Rapid Detection of *Pseudo-nitzschia* Species Using Whole-cell Fluorescence *in situ* Hybridization (FISH)

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ABSTRACT

*Pseudo-nitzschia* comprised of thirty-four known species with half of them reported to be domoic acid (DA) producers, DA is a neurotoxin associated with Amnesic Shellfish Poisoning (ASP). Species identification of *Pseudo-nitzschia* mainly depends on detailed observations of the fine ultrastructure of frustules which is indistinguishable without the aids of electron microscope. In this study, a molecular approach for rapid detection of *Pseudo-nitzschia* spp. by using whole-cell fluorescence *in situ* hybridization (FISH) was developed. Cultured and natural samples of *Pseudo-nitzschia* used in this study were identified by transmission and scanning electron microscopy (TEM & SEM). Species-specific LSU rRNA oligonucleotide probes that is specific to *P. pungens* and *P. brasiliana* were designed in silico based on sequences obtained in this study using SILVA database. Synthesized *P. pungens* species specific probe PuD1 was tested on both cultured and field samples together with UniC (+ve control) and UniR probe (-ve control). Efficient of probe PuD1 in detection is consistently more than 93%. Probe PuD1 coupled with FISH method is equally efficiency in cell enumeration compared to traditional method with low discrepancy (<20%). This rapid detection method proven its efficiency and should be adopted in the HABs monitoring program for detection of harmful and potential harmful species of *Pseudo-nitzschia*.

Key words: *Pseudo-nitzschia*, fluorescent *in situ* hybridization (FISH), LSU rRNA oligonucleotide probe, cell enumeration, rapid detection

INTRODUCTION

Amnesic Shellfish Poisoning (ASP) is a type of mollusks contamination caused by bioaccumulation of domoic acid (DA) which is a naturally occurring marine toxin. ASP was first reported in year 1987 due to contaminated blue mussels (*Mytilus edulis*) (Bates et al. 1989). The causative organism of the event was identified later as diatom *Pseudo-nitzschia multiseries* (previously known as *Nitzschia pungens* f. *multiseries*).

Identification of *Pseudo-nitzschia* to species level required detail observation of cell ultrastructure under electron microscopy (Hasle & Syvertsen 1997; Fryxell & Hasle 2003). However, sample preparation and species identification required well trained taxonomical expertise.

Molecular approaches have been widely applied to compliment the traditional approach in monitoring of HABs species. In this study, molecular approach of whole cell fluorescence *in situ* hybridization (FISH) was used to detect *Pseudo-nitzschia* species. Optimization of the whole cell FISH on cultured and natural samples of *Pseudo-nitzschia* species. UniC probe was act as positive and UniR probe act as negative control according to Miller & Scholin (2000).

MATERIALS AND METHODS

Clonal cultures of *Pseudo-nitzschia* were kept at 25°C under 12:12 hour light/dark photoperiod with light intensity of 100 µmol photons m⁻² s⁻¹ in temperature controlled cold white fluorescent incubator (SHELAB, USA). Cultures of *Pseudo-nitzschia* used in FISH optimization include strains PnSb60, PnSb62, PnSb64, PnSb66, PnSm07 and PnSm09. For species identification, samples of *Pseudo-nitzschia* were treated with acid for identification under electron microscopy observations as described in Bargu et al. (2002). For SEM, samples were sputter coated with gold palladium using JEOL sputters coater (Kosijaya Didactic, Malaysia) and examined under a JEOL JSM-6390LA (JEOL, Japan) SEM. While for TEM, the acid-cleaned materials were mounted on a square mesh VECO copper grid and examined under a JEOL JEM-1230 TEM (JEOL, Japan). Black and white micrographs were taken using Gatan Digital Micrograph (DM) Software with Erlangshen ES500W camera (Gatan, USA).

Mid-expontial clonal cultures of *Pseudo-nitzschia* were harvested and the genomic DNA was extracted with 2× CTAB (cetyl-trimethyl ammonium bromide) buffer as described in Vinod (2004). Domains 1 and 3 (D1-D3) of the large subunit (LSU) ribosomal RNA gene (rDNA) was amplified using
primer pair, D1R, 5’-ACC CGC TGA ATT TAA GCA TA-3’ and D3Ca, 5’-CTG ACG TGC AAA TCG TTC GT-3’ (Vivantis Technologies, Malaysia) (Scholin et al. 1994). The PCR products were purified using Wizard® PCR Preps DNA purification kit (Promega, Madison, WI, USA) according to manufacturer’s instructions. Sequencing was carried out using an ABI 377 automated sequencer (PE Applied Biosystems, Foster City, CA, USA).

