Development of *Pseudo-nitzschia* species-specific oligonucleotide primers for ribosomal intergenic spacer analysis

Sing-Tung Teng¹, Hong-Chang Lim², Po-Teen Lim³, Chui-Pin Leaw¹

¹ Institute of Biodiversity and Environmental Conservation,  
² Faculty of Resource Science and Technology, Universiti Malaysia Sarawak  
Corresponding email: cpleaw@ibec.unimas.my

ABSTRACT
A study was carried out to determine the community structure of *Pseudo-nitzschia* species based on ribosomal intergenic spacer analysis (RISA). A molecular approach was carried out by using the secondary structure of the internal transcribed spacers (ITSs) transcripts to characterize the genetic variability of *Pseudo-nitzschia* species. Clonal cultures used were grown in SWII medium at 26°C under 14: 10 hour light:dark photo cycle. Genomic DNA was extracted and used for ITS region amplification and sequencing. The sequences obtained were used to predict the RNA secondary structure. Sequence-structure alignment was performed with related taxa to identify the sequence signatures. *Pseudo-nitzschia* species- and genus-specific oligonucleotide primers were designed in silico based on the secondary structure information. The designed specific primers were tested for the development of a genotyping system.

Key words: *Pseudo-nitzschia*, ribosomal intergenic spacer analysis, species-specific oligonucleotide primers, genotyping

INTRODUCTION
Amnesic Shellfish Poisoning (ASP) is a type of shellfish poisoning in human that caused by the diatom, *Pseudo-nitzschia*. This type of poisoning is caused by the neurotoxin, domoic acid (DA). In *Pseudo-nitzschia* taxonomy, species was discernable by detailed morphological characteristics of the frustules (Hasle 1965; Hasle 1994). However morphological observations on fine structures often rely on the advanced electron microscopy (EM) (Hasle & Syvertsen 1997).

Molecular characterization using the ribosomal intergenic spacer analysis (RISA) targeting the internal transcribed spacer (ITS) region is one of the recent approaches that have been applied to characterize the species of *Pseudo-nitzschia* (Hubbard et al. 2008). In this study, we aim to characterize the species of *Pseudo-nitzschia* in Malaysian waters using this approach. The first ITS (ITS1) of the ribosomal RNA genes were amplified and sequenced from the strains of *Pseudo-nitzschia* established in this study. Sequences of ITS1 with the secondary structure information were used to design the species- and genus-specific primer pairs. The specific primers were used to test on the culture samples for molecular identification while the genus-species primers were used to develop a genotyping system for immediate characterization of *Pseudo-nitzschia* populations in the environment.

MATERIALS AND METHODS
Late exponential phase cultures were harvested for genomic DNA extraction. In brief, cently-trimethyl-ammonium-bromide (CTAB) organic lysis method was used to lyse the cells. Alcohol was used to precipitate DNA. The DNA pellet was dissolved in TE buffer and stored at -20°C. Ribosomal gene amplification of the ITS region of *Pseudo-nitzschia* species was performed by using primers ITS1 and ITS4 (White et al. 1990). ITS region (ITS1, 5.8S and ITS2) was amplified by using Appendorf mastercycler with thermal-conditions of 94°C for 2 min, 35 cycles of 95°C for 30 s, 51°C for 30 s, and 72 °C for 60 s, with a final 72 °C extension for 10 min. The PCR product were purified and sent to 1st BASE for sequencing.

Sequences obtained were initially examined using Sequence Scanner ver 1.0 (Applied Biosystem, USA) for good quality sequences. Sequences of both strands were manually checked by eyes. The ambiguous bases were determined based on the IUPAC nucleotide genetic code.

The sequences were used to blast in BLAST (Alchul et al. 1990). Closely related sequences were retrieved from the Genbank database. The termini of the transcribed regions were determined based on the annotation of sequences in the database.

Prediction of RNA secondary structure was performed by using two approaches; i.e. free energy minimization and homologous modeling. For homologous modeling, the ITS1 transcript of *P. multisiria* (Casteleyn et al. 2008) and *P. pungens* (D’Alelio et al. 2008) was used as templates.

The program BioEdit ver. 7.0 was used to convert the ITS1 region to RNA sequences. The RNA sequences were used to predict the RNA secondary structure with free energy minimization based on dynamic programming using RNAstructure ver. 5.0 (Mathews et al. 2004). The ITS1 RNA sequences with secondary structure information in Nussinov format were saved as FASTA for further analysis.
In silico primer design was performed using Primer3 plus and Integrated DNA Technologies. Three primer pairs were developed. Confirmatory test was performed using BLAST. The primer specificity was tested using genomic DNA from *Pseudo-nitzschia* and common algae species.

RESULTS AND DISCUSSION
A total of 35 cultures of *Pseudo-nitzschia* species were established and used in this study. Twenty-five secondary structures of ITS1 transcript were constructed from *Pseudo-nitzschia* species from own sequences and sequences obtained in the GenBank database. The secondary structure of ITS1 transcript was initially modeled from the ITS1 nucleotide sequences of *P. multistriata* and *P. pungens* which were later used as templates for subsequent homologous modeling.

For species-specific primer design, Pungens II F/R and Brasiliana I F/R were *in silico* designed based on the species-specific/signature regions. Pungens II F/R was designed to target the *P. pungens* with RISA length of 136bp and Brasiliana I F/R for *P. brasiliana* with RISA length of 162bp (Table 1). The primer locations were annotated in the secondary structures of *P. pungens* (Figure 1A) and *P. brasiliana* (Figure 1B).

