MORPHOLOGICAL CHARACTERIZATION AND MOLECULAR DETECTION OF POTENTIAL HARMFUL DIATOM, *PSEUDO-NITZSCHIA* FROM MALAYSIAN WATER

Teng Sing Tung

Master of Science
2012
MORPHOLOGICAL CHARACTERIZATION AND MOLECULAR DETECTION OF POTENTIAL HARMFUL DIATOM, *PSEUDO-NITZSCHIA* FROM MALAYSIAN WATER

TENG SING TUNG

Thesis submitted in fulfilment for the degree of Master of Science (Marine Biotechnology)

Supervisor: Dr Leaw Chui Pin

Co-supervisor: Dr Lim Po Teen

Institute of Biodiversity and Environmental Conservation
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak

2012
DECLARATION

I hereby declare that the work in this thesis is my own except for quotations and summaries which have been duly acknowledged.

August 2012

TENG SING TUNG
10021625
ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and appreciation to all whose have contributed to the completion of this dissertation. Their participation made this thesis possible complete in time.

First of all, my special thanks are due to my supervisors, Dr Leaw Chui Pin, and Dr Lim Po Teen. I will always be indebted to them for their earnest supervision and guidance on this project throughout past two years. Secondly, I would like to thank Zamalah postgraduate scholarship UNIMAS, MOHE-FRGS grants to Dr Lim Po Teen and E- Science to Dr Leaw Chui Pin for financials support provided in this research. Special thanks to Dr. Stephen S. Bates for his valuable suggestions and English revision of manuscript.

I would also like to thank member of UNIMAS Harmful Algal Blooms Research Group (MEOHAB): Lim Hong Chang, Tan Toh Hii, Fareha Haji Hilaluddin, Siti Zubaidah Kamarudin, Hii Kieng Soon and final year undergraduates students whom generously to share personal knowledge and experiences to complete this research and dissertation. I am externally grateful to the faculty, staffs at laboratories of Kak Rahah, Abang Hasri, and also science officer Madam Ting Woei from Institute of Biodiversity and Environmental Conservation (IBEC) and Department of Aquatic Sciences for their assistances especially in term of technical problems.

Special thanks express to my Mum, Dad, brother Sing Nan and Sister Ching Ching for your encouragement and supportive. Lastly, I would like to thank my life partner, Ching Sang Sang for her love and support in past two years, it means a lot to me.
MORPHOLOGICAL CHARACTERIZATION AND MOLECULAR DETECTION OF DIATOM, PSEUDO-NITZSCHIA SPECIES (BACILLARIOPHYCEAE) IN MALAYSIA

Teng Sing Tung
Institute of Biodiversity and Environmental Conservation,
Universiti Malaysia Sarawak

Abstract

The diatom, *Pseudo-nitzschia* is a genus that associated with harmful algal blooms (HABs) worldwide. One third of the species produce the neurotoxin, domoic acid (DA) which is a causative agent of amnesic shellfish poisoning (ASP). Research interest in toxic *Pseudo-nitzschia* thrived after the outbreak of the first ASP case stroked Canada in 1987. Following the incidence, numerous studies have been undertaken throughout the open oceans. However, occurrences of *Pseudo-nitzschia* spp. were reported as species complex/complexes due to the taxonomic uncertainty in the genus. This has lead to enormous confusion in species identification. In this study, a thorough field survey focused on *Pseudo-nitzschia* species were undertaken to investigate the spatial distribution pattern and species composition in Malaysian waters. Samplings covered locations in the Andaman Sea, the Straits of Malacca, the South China Sea, Sulu Sea and Celebes Sea. Plankton samples were collected from 17 locations using a 20 μm-mesh plankton net haul. Samples were subjected to acid wash treatment before detailed morphological observations under transmission electron microscope (TEM). Identification of *Pseudo-nitzschia* species was based on the frustules morphology with morphometric measurements. In total, 22 species were identified, in which 16 were new records in Malaysia, and a new morphotype discovered. Among the toxic species found, including *P. brasiliana*, *P. caciantha*, *P. calliantha*, *P. cuspidata*, *P. delicatissima*, *P. multistriata*, *P. pseudodelicatissima*, *P. pungens*, and *P. turgidula* were previously reported to associate with ASP events worldwide.