A total of 57 strains of *Pseudo-nitzschia* sequences were retrieved from GenBank and SILVA database and 12 strains in this study, were aligned by using ClustalX (Thompson et al. 1997). The signature sequences of LSU rDNA sequences were manually analyzed using multiple sequence alignment files. Region with at least one mismatch was observed and examined. The probe parameters such as probe length, melting temperature (Tm), GC content, Gibbs’ free energy (ΔG) and E value were obtained by using the web interface, Integrated DNA Technologies (http://eu.idtdna.com/analyze/Applications/OligoAnalyzer). The specificity of probes designed was further verified by confirmatory test using BLAST search (Altschul et al. 1990).

Whole cell FISH hybridization was carried out according to Miller & Scholin (2000). Cell pellet of *Pseudo-nitzschia* was fixed with freshly prepared modified saline ethanol. About 500 µL of hybridization buffer (20 mL of 25× SET, 0.1 mL Nonidet P-40, 40 mL of 100% formamide and 0.24 mL of Poly A) was added, followed by 12 µL of PuD1 probe; UniC probe (+ve control) or UniR probe (-ve control) incubated at 45°C for 1 hr. Samples were mounted on glass slide and observed using IX51 Olympus inverted microscope.

**RESULTS AND DISCUSSION**

The two *Pseudo-nitzschia* species used in this study was confirmed by ultrastructural observation, as *Pseudo-nitzschia brasiliana* and *P. pungens*. Detailed morphological observations of both species were showed as below (Figure 1).

![Figure 1](image)

Figure 1 (A, C, E-F) *P. brasiliana*; (B, D, G-H) *P. pungens*. [Scale bar: A-D = 10 µm; E & G = 1 µm; F & H = 0.5 µm]

Previous studies of Miller and Scholin (1998) proposed eight *Pseudo-nitzschia* species-specific oligonucleotide probes based on LSU rRNA. However, the probes that designed were based on the sequence of *Pseudo-nitzschia* species found in the subtropical Mediterranean geographical region (Miller & Scholin 1996). The probes that designed may not be applicable to *Pseudo-nitzschia* species from other geographical regions (Rhodes et al. 1998).

A few parameters for in silico probe design have been adopted in the present study. These included the melting temperature, GC content, length of probes, the secondary structure information and the Gibb’s free energy. Melting temperature of probe sequence was suggested equal or more than 57°C by using nearest neighbor method (NN) (Hugenholtz et al. 2001), and not to exceed 67°C because the tendency for secondary annealing may occurred (Marras et al. 2005). Length of the probe should be in the range of 18-24 bp. This range of length is long enough for adequate specificity and short enough for probe to bind easily to the template at the annealing temperature (Pernthaler et al. 2001). A confirmatory test was performed to evaluate the probe specificity by comparing the E-value.

Three variable signature regions of *P. pungens* were detected from the 720 nucleotide length of 69 sequences of LSU rRNA *Pseudo-nitzschia* species that had been multiple-aligned (Table 1). The proposed oligonucleotide probes of *P. pungens* consist of 23 nt probe length. Set 3 had suitable hairpins melting temperature below 40°C, with lower probability in formation of secondary structure. Set 3 also had a higher delta G, GC content and the most mismatch position (nine). The more mismatches position found in *P. pungens* with other species, the proposed probe are expected to be
more specific. Thus, the probe Set 3 \([5´-GCA AGT CCA CAG CGC CCA GAC CA-3´]\) was proposed as the most potential probe sequence for *P. pungens* species specific probe. The probe was designated L-S-P.pu-0378-b-A-23 as according to the Probe Nomenclature (Alm et al. 1996).

The proposed oligonucleotide probe was compared with the *P. pungens* species specific PuD1 probe by Scholin et al. (1996). The sequence of PuD1 probe is \(5´-ATG ACT CAC TTT ACC A-3´\) with only 16 nucleotides. The GC content of the sequence was low, only 37.5% while the T_m was 43.3°C. Based on BLAST result, the sequence of PuD1 probe \(5´-ATG ACT CAC TTT ACC A-3´\) with E-value of 15 with 10 hits of *P. pungens*, however the sequence also hit on other species such as the marine bacteria, *Shewanella putrefaciens* and *Shewanella blatica*. In this study, the same region was found to be the possible probe regions. The second set, Set 2 \([5´-ATG ACT CAC TTT ACC AGG CGG AC-3´]\) was proposed but with longer nucleotide length, which had same signature region as PuD1 probe. The probe length in this study was longer and it showed higher T_m, higher GC content, higher delta G and thus more stable compared to PuD1 probe.