PnAll F/R (Hubbard et al. 2008) and PnITS1 F/R were used as genus-specific primer pairs to target *Pseudo-nitzschia* species in the environment. PnAll F/R and PnITS1 F/R were designed based on genus specific region. PnAll F/R targeted *P. pungens* with RISA length of 142bp for *P. pungens* type I, and 143bp for *P. pungens* type II. PnITS1 F/R targeted *P. pungens* with RISA length of 296bp (Table 1).

![Figure 1: Secondary Structure of ITS1 transcript of *Pseudo-nitzschia pungens* (A) and *P. brasiliana* (B) modeled with conserved structures of four helices (I-V). Location of species-specific primer pairs is indicated as red line.](image)

Species-specific primers Pungens II F/R and Brasiliana I F/R were tested at genus- and species-level specificity. Both species-specific primers successfully amplified only the target species. Pungens II F/R yielded a product of 136bp when amplifying *P. pungens* clonal cultures (Figure 2) and resulted in multiple bands or different size of PCR products in non-target species (Figure 2). Brasiliana I F/R amplified target species, *P. brasiliana* with a product size of 162bp in length (Figure 3). The non-target species resulted in multiple bands in the agarose gel electrophoresis (Figure 4). This is consistent with the predicted lengths for RISA.

Genus-specific primer pairs, PnITS1 F/R and PnAll F/R (Hubbard et al., 2008) were used to test on field samples collected from Santubong estuarine water. Total genomic DNA was isolated from field samples and used for amplification. PCR product of PnAll F/R showed the presence of *P. pungens* with 142/143bp in Santubong samples (Figure 4). The result was further proven by the primer pair designed in this study, PnITS1 F/R, which showed the presence of *P. pungens* with a product of 296bp in the samples (Figure 4).
Table 1: *Pseudo-nitzschia* RISA specific primers developed in this study.

<table>
<thead>
<tr>
<th>Primer (5' –&gt; 3')</th>
<th>Length</th>
<th>Tm (°C)</th>
<th>GC (%)</th>
<th>Target species</th>
<th>RISA length</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pungens II F GCGTTGCTGCCATTCTTACGATGTTGAACCTGG</td>
<td>33</td>
<td>63.9</td>
<td>48.5</td>
<td><em>P. pungens</em></td>
<td>136bp</td>
</tr>
<tr>
<td>Pungens II R AGTGCCAGCAATAGAGTGGATG</td>
<td>29</td>
<td>61.9</td>
<td>48.3</td>
<td><em>P. pungens</em></td>
<td></td>
</tr>
<tr>
<td>Brasiliana I F CGAATTCAGGACCTAGGAGCTTCGC</td>
<td>27</td>
<td>62</td>
<td>55.6</td>
<td><em>P. brasiliana</em></td>
<td></td>
</tr>
<tr>
<td>Brasiliana I R CCGAGGGAGCAGACACACCCAA</td>
<td>22</td>
<td>61.2</td>
<td>59.1</td>
<td><em>P. brasiliana</em></td>
<td></td>
</tr>
<tr>
<td>PnITS1F AAGGATCCATTACACACCGATC</td>
<td>21</td>
<td>54.5</td>
<td>45.5</td>
<td>All <em>Pseudo-nitzschia</em> species</td>
<td></td>
</tr>
<tr>
<td>PnITS1R ATCCACCGSTGAAAGTTGAAT</td>
<td>20</td>
<td>54.5</td>
<td>42.9</td>
<td>All <em>Pseudo-nitzschia</em> species</td>
<td></td>
</tr>
</tbody>
</table>

The results in this study based on species- and genus-specific primer pairs targeting *Pseudo-nitzschia* species revealed promising results when applying on both cultured and environmental samples. This method has proven its application as a potential genotyping system for immediate characterization of *Pseudo-nitzschia* population and monitoring purpose for detection of potentially harmful species in the environmental samples. Species-specific primers could be used as a DNA barcoding tool for identifying *Pseudo-nitzschia* species. Since species identification of *Pseudo-nitzschia* species based on morphology require taxonomic expertise with the aids of advanced electron microscopy. Primer synthesis is much cheaper, faster and easier.

Figure 2: Gel image of PCR products amplified with Pungens II F/R. M, 50 bp ladder (Fermentas, USA); 1, PnSm24 (*P. brasiliana*); 2, PnSm25 (*P. brasiliana*); 3, PnPd10 (*P. brasiliana*); 4, PnKk24 (*P. pungens*); 5, PnPd14 (*P. brasiliana*); 6, Pnsb60 (*P. pungens*); 7, PnKk36 (*P. cuspidata*); 8, AmKB04 (*Alexandrium minutum*); 9, control.

Figure 3: Gel image of PCR products amplified with Brasiliana I F/R. M, 50 bp ladder (Fermentas, USA); 1, PnKk24 (*P. pungens*); 2, PnSm24 (*P. brasiliana*); 3, PnSm25 (*P. brasiliana*); 4, PnPd10 (*P. brasiliana*); 5, PnPd14 (*P. brasiliana*); 6, Pnsb60 (*P. pungens*); 7, Pnsb58 (*P. circumpora*); 8, PnKk36 (*P. cuspidata*); 9, AmKB04 (*Alexandrium minutum*); 10, control.
Figure 4: Gel image of PCR products amplified using different genus-specific primer pairs to target *P. Pungens* in this study. M, 50 bp ladder (Fermentas, USA); lane 1 and 2, PnITS1F/R; 3 and 4, PnAllF/R.

REFERENCES