In order to aid the species identification of this complex species, molecular approaches have been introduced in *Pseudo-nitzschia* taxonomy. Molecular phylogenetic analyses are applied based on a genetic marker to resolve the *Pseudo-nitzschia* species complex/complexes. In this study, whole cell fluorescence in situ hybridization (FISH), a rapid molecular detection tool was applied on both field and cultured samples. Two toxic species, *P. pungens* were used as model species in FISH optimization and on-field application. Species-specific probes were designed in silico. Specific probes were tested in silico based on complete 28S rRNA secondary structure information. The accessibilities of probes were tested in silico based on complete 28S rRNA secondary structure information. The probes were successfully optimized on clonal cultures and natural spiked samples. Whole-cell FISH was proven as a potential rapid detection tool for monitoring of toxic *Pseudo-nitzschia* species in Malaysia. This study has provided further insight and better understanding on *Pseudo-nitzschia* taxonomy using both the morphological and molecular approaches. The finding of a new morphotype warrants further investigation to obtain genetic evidence. FISH method with species specific probes on targeted *Pseudo-nitzschia* species are proposed as monitoring tools for the related agency in Malaysia.

Key words: *Pseudo-nitzschia*, Amnesic Shellfish Poisoning (ASP); taxonomy; frustules morphology; fluorescence in situ hybridization; oligonucleotide probes.
PENCIRIAN MORFOLOGI DAN PENGESANAN MOLEKULAR DIATOM, 
*PSEUDO-NITZSCHIA* SPESIES (BACILLARIOPHYCEAE) DI MALAYSIA

Teng Sing Tung
Institute of Biodiversity and Environmental Conservation, 
Universiti Malaysia Sarawak

Abstrak


Kata kunci: *Pseudo-nitzschia*, Kerang-kerangan Keracunan Amnesic (ASP); taksonomi; frustules morfologi; hibridisasi in situ pada pertarungan (FISH); oligonukleotida prob.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>DECLARATION</td>
<td>ii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>iii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>iv</td>
</tr>
<tr>
<td>ABSTRAK</td>
<td>v</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>x</td>
</tr>
</tbody>
</table>

## CHAPTER I INTRODUCTION

1.1 General introduction

1.2 The pennate diatom, *Pseudo-nitzschia*

1.2.1 Taxonomy of *Pseudo-nitzschia*

1.2.2 Genetics of *Pseudo-nitzschia*

1.2.3 Reproduction of *Pseudo-nitzschia*

1.2.4 Toxicology of *Pseudo-nitzschia*

1.3 *Pseudo-nitzschia* in the Southeast Asian region

1.4 Objectives of the study

## CHAPTER II *PSEUDO-NITZSCHIA* (BACILLARIOPHYCEAE) IN MALAYSIA: A RECORD OF TAXA FROM FIELD INVESTIGATION, WITH DESCRIPTIONS OF NEW MORPHOTYPE

2.1 Introduction

2.2 Materials and Methods
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.2.1</td>
<td>Field sampling</td>
<td>14</td>
</tr>
<tr>
<td>2.2.2</td>
<td>Species identification</td>
<td>14</td>
</tr>
<tr>
<td>2.2.3</td>
<td>Morphological character coding and phylogenetic analysis.</td>
<td>15</td>
</tr>
<tr>
<td>2.3</td>
<td>Results</td>
<td>20</td>
</tr>
<tr>
<td>2.3.1</td>
<td>Species diversity</td>
<td>20</td>
</tr>
<tr>
<td>2.3.2</td>
<td>Species diagnosis</td>
<td>21</td>
</tr>
<tr>
<td>2.3.3</td>
<td>Description of a new morphotype</td>
<td>46</td>
</tr>
<tr>
<td>2.3.4</td>
<td>Phylogenetic analyses</td>
<td>48</td>
</tr>
<tr>
<td>2.4</td>
<td>Discussion</td>
<td>50</td>
</tr>
<tr>
<td>2.4.1</td>
<td>Species richness and geographical distribution</td>
<td>50</td>
</tr>
<tr>
<td>2.4.2</td>
<td><em>Pseudo-nitzschia</em> morphology</td>
<td>51</td>
</tr>
<tr>
<td>2.5</td>
<td>Conclusion</td>
<td>65</td>
</tr>
</tbody>
</table>

**CHAPTER III**

RAPID DETECTION OF *PSEUDO-NITZSCHIA* SPECIES BY WHOLE CELL FLUORESCENCE IN SITU HYBRIDIZATION (FISH)

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Introduction</td>
<td>66</td>
</tr>
<tr>
<td>3.2</td>
<td>Materials and Methods</td>
<td>68</td>
</tr>
<tr>
<td>3.2.1</td>
<td>Sampling and culture establishment</td>
<td>68</td>
</tr>
<tr>
<td>3.2.2</td>
<td>Genomic DNA extraction</td>
<td>68</td>
</tr>
<tr>
<td>3.2.3</td>
<td>Gene amplification of the nuclear-encoded large subunit ribosomal RNA gene</td>
<td>69</td>
</tr>
<tr>
<td>3.2.4</td>
<td>Taxon sampling</td>
<td>71</td>
</tr>
<tr>
<td>3.2.5</td>
<td>Determination of the termini for</td>
<td>72</td>
</tr>
</tbody>
</table>
LSU rDNA

