



Faculty of Resource Science and Technology

Morphology and Molecular Characterization of Marine Dinoflagellates (Dinophyceae)

Kon Nyuk Fong (23773)

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This project is submitted in partial fulfillment of the requirements for the degree of
Bachelor of Science with Honours
(Resource Biotechnology)

Supervisor: Dr Leaw Chui Pin
Co-supervisor: Dr Lim Po Teen

Resource Biotechnology Programme
Department Of Molecular Biology

Faculty of Resource Science and Technology
University Malaysia Sarawak

2012

DECLARATION

I hereby declare that this thesis is based on my original work except for quotation and citation, which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UNIMAS or other institutions.

Kon Nyuk Fong
Resource Biotechnology Programme
Department of Molecular Biology
Faculty of Resource Science and Technology
University Malaysia Sarawak

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LIST OF ABBREVIATIONS

CFP	Ciguatera Fish Poisoning
CTAB	Cetyltrimethylammonium Bromide
CTX	Ciguatoxin
DSP	Diarrhetic Shellfish Poisoning
ITS	Internal Transcript Spacer
LSU	Large Subunit
PCR	Polymerase Chain Reaction
PSP	Paralytic Shellfish Poisoning
SEM	Scanning Electron Microscope
SSU	Small Subunit
STX	Saxitoxin

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Morphology and Molecular Characterization of Marine Dinoflagellates (Dinophyceae)

Kon Nyuk Fong

Faculty of Resource Science and Technology
Universiti Malaysia Sarawak

ABSTRACT

There is a wide diversity of dinoflagellates inhabiting the complex nature of the marine environment. Marine dinoflagellates are potentially responsible for toxic red tides which can cause harmful effects to the surrounding ecosystem, marine life as well as human health. In this study, marine dinoflagellates were characterized based on morphology and molecular information. Field samplings were carried out at Samariang, Sarawak and clonal cultures were established. Morphological observations of cultured samples were carried out using epifluorescence microscope. The cultures established were subjected to genomic DNA isolation for molecular characterization. Gene amplification was performed to amplify the nuclear-encoded ribosomal RNA gene (rDNA). The amplicons obtained were subjected to further purification prior to DNA sequencing. In further, the nucleotide sequences obtained were analysed and used in phylogenetic reconstruction. Sequence signatures of species found were assigned for DNA barcoding. A species inventory of benthic dinoflagellates in Samariang with molecular signatures as potential DNA barcode was obtained in this study. The results of the present study showed that the Kudat isolate resembled *C. malayensis* with identical morphological features. The phylogenetic tree constructed for *Coolia* species showed that the Kudat isolate was claded together with other *C. malayensis* from Langkawi Island, Port Dickson and Sabah. In addition, three *Prorocentrum* species from Samariang and one from Kudat were identified. Their phylogenetic tree showed that they are claded into respective species clades which further supported the morphology evidence. Furthermore, one *Alexandrium* species was identified from Kuala Abai, Sabah. The cells had rhomboidal first apical plate (1'), wide sixth precingular plate (6'') and posterior sulcal plate (Sp) that was wider than longer. The phylogenetic tree constructed for *Alexandrium* species further proved that the isolate was *Alexandrium tamutum*. Therefore, the morphologies and molecular data obtained were well support each other.

Key words: Marine dinoflagellates, morphology, epifluorescence microscope, phylogenetic, DNA barcoding.

ABSTRAK

Terdapat pelbagai jenis dinoflagellates didapati mendiami persekitaran laut yang kompleks. Dinoflagellates marin berkemungkinan menyebabkan air pasang merah bertoksik yang mempunyai kesan bahaya terhadap ekosistem sekeliling, hidupan laut serta kesihatan manusia. Dalam kajian ini, dinoflagellates marin dikenal pasti berdasarkan morfologi dan maklumat molekulnya. Sampel diambil di Samariang, Sarawak dimana kultur klon telah ditubuhkan. Pemerhatian terhadap morfologi dijalankan dengan menggunakan mikroskop epifluorescence. Genomik DNA kultur klon diekstrak untuk pencirian molekul. Ampikasi gen dijalankan untuk memperbanyakkan RNA ribosome (rDNA) yang dikodkan dalam nuklear. Amplicons yang diperolehi akan ditapis sebelum penjujukan DNA. Seterusnya, jujukan nukleotida yang diperolehi perlu dianalisa dan digunakan dalam pembinaan semula filogenetik. Turutan tandatangan spesies yang diperolehi telah dijadikan DNA kod bar. Inventori spesies dinoflagellates bentik di Samariang telah diperolehi yang mempunyai tandatangan molekul berpotensi dijadikan DNA bar kod. Keputusan kajian menunjukkan kultur Kudat serupa dengan *C. malayensis* dalam ciri-ciri morfologikal. Pokok filogenetik yang dibina untuk spesies *Coolia* menunjukkan kultur Kudat yang diasingkan diletakan dalam satu kumpulan bersama dengan *C. malayensis* lain dari Pulau Langkawi, Port Dickson dan Sabah. Sebagai tambahan, tiga spesies *Prorocentrum* dari Samariang dan satu spesies dari Kudat telah dikenal pasti. Pokok filogenetik menunjukkan bahawa mereka dikumpulkan ke dalam spesies kumpulan masing-masing. Ini juga yang menyokong bukti morfologi. Tambahan pula, salah satu spesies *Alexandrium* telah dikenal pasti dari Kuala Abai, Sabah. Sel-sel itu mempunyai rhomboidal plat apikal pertama (1'), luas enam precingular plat (6'') dan plat posterior sulkus (Sp) yang luasnya lebih panjang. Pokok filogenetik yang dibina untuk spesies *Alexandrium* membuktikan lagi kultur tersebut ialah *Alexandrium tamutum*. Oleh itu, data morfologi dan molekul yang diperolehi menyokong satu sama lain.