To date, none of the *P. brasiliana* species specific probe has been reported. In this study, two signature region of *P. brasiliana* was detected from the 720 nucleotides length of 69 sequences of LSU rRNA *Pseudo-nitzschia* species. The first signature region was found in nucleotide position of 27 – 49 (Set 1) while the second signature region was found in nucleotide position of 133 – 155 (Set 2). The proposed oligonucleotide probes of *P. brasiliana* consist of 23 nt probe length. Set 2 had higher melting temperature (65.1°C) and higher GC content (65.2%) (increase the stability of structure) compared to Set 1. Thus, Set 2 probe was proposed as the most suitable signature sequence for *P. brasiliana* species specific probe and was designated L-S-P.br-0129-b-A-23 as according to the Probe Nomenclature (Alm et al. 1996).

Table 1: Proposed oligonucleotide probes of *Pseudo-nitzschia pungens* with nucleotide length, melting temperature (T_m), GC content (%), delta G (ΔG), and E-value.

<table>
<thead>
<tr>
<th>Set</th>
<th>Probe Sequence 5´-3´</th>
<th>Signature region 5´-3´</th>
<th>Probe length</th>
<th>T_m (°C)</th>
<th>GC%</th>
<th>ΔG (kcal/mol)</th>
<th>E-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>TTT ACC AGG CGG ACG GGA GTG GAC ACU CCC GUC CGC CUG GUA AA</td>
<td>23</td>
<td>63.0</td>
<td>60.9</td>
<td>0.7</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>ATG ACT CAC TTT ACC AGG CGG GUC CGC CU G GUA AAG UGA GUC AU</td>
<td>23</td>
<td>58.9</td>
<td>52.2</td>
<td>0.0</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>GCA AGT CCA CAG CGC CCA GAC UGG UCU GGG CGC UGU GGA CTT GC</td>
<td>23</td>
<td>66.2</td>
<td>65.2</td>
<td>0.9</td>
<td>0.002</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Proposed oligonucleotide probes of *Pseudo-nitzschia brasiliana* with nucleotide length, melting temperature (T_m), GC content (%), delta G (ΔG) and E-value.

<table>
<thead>
<tr>
<th>Set</th>
<th>Probe Sequence 5´-3´</th>
<th>Signature region 5´-3´</th>
<th>Probe length</th>
<th>T_m (°C)</th>
<th>GC%</th>
<th>ΔG (kcal/mol)</th>
<th>E-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>TAG AGC GCA GAT TCA CAT CCT UCA GGA UGU GAA UCU GCG GA</td>
<td>23</td>
<td>57.8</td>
<td>47.8</td>
<td>0.0</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>TCA CTC AAC CAG GCG GAC GGG CUC CC G UCC GCC UGU UUG AG</td>
<td>23</td>
<td>65.1</td>
<td>65.2</td>
<td>0.7</td>
<td>0.002</td>
<td></td>
</tr>
</tbody>
</table>

Application of FISH on the Probe PuD1 showed specificity towards *P. pungens* and not to other *Pseudo-nitzschia* and *Nitzschia longissima* tested in this study. Cells that successfully hybridized with the FITC-labeled probe appeared as green fluorescence (Figure 2). It can be easily distinguished from the non-labeled cells. All *Pseudo-nitzschia* species (*P. brasiliana* and *P. pungens*) and *N. longissima* showed green fluorescence color when applied with positive control probe (Table 3).
Figure 3: Micrographs of cultured cells treated with PuD1 probe (Pseudo-nitzschia pungens specific probe) (A-B). Autofluorescence of cells showing chloroplast content (A’-B’) and bright field micrographs (A’’-B’’). Species tested are Pseudo-nitzschia pungens (A), Pseudo-nitzschia brasiliana (B). Only Pseudo-nitzschia pungens showed green fluorescence.

Table 3: Whole cell hybridization probe reactivity for Pseudo-nitzschia species and Nitzschia species. Each “+” indicates a strong probe reaction and “-” indicates no reaction was detected.

<table>
<thead>
<tr>
<th>Species</th>
<th>Culture source</th>
<th>PuD1 probe</th>
<th>UniC probe</th>
<th>UniR probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. pungens</td>
<td>PnSb60</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>PnSb62</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>PnSb64</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>PnSb66</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>P. brasiliana</td>
<td>PnSm07</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>PnSm09</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>N. longissima</td>
<td>N72</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

CONCLUSION
Species-specific signature sequences of P. pungens and P. brasiliana were successfully determined, and potential species-specific oligonucleotide probes were proposed for rapid detection by using whole-cell fluorescent in situ hybridization.

REFERENCES