3.2.6 LSU rRNA secondary structure prediction 72
3.2.7 LSU rRNA phylogenetic analysis 73
3.2.8 In silico oligonucleotide probes design and probe synthesis 73
3.2.9 Whole-cell fluorescence in situ hybridization (FISH) 75

3.3 Results and Discussion 76
3.3.1 Algal culture 76
3.3.2 Secondary Structures of LSU rRNA (D1-D3) 77
3.3.3 Phylogenetic Inferences 78
3.3.4 Species-specific probe of Pseudo-nitzschia pungens 82
3.3.5 Probe optimization 89

3.4 Conclusion 96

CHAPTER IV CONCLUSION 97

REFERENCES 100

APPENDICES 118
A Distribution of character states among taxa 118
B Complete sequences of LSU rDNA (D1-D8) 120
C Nucleotide sequences of LSU rDNA (D1-D3) 121
D List of publications 122
LIST OF TABLE

Table 2.1 Morphological characters and the character states used in this study to develop the cladogram (Fig. 2.28).  
Table 2.2 Morphometric measurements of *Pseudo-nitzschia* species in Malaysian coastal waters obtained from this study. All measurements were taken by TEM. CIS, central interspace, *n*, number of cells examined. Species with asterisks are reported as toxic. *a* Poroid structure with perforation (+) or non-perforation (-). Numbers in parenthesis denote numbers of perforated sectors within poroids.

Table 3.1 Primers used to amplify LSU rRNA of *Pseudo-nitzschia*.  
Table 3.2 Compositions of PCR reagents used in this study.  
Table 3.3 Thermal cycling steps used in this study.  
Table 3.4 Positive and negative control probes used in this study.  
Table 3.5 *Pseudo-nitzschia* used in this study, with species, strains and isolation localities.  
Table 3.6 Potential signature regions for *Pseudo-nitzschia pungens* species-specific probe design.  
Table 3.7 Proposed oligonucleotide probe of *P. pungens* with probe name, position, signature region, probe sequence, melting temperature (Tm), GC content, Gibb’s free energy (ΔG°), and hybridization efficiency (HE).  
Table 3.8 Probe mismatch analysis of the *P. pungens* species-specific probe, L-S-Ppun-405-A-18, showing binding sites on target and non-target species.
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.1</td>
<td>A time line of the studies of <em>Pseudo-nitzschia</em>. (A) year 1900-1999, (B) 2000 – 2012.</td>
<td>5</td>
</tr>
<tr>
<td>Figure 1.2</td>
<td>Silica frustule of <em>Pseudo-nitzschia</em> sp. shown in three different views. The cell in valve view is made purposely larger than actual <em>Pseudo-nitzschia</em> cells to show poroids and striae.</td>
<td>6</td>
</tr>
<tr>
<td>Figure 2.1</td>
<td>Map showing 17 sampling locations along the coasts of Malaysia.</td>
<td>14</td>
</tr>
<tr>
<td>Figure 2.2</td>
<td>Poroid morphology of <em>Pseudo-nitzschia</em> species. TEM. (A-F) One row of poroids. (A) 4-6 perforation sectors with wide space at the center, bar = 0.2 µm. (B) 2-4 perforation sectors with central dot, and more rounded poroid edge, bar = 0.5 µm. (C) 2 perforation sectors, bar = 0.1 µm. (D) Four perforation sectors with narrow central space, bar = 0.1 µm. (E) &gt;7 perforation sectors with more than one centre dot, bar = 0.2 µm. (F) 4-7 perforation sectors with central dot, bar = 0.2 µm. (G-I) Two rows of poroids. (G) 4-6 perforation sectors with split in the middle of the poroids, bar = 0.2 µm. (H) Hexagonal poroids without sector perforation, bar = 0.2 µm. (I) Round poroids without sector perforation, bar = 0.1 µm.</td>
<td>19</td>
</tr>
<tr>
<td>Figure 2.3</td>
<td>Spatial distribution of <em>Pseudo-nitzschia</em> species in Malaysian coastal waters.</td>
<td>20</td>
</tr>
<tr>
<td>Figure 2.4</td>
<td>Species composition of known toxic/potentially toxic and non-toxic <em>Pseudo-nitzschia</em> species found in Malaysian waters. Abbreviations of sampling locations: Stb, Santubong; Sm, Samariang; Ge, Gerigat; Kb, Kabong; Bin, Bintulu; KP, Kuala Penyu; KB, Kota Belud; KK, Kota Kinabalu; Ku, Kudat; PB, Pulau Bangi; Sem, Semporna; JB, Johore Bharu; Mu, Muar; PD, Port Dickson; TB, Teluk Batik; QB, Queen Bay; KT, Kuala Terengganu.</td>
<td>21</td>
</tr>
<tr>
<td><strong>Figure 2.5</strong> <em>Pseudo-nitzschia pseudodelicatissima</em>. TEM. (A) Acid-cleaned valve showing linear valve shape, bar = 20 µm. (B and C) Apices, bar = 1.0 µm. (D) Centre of valve showing central interspace, bar = 2.0 µm. (E) Striae and one row poroids, bar = 0.2 µm. (F) Close up of poroids structure showing poroids with 2 hymen perforation sectors, bar = 0.1 µm.</td>
<td>23</td>
<td></td>
</tr>
</tbody>
</table>
Figure 2.6  *Pseudo-nitzschia* cf. *cuspidata*. TEM. (A) Acid-cleaned valve showing lanceolate valve shape, bar = 10 μm. (B) Centre of valve showing central interspace, bar = 0.5 μm. (C) Close up of poroids structure with two hymen perforated sectors, bar = 0.2 μm.

Figure 2.7  *Pseudo-nitzschia* caciantha. TEM. (A) Acid-cleaned valve showing lanceolate valve shape, bar = 10 μm. (B and C) Apices, bar = 2.0 μm (B), bar = 0.5 μm (C). (D) Centre part of valve showing the central interspace, bar = 2.0 μm. (E) Striae and poroids, bar = 0.5 μm (F) Close up of poroids with hymenate structure, bar = 0.2 μm.

Figure 2.8  *Pseudo-nitzschia* calliantha. TEM. (A) Acid-cleaned valve showing linear valve shape, bar = 10 μm. (B) Apices, bar = 2.0 μm (C) Centre part of valve, bar = 2.0 μm. (D) Details of striae and poroids, bar = 2.0 μm. (E) Central interspace, bar = 0.5 μm. (F) Close up of poroids with 4-10 hymen perforation sectors, bar = 0.2 μm.

Figure 2.9  *Pseudo-nitzschia* mannii. TEM. (A and B) Linear valve, bar = 10 μm. (C and D) Apices, bar = 1.0 μm (C) and bar = 2.0 μm (D). (E) Central part of valve, bar = 2.0 μm. (F) Central interspace, bar = 0.5 μm. (G) Close up of poroids with mainly 4-5 hymenate sectors, bar = 0.2 μm.

Figure 2.10  *Pseudo-nitzschia* similica. TEM. (A) Linear valve, bar = 10 μm. (B) Apices, bar = 2.0 μm. (C) Centre part of valve, bar = 2.0 μm. (D) Valve showing central interspace, bar = 2.0 μm. (E) Detail of central interspace showing central nodule, bar = 1.0 μm. (F) Close up of poroids, bar = 0.2 μm.

Figure 2.11  *Pseudo-nitzschia* circumpora. TEM. (A) Lanceolate valve, bar = 10 μm. (B) Apices, bar = 0.5 μm. (C) Central part of valve showing the central interspace, bar = 1.0 μm. (D and E) Close up of poroids, bar = 0.5 μm (D) and 0.1 μm (E).

Figure 2.12  *Pseudo-nitzschia* hasleana. TEM. (A) Lanceolate valve, bar = 10 μm. (B and C) Apices, bar = 2.0 μm. (D) Central part of valve, bar = 2.0 μm. (E) Valve showing central interspace. Note striae and poroid structure of proximal mantle, bar = 0.5 μm. (F) Close up of poroids hymen perforation, bar = 0.2 μm.
Figure 2.13 *Pseudo-nitzschia inflata*. TEM. (A and B) Lanceolate valves, bar = 10 µm. (C and D) Apices, bar = 2.0 µm. (E and F) Poroids structure at valve ends, bar = 0.5 µm. (G) Central interspace, bar = 2.0 µm. (H, I and J) Striae structure with 1-2 rows of poroids, bar = 0.5 µm (H), 0.1 µm (I) and 0.2 µm (J).

Figure 2.14 *Pseudo-nitzschia subfraudulenta*. TEM. (A) Lanceolate valve, bar = 10 µm. (B) Apices, bar = 5.0 µm. (C) Central interspace, bar = 2.0 µm. (D) Striae structure with two perforated rows of poroids, bar = 0.5 µm. (E) Abnormal 1 row of poroids per striae, bar = 0.5 µm. (F) Cingular band, bar = 2.0 µm.