Kata kunci: Dinoflagellates marin, morfologi, mikroskop epifluorescence, filogenetik, DNA barkod.

1. INTRODUCTION

Dinoflagellates are eukaryotic protists that exist as plankton in marine and fresh water. Marine dinoflagellates that live and swim freely in the water column are called planktonic, while other that live attached to sediment, sand, corals or macroalgal surfaces are called benthic.

Identifying harmful dinoflagellates species has become increasingly important due to the increase in red tides, fish kills and shellfish poisoning events reported from coastal marine ecosystem. Dinoflagellate ‘blooms’ can cause discoloration of the water known as red tides and cause harmful effects to the surrounding ecosystem, marine life as well as human health. If the dinoflagellate ‘blooms’ involve the toxic species, the toxins can be transferred to human via fish or shellfish consumption, resulting in fish or shellfish poisoning. Examples of poisoning syndromes are paralytic, neurotoxic, diarrhetic, amnesic shellfish poisoning and ciguatera fish poisoning (PSP, NSP, DSP, ASP and CFP) (Alpermann, 2009). The increasing nutrient concentration at coastal sites can stimulate macroalgae growth, which leads to more substrate for toxigenic dinoflagellates (Littler and Littler, 1984). For example, there is increased number of published studies on the blooms of *Ostreopsis* species since 2000.

Several species of benthic dinoflagellates produce polyether toxins (maitotoxin, ostreopsin, cooliatoxin etc.) which may cause CFP. These toxins can be sequestered in shellfish and bottom-feeding fish (Shears and Ross, 2009). Dinoflagellates in the genera *Gambierdiscus*, *Ostreopsis*, *Coolia*, *Prorocentrum* and *Amphidinium* have been reported in the ciguatera endemic areas (Ballantine et al., 1985; Carlson & Tindall, 1985; Bomber & Aikman, 1989; Bourdeau et al., 1995; Faust, 1995). The symptoms associated with ciguatera include gastrointestinal and neurological disorders and sometimes death (Vila et al., 2001).

It is interestingly to document the occurrence of planktonic or benthic dinoflagellates in Sarawak water especially those that are potentially toxic. Most of the previous studies were carried out mainly in the Peninsular Malaysia and Sabah (Leaw et al., 2001; Leaw et al., 2011), only limited study was carried out in Sarawak. The main objective of this study is to identify various marine dinoflagellates based on morphological and molecular information.

In this study, morphology of marine dinoflagellates was investigated and molecular information characterized. Samplings were carried out at Samariang, Kuching and clonal cultures of the two dinoflagellates species were established. Both established culture and samples from UNIMAS Harmful Algae Culture Collection were observed under epifluorescence microscope. Clonal cultures were further analyzed based on the nucleotide sequences of ribosomal RNA genes (rDNA). Genomic DNA was extracted, and the rDNA amplified prior to sequencing. The sequences obtained were analyzed and used for phylogenetic reconstruction.

2.0 LITERATURE REVIEW

2.1 Marine dinoflagellates

Dinoflagellates species inhabit a wide range of habitat: from pelagic to benthic, from temperate to tropical seas and from estuaries to fresh water (Faust and Gulledge, 2002).

Majority of dinoflagellates (nearly 90%) are marine planktonic or benthic forms, with highest diversity in tropical waters (Taylor, 1987).

2.1.1 Planktonic dinoflagellates

Dinoflagellates that live and swim freely in the seawater column are called planktonic dinoflagellates. They move in twisting or spiraling motion propelled by two dimorphic flagella. Their distributions are determined by temperature, salinity or depth (e.g. Lim and Ogata, 2005; Lim et al., 2006). Species identification of dinoflagellates is mainly based on their divergence in morphology and size as well as surface ornamentation (pores, areolae, spines, ridges, etc.). The shape, size and position of nucleus are also the important features for species characterization.

Dinoflagellates possess a large unique nucleus called dinokaryon which contains condensed chromosomes and lack nucleosomal histone. Dinoflagellates mainly reproduce asexually by binary fission. Some species reproduce sexually and form resting cysts. The cysts live attached to sediments for a period of time after which will germinate to cause blooms (Spector, 1984).

2.1.2 Benthic dinoflagellates

Benthic or epiphytic dinoflagellates are microscopic algae that live attached to sand particles, corals, seaweeds, and mangroves by producing mucilage. Some also have been found to be associated with brown and red algae, dead corals and sediments (Okolodkov et

al., 2007). They play an important ecological role in marine environment as primary producers and also contribute to marine foodwebs. According to Okolodkov et al. