA, USA). Sequencing for each sample was carried out on both strands. The ITS rDNA sequences obtained in this study were deposited in GenBank, NCBI (Table 1).

Secondary structure prediction and sequence-structure alignment

ITS1 transcript of *P. pungens* was modeled by homologous modeling using ITS2 Database (Koetschan at al., 2010) based on structure of Casteleyn et al. (2008). 5.8S rRNA was referred to the model in Gottschling and Plotner (2004). ITS2 transcript was predicted by free energy minimization using RNAstructure 5.02 (Mathews et al. 2004). The RNA structure were redrawn and edited by VARNA 3.7 (Darty et al. 2009). ITS sequence-structure information was synchronously aligned using 4SALE (Seibel et al. 2006, 2008).

Population genetic analyses

Haplotype analysis was conducted using DnaSP v5 (Librado and Rozas 2009). Indices of *F*-distribution in population genetics, F_{ST} (Wright 1969), average pairwise differences within and between populations, and minimum spanning tree analysis (Rohlf 1973) were generated by Arlequin 3.5.1.2 (Excoffier and Lischer 2010).

Table 1. Strains of *Pseudo-nitzschia pungens* used in this study.

Strain	Sampling location	Date of collection	GenBank accession no.
PnSb48	Santubong, Sarawak	8 September 2009	HQ111414
PnSb52	Santubong, Sarawak	8 September 2009	HQ111415
PnSb53	Santubong, Sarawak	8 September 2009	HQ111416
PnMt45	Muara Tebas, Sarawak	7 May 2009	HQ111412
PnKd03	Kudat, Sabah	9 September 2009	HQ111417
PnKd05	Kudat, Sabah	9 September 2009	HQ111418
PnKd06	Kudat, Sabah	9 September 2009	HQ111419
PnKd07	Kudat, Sabah	9 September 2009	HQ111420
PnKd10	Kudat, Sabah	9 September 2009	HQ111421
PnKd12	Kudat, Sabah	9 September 2009	HQ111422
PnKd18	Kudat, Sabah	9 September 2009	HQ111423
—	China		AY544769
PO3	Catalan Coast, Spain		DQ990370
CTB1	Cape Tormentine, Canada		EU599141
—	Hai Phong, Vietnam		DQ166533
KBH2	Khan Hoa Bay, Vietnam		DQ062665
P-24	Costa Nova, Portugal		AY257845
Alfa 3	Portugal		EU684235

Profile neighbor joining (PNJ) tree of the ITS transcripts was reconstructed by using program ProfdistS 0.9.8 with bootstrap value set to 1000 replicates, Distance Correction Model=General Time Reversal (GTR) and Ratematrix Q=Q_ITS2 (Müller et al. 2004, Friedrich et al. 2005, Rahmann et al. 2006, Wolf et al. 2008).

Results and Discussion

Morphological observation

Species identification of the eleven strains of *Pseudo-nitzschia pungens* obtained in this study was confirmed through light and electron microscopy and documented in Lim et al. (2011). The morphometric data of *P. pungens* strains obtained in this study is as shown in Table 2.

Structural information of ITS transcript

The transcript folding of ITS1-5.8S-ITS2 rRNA in *P. pungens* (PnMt45) are shown in Fig. 2. Common structure of the transcript was observed in all strains analysed. In ITS1 transcript, a multi-branch loop with five helices (I–V) was identified. Helix V is the longest helix with more than 17 paired bases. In ITS2 transcript, the loop is closed, forming a proximal stem at the base of ITS2. The ITS2 transcript consisted of four helices (I–IV) and one pseudo-helix IIa. Helix III is the longest helix among the helices. The universal motifs such as pyrimidine–pyrimidine (U–U) mismatch at the base of helix II, AAA motif between helix II and helix IIa and UGGU motif at the end apices of helix III (Gottschling and Plotner 2004, Schultz et al. 2005, Coleman 2009, Koetschan et al. 2009) was observed in the secondary structures of all strains predicted. Despite the structural conservation, the sequence-structure divergence in *P. pungens* strains is relatively high. A total of 11 single nucleotide substitutions (SNPs), one insertion and four HCBCs were found in the ITS1 transcript; two base changes were found in 5.8S transcript. The highest sequence-structure divergence was found in the ITS2 transcript, where 16 SNPs, four HCBCs and one CBC were observed (Fig. 2).