Figure 2.15 *Pseudo-nitzschia delicatissima/ arenysensis*. TEM. (A) Lanceolate valve, bar = 5.0 µm. (B, and C) Apices, bar = 0.5 µm (D) Central interspace, bar = 0.5 µm. (E) Close up of striae with two rows of poroids, bar = 0.2 µm.

Figure 2.16 *Pseudo-nitzschia decipiens*. TEM. (A) Lanceolate valve, bar = 10 µm. (B, and C) Apices, bar = 2.0 µm. (D) Central part of valve, bar = 2.0 µm. (E) Central interspace, bar = 0.5 µm. (F and G) Close up of striae structure with two rows of poroids. Poroids are hexagonal, bar = 0.2 µm.

Figure 2.17 *Pseudo-nitzschia dolorosa*. TEM. (A) Lanceolate valve, bar = 10 µm. (B) Apices, bar = 1.0 µm. (C) Striae structure with 1-2 rows of poroids; note central interspace, bar = 1.0 µm. (D) Detail of poroids, bar = 0.2 µm.

Figure 2.18 *Pseudo-nitzschia lineola*. TEM. (A) Lanceolate valve, bar = 10 µm. (B) Apices, bar = 0.5 µm. (C) Part of valve showing striae structure, bar = 2.0 µm. (D) Detail of striae and poroids, bar = 0.5 µm.

Figure 2.19 *Pseudo-nitzschia micropora*. TEM. (A) Lanceolate valve, bar = 5.0 µm. (B, and C) Apices, bar = 0.5 µm. (D) Central part of valve, note absence of central interspace, bar = 0.5 µm. (E) Detail of striae and poroids, bar = 0.2 µm.

Figure 2.20 *Pseudo-nitzschia multistriata*. TEM. (A) Lanceolate valve, bar = 10 µm. (B, C) Apices, bar = 2.0 µm. (D) Central part of valve; note absence of central interspace, bar = 2.0 µm. (E) Detail of striae with uneven row arrangement of poroids, bar = 0.2 µm.
Figure 2.21  *Pseudo-nitzschia americana*. TEM. (A) Linear to lanceolate valve, bar = 2 µm. (B, C) Broad rounded apices, bar = 0.5 µm. (D, E) Striae structure; note two different patterns of striae structure; normal striae arrangement (D), bar = 0.5 µm, and abnormal striae arrangement (E), bar = 0.2 µm. (F, G) Cingulum band, bar = 0.5 µm (F) and 0.2 µm (G).

Figure 2.22  Fig. 22. *Pseudo-nitzschia linea*. TEM. (A) Linear valve, bar = 2.0 µm. (B) Central part of valve, bar = 0.5 µm. (C, D) Apices, bar = 0.5 µm. (E) Poroids and striae structures, bar = 0.2 µm. (F) Cingular band with pattern 2 × 4-5, bar = 0.2 µm.

Figure 2.23  *Pseudo-nitzschia brasiliiana*. TEM. (A) Lanceolate valve, bar = 5.0 µm. (B, C) Apices, bar = 1.0 µm (B), 2.0 µm (C). (D) Central part of valve, bar = 2.0 µm. (E, F and G) Clear perforation of poroids with two rows and an incomplete third row of poroids, bar = 0.2 µm (E), 0.5 µm (F), and 0.2 µm (G).

Figure 2.24  *Pseudo-nitzschia pungens*. TEM. (A, B) Lanceolate valve, bar = 10 µm (C, D) Apices, bar = 2.0 µm. (E) Central part of valve with two rows of poroids, bar = 2.0 µm. (F) Striae structures, bar = 2.0 µm.

Figure 2.25  *Pseudo-nitzschia cf. pungens*. TEM. (A, B) Lanceolate valve, bar = 20 µm. (C, D) Apices, bar = 2.0 µm. (E, F and G) Striae structure, showing uneven arrangement of fibulae and striae, bar = 2.0 µm. (H) Poroids structure, showing incomplete third row of poroids, bar = 0.5 µm. (I) Cingular band with round poroids, bar = 0.2 µm.

Figure 2.26  *Pseudo-nitzschia turgidula*. TEM. (A) Lanceolate valve, bar = 10 µm. (B, C) Apices, bar = 2.0 µm. (D) Central part of valve, bar = 2.0 µm. (E) Central interspace, bar = 0.5 µm. (F) Poroids structure, showing two rows of poroids, bar = 0.2 µm.

