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

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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, centyl-trimetyl-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 ^{o}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. multistriata* (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.