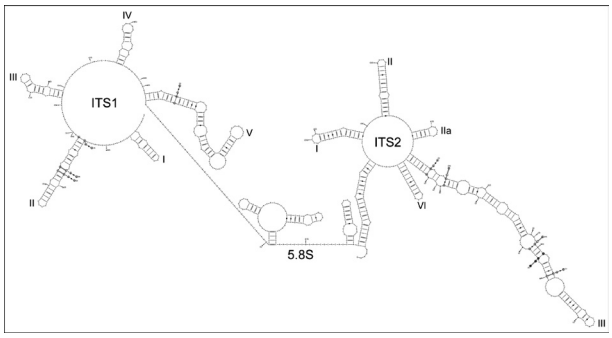

Fig. 2. ITS1-5.8S-ITS2 rRNA secondary structure model of *Pseudo-nitzschia pungens*. Major helices are labeled as I–V, I–IV and IIa in ITS1 and ITS2 transcripts respectively. Bold letters on base-pair changes indicate compensatory base changes, CBC (white) and hemi compensatory base changes, HCBCs (black). Single nucleotide substitutions (SNPs) are shown in arrows. Numbers in brackets indicate base changes occurred among sequences.

Inter- and intrapopulation structure of P. pungens

The *P. pungens* populations from Kuching and Kudat were genetically structured. Genetic homogeneity was observed in Kuching population (n=4). However the Kudat population showed some degree of intra-strain variability (0.69%, n=7; Table 3). The notably high genetic divergence within the Kudat population could be speculated by the inflow of different genotypes during water exchange between the Sulu Sea and the southeastern South China Sea.

Genetic heterogeneity of 1.45% was recorded between populations of Kuching and Kudat (n=11). In our PNJ analysis, Kudat strains were clustered together with one strain from Vietnam (KBH2) forming group 1 (G1) while Kuching strains were grouped together forming group 2 (G2) with the Vietnamese (DQ166533) and Chinese strains (AY544769) formed a sister basal to G2 (Fig. 3A). Kuching populations appeared genetically distinct from G1. However the F_{ST} value (0.72) could not strongly support the absence of gene flow between the two groups. It is likely that certain degree of ge-

Table 2. Morphometric measurements of *Pseudo-nitzschia pungens* strains obtained in this study.

Strain	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	
PnMt45	67–80 [20]	3.0–3.5 [20]	12–13 [4]	absent	11–14 [5]	3–4 [3]	2 (3) [4]	1/4
PnSb48	84–88 [30]	3.5–4.1 [30]	13–15 [5]	absent	12–14 [5]		2	1/4
PnSb52	62–64 [30]	3.8–4.2 [30]	13–14 [8]	absent	11–13 [8]	3–4 [7]	2 [4]	1/4
PnSb53	81–84 [30]	3.4–4.0 [30]	12–13 [10]	absent	11–13 [10]	4 [4]	2 [4]	1/4
PnKd03	117–128 [10]	3.2–4.1 [10]		absent				
PnKd10	98–101 [30]	3.2–3.9 [30]	12–14 [4]	absent	12–14 [4]	4 [6]	2 [3]	1/4
PnKd12	120–125 [20]	3.3–4.2 [20]		absent				
PnKd18	113–117 [30]	3.0–3.5 [30]		absent				

Table 3. Genetic distances in ITS region within *Pseudo-nitzschia pungens*. *P. pungens*

P. pungens	Kuching	Kudat	Vietnam	Portugal	China	Spain	Canada
Kuching (n=4)	0	0.6959	0.7241	1.0000	1.0000	1.0000	1.0000
Kudat (n=7)	1.45	0.69	0.0567	0.6313	0.7177	0.7826	0.7813
Vietnam (n=2)	1.11	0.86	1.11	0.6000	0.4286	0.6364	0.6364
Portugal (n=2)	1.25	1.51	1.39	0	1.0000	1.0000	1.0000
China (n=1)	2.08	2.46	1.94	2.50	0	1.0000	1.0000
Spain (n=1)	2.91	3.19	3.05	2.91	4.16	0	1.0000
Canada (n=1)	2.91	3.17	3.05	2.77	4.16	0.14	0