Figure 2.27  *Pseudo-nitzschia* sp. Port Dickson. TEM. (A) Lanceolate valve, bar = 20 µm. (B, C) Apices, bar = 2.0 µm. (D, E) Central part of valve, bar = 2.0 µm. (F) Center interspace, bar = 0.5 µm. (G) Poroids structure showing hymen perforation sectors, bar = 0.2 µm. (H) Cingular band with 1-2 × 2-3 (1 split to many), bar = 0.5 µm.
Figure 2.28 Unweighted cladogram of majority-rule tree based on *Pseudo-nitzschia* morphological characters. Terminal labels indicate morphospecies. Columns to the right of the tree indicate characters mapping. See Table 2 for details of characters and their states.

Figure 2.29 Key to species of *Pseudo-nitzschia* in Malaysian waters.

Figure 2.30 Morphometric variation between *Pseudo-nitzschia* sp. Port Dickson (PD) and species in Clade I: *pseudodelicatissima* group: cac, *P. caciosantha*, cal, *P. calliantha*, cus, *P. cuspidata*, cir, *P. circumpora*, pde, *P. pseudodelicatissma*, man, *P. mannii*, has, *P. hasleana*, fry, *P. fryxelliana*. Valve width (A); density of fibulae (B) and interstriae (C) in 10 μm; density of poroids in 1 μm (D); sector perforations in the poroids (E); and band striae in 10 μm (F).

Figure 2.31 (A) Frustules of *P. brasiliiana* (a), *P. americana* (b), and *P. linea* (c) in valve view showing the transapical axes in the apices (x) and the maximum widths (y). Comparison of ratios of x and y (B), and the variation of x and y in the three species (C).

Figure 3.1 Secondary structure of domain 1 to domain 3 of the LSU ribosomal RNA of *Pseudo-nitzschia pungens*. Strands in grey indicate regions with high variability.

Figure 3.2 Maximum likelihood topology inferred from complete sequences of LSU rDNA. Note the position of *Pseudo-nitzschia* in red branches. Scale bar represents 0.1 substitutions/sites.

Figure 3.3 Phylogenetic inferences of the D1-D3 of LSU Ribosomal RNA gene in *Pseudo-nitzschia* species. Trees were inferred by maximum likelihood (ML) and Bayesian analysis (B1), with ML bootstrap values of 100 replications and posterior probability indicate in the internal nodes. Scale bar represents 0.03 substitutions/sites.

Figure 3.4 Secondary structure of C1 helix constructed by 4SALE showed variability of helix C1 and hybridization efficiency increased along helix C1a.
Figure 3.5 Sequences logos of signature regions for *Pseudo-nitzschia pungens* predicted in this study. A, L-S-Ppun400-A-18; B, L-S-Ppun399-A-18; and C, L-S-Ppun405-A-18.

Figure 3.6 Hybridization efficiency of *P. pungens* species-specific probe, L-S-Ppun-405-A-18, against target and non-target species.

Figure 3.7 Fluorescent micrographs of whole-cell FISH using centrifugation system. (A) Cells of culture sample (PnSb62) treated with UniC probe (universal positive control probe) showing green fluorescence. (C) Cells treated by UniR (negative control probe) showing yellowish fluorescence. (B and D) Cells with auto-fluorescence.

Figure 3.8 Fluorescent micrographs of whole-cell FISH using centrifugation system. (A) Cells of *P. pungens* culture, PnSb62, treated with the species-specific probe, L-S-Ppun-405-A-18, showing positive green fluorescence. (B) Cells with auto-fluorescence. (C) Spiked samples (cultured cells + environmental samples) treated the probe showing green fluorescence from *P. pungens* cells, but not the non-target species (arrows).

Figure 3.9 Fluorescent micrographs of whole-cell FISH using centrifugation system. Cells of *P. brasiliana* (A), *P. caciantha* (C), and *P. circumpora* (E) treated with the *P. pungens* species-specific probe showing no fluorescence. (B, D and F) Cells with auto-fluorescence, *P. brasiliana* (B), *P. caciantha* (D), and *P. circumpora* (F).
1.1 General introduction

Microalgae had been received great attentions for the past few decades since the discovery of marine toxic microalgae that are harmful to ecosystem health. Harmful Algal Bloom (HAB), a scientific term used to describe the natural phenomena of increasing in cell density and caused deleterious effects to the environmental life. There were several marine toxins that have been discovered and result in different impact to the environments (Table 1.1).

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Impact</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saxitoxin</td>
<td>Paralytic Shellfish Poisoning (PSP) (Dell'Aversano et al., 2008)</td>
</tr>
<tr>
<td>Domoic acid</td>
<td>Amnesic Shellfish Poisoning (ASP) (Clayden et al., 2005)</td>
</tr>
<tr>
<td>Okadaic acid</td>
<td>Diarrhetic Shellfish Poisoning (DSP) (MacKenzie et al., 2005)</td>
</tr>
<tr>
<td>Azaspiracids</td>
<td>Azaspiracid Shellfish Poisoning (AZP) (Twiner et al., 2004)</td>
</tr>
<tr>
<td>Ciguatera</td>
<td>Ciguatera Fish Poisoning (CFP) (Withers, 1982)</td>
</tr>
<tr>
<td>Brevetoxin</td>
<td>Neurotoxic Shellfish Poisoning (NSP) (Watkins et al., 2008)</td>
</tr>
</tbody>
</table>
1.2 The pennate diatom, *Pseudo-nitzschia*

Research interests of the pennate *Pseudo-nitzschia* have increased in last two decades due to the health impact. Domoic acid, causative of the Amnesic shellfish poisoning (ASP) since the first outbreak of the ASP cases at Prince Edward Island, Canada in 1987 that caused by the *Pseudo-nitzschia multiseries* (Bates et al., 1989). There were numerous studies had been done on *Pseudo-nitzschia* physiological ecology, phylogeny and toxicity to obtain better understanding on *Pseudo-nitzschia* (Bates et al., 1996; Lundholm et al., 2002b; Fehling et al., 2004; Lundholm et al., 2006; Amato and Montresor, 2008; Lim et al., In press). However, previous researches were only concentrated at the North Atlantic Sea, including Mediterranean Sea. There was still lack of researches on the Pacific Ocean especially the South China Sea. Only a few species were reported in the tropical South China Sea coastline (e.g. Skov et al. 2004; Lim et al., 2012a; Lim et al., In press). *Pseudo-nitzschia* species still remained unresolved in their taxonomy due to the occurrences of species complexes (Lundholm et al., 2002b; Lundholm et al., 2003; Lundholm et al., 2006; Lundholm et al., 2012; Lim et al., In press). Hence, molecular tools were used to further infer and resolve the cryptic diversity in the *Pseudo-nitzschia* complex (Miller and Scholin, 1998; Lundholm et al., 2002a; Lim et al., 2012a; Lundholm et al., 2012).

1.2.1 Taxonomy of *Pseudo-nitzschia*

The genus *Pseudo-nitzschia* (Bacillariophyceae) was first delineated from the genus *Nitzschia* (Hasle, 1994) by their ability to form stepped chains, however, with the exception of *P. americana* (Hernández-Becerril, 1998; Orlova and Shevchenko, 2002; Stonik et al., 2011) and *P. antarctica* (Scott and Thomas, 2005).
Chain length was species dependent. Even though species that form chains sometimes appears as single cells. For examples a long cell chain *Pseudo-nitzschia brasiliana* was separated into single cells, and sink to the bottom due to nutrient depletion at the stationary phase (Lundholm et al., 2002b). On the other hand, *P. galaxiae* lost its chain-forming ability after established in culture (Lundholm and Moestrup, 2002). *Pseudo-nitzschia pungens*, long chains forming species, sometimes forms spiral in young culture. Some old cultures eventually lost this ability when stepped chains gradually changed to stacked chain (ribbon–shaped) due to reduction of the cell length (Chepurnov et al., 2005).

Observation of *Pseudo-nitzschia* cells under a light microscope can only permit the identification to the group level (*seriata delicatissima*-group). *Seriata*-(width > 3 µm) and *delicatissima*-(width < 3 µm) groups were first adopted in the classification of *Pseudo-nitzschia* spp. based on the width of cell valves by Hasle and Syvertsen (1997) (Figure 1.1).

Taxonomy of *Pseudo-nitzschia* was further refined by the improvement of scanning and transmission electron microscopy (SEM and TEM) to obtain the ultrastructure of cell frustules. There were several main characters used in the taxonomy of *Pseudo-nitzschia* (Lundholm and Moestrup, 2002; Amato and Montresor, 2008; Lim et al., 2012a; Lim et al., In press). They are shapes of valve, cells overlapping, central interspace, valve width, valve length, and poroid morphology such as densities of striae and fibulae. This taxonomy evolution has resolved the species confusion, and numerous novel species emerged in 1996 - 2012 (Figure 1.1).