Upper diagonal: Population pairwise *F*-statistic, F_{ST}
Diagonal elements: Average number of pairwise genetic differences within population (P_iX) [%]
Lower diagonal: Average number of pairwise genetic differences between populations (P_iXY) [%]

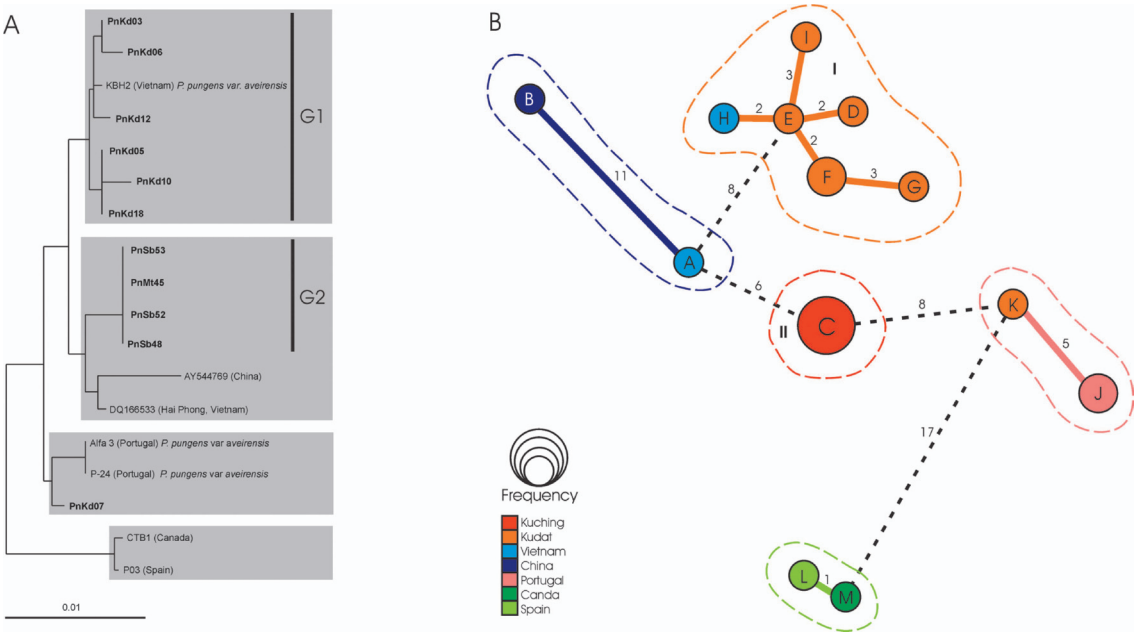

Fig. 3. Profile neighbor joining tree of *Pseudo-nitzschia pungens* revealed by ITS sequences with structural information. Sequences obtained in this study are in boldface (A). Minimum spanning tree of *P. pungens* showing 13 haplotypes. Each circle represents location, and scaled with sample numbers as frequency. Numbers indicate connection length (B).

netic exchange occurred between the two populations due to the current circulations in the South China Sea.

It is interesting to note that a strain from Kudat (PnKd07) was grouped together with Portugal strains (Alfa3 and P-24) (Fig. 3). The Portugal strains have been identified as variety *aveirensis* (Casteleyn et al. 2009). Species variety of *P. pungens* has been widely used in species identification and population studies (Casteleyn et al. 2010); however lack of morphological information on the variety of *P. pungens* strains used in this study could not provide any corroborative evidence that the varieties of *P. pungens* may possess any genetic variability. The populations of Kuching and Kudat may belong to two different varieties. Clearly, detailed morphological observation of the *P. pungens* particularly the ultrafeature of valvocopula is needed.

The present study shows that population genetic structuring of *Pseudo-nitzschia pungens* from Borneo possessed certain degree of concordance to the biogeographic clines, indicating that the species is geographically structured, forming distinct clustering in the South China Sea population. However, more isolates from different biogeographical regions in the South China Sea are needed to gain better insights into the population dynamic of the species.

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