Attention has been given to finding distinctive morphological characters in delineating species of *Pseudo-nitzschia*, and features such as poroid hymen and cingular band characters, density of striae in the cingular band, and the pattern of cingular band
were used in species delimitation in 2002-2012 (Lundholm et al., 2002b; Lundholm et al., 2003; Lundholm et al., 2006; Lundholm et al., 2012; Lim et al., In press) (Figure 1.1). The discovery of *P. americana*, *pseudodelicatissima* and *delicatissima* complexes was based on the characters of cingular band and poroid hymen.

Current taxonomic features used in identifying species of *Pseudo-nitzschia* are characters that can be observed in the girdle view and valve view (Figure 1.2). These included the valve view characters, such as striae, fibulae, central interspace and poroids. Cingulum band pattern is one of the important characters to delineate species in the complex. Cross sectional view of valve to reveal the ultrastructure is not commonly used in species identification; there was only one report on the ultrastructure of *P. galaxiae* as described in Lundholm and Moestrup (2002).
Figure: 1.1: A time line of the studies of Pseudo-nitzschia. (A) year 1900 - 1999, (B) 2000 - 2012.
Figure 1.2: Silica frustule of *Pseudo-nitzschia* shown in three different views, girdle view (A), valve view (B) and cross sectional view (C). The cell in valve view is made purposely larger than actual *Pseudo-nitzschia* cells to show poroids and striae. (Source: Lelong et al., 2012)
1.2.2 Genetics of *Pseudo-nitzschia*

Before early 20th century, identification of *Pseudo-nitzschia* species was solely based on the morphological characters. However, molecular studies started to couple with the morphological characterization and become one of the important evidence to support species delineation.

The nuclear encoded of large subunit (LSU) ribosomal DNA (rDNA) phylogeny was the earliest molecular studies that used to support *Pseudo-nitzschia* cryptic species description (Lundholm et al., 2002a; Lundholm et al., 2006; Lundholm et al., 2012; Lim et al., In press). The genetic markers used subsequently increased to regions such as the internal transcribed spacers (ITS) of the nuclear encoded rDNA (Lundholm et al., 2006; Amato et al., 2007; Kaczmarska et al., 2008); the mitochondrial cytochrome c oxidase gene (Kaczmarska et al., 2008; Lundholm et al., 2012) and the plastids RUBISCO gene were used in inferring the phylogeny of *Pseudo-nitzschia* (Amato et al., 2007; Casteleyn et al., 2010; Lundholm et al., 2012). The ITS region is a powerful and most common region used to infer the phylogeny of *Pseudo-nitzschia*. Furthermore, compensatory base change (CBC) and hemi compensatory base change (HCBC) of the ITS2 secondary structure agreed with the sexual compatible theory (Amato et al., 2007; Lundholm et al., 2012). Currently, the combination of the D1 - D3 of LSU rDNA, ITS and *rbcL* gene was used to construct the phylogenetic relationship of *P. pungens* varieties and species in the *P. pseudodelicatissima* complex (Lundholm et al., 2012).

Besides molecular phylogenetic studies, several molecular detection methods were developed to assist the non-expertise organization in species identification. Whole cell fluorescence *in situ* hybridization (FISH) can be employed to detect and localize the presence and absence of a specific DNA sequences. In HABs research, the LSU rDNA and ITS region were the common target DNA region used in FISH (Scholin et al.,
1996a; Scholin et al., 1996b; Miller and Scholin, 2000). This technique has been proven as a rapid detection tool to identify and detect species in both cultured and environmental samples, in a considerable short time, low cost and need no expertise.

Automated ribosomal intergenic spacer analysis (ARISA) is one of the rapid detection methods that is adopted from the bacterial research field. ARISA is an analysis included normal PCR and fragment analysis. ARISA is used to study the temporal and spatial community structure of the environmental samples (Bailey, 2008). ITS and LSU of rDNA are always the target region to use in ARISA analysis. ARISA is also used to detect the species occurrences in environmental samples without the difficulty in isolation and establishment of cultures. In a study of Hubbard et al. (2008), they detected nine distinct fragment lengths in the environmental samples. However, the development of clone library is necessary, in order to differentiate between species and to determine genotype within the species.

Microsatellite analysis is also a common molecular tool used to determine the populations that possess cryptic species, varieties and hybrids (Adams et al., 2009; Casteleyn et al., 2009; Casteleyn et al., 2010). However, this molecular tool is based on clonal cultures because the analysis can only be performed on clones that survive in the culture establishing process. The approach has been adopted in studying the population structures of P. pungens varieties (Adams et al., 2009; Casteleyn et al., 2010). Recently the analysis was used to infer P. multisiriata dynamics over seasonal blooms, genetic structure and changes of genetic diversity across the sexually produced progenies (Tesson et al., 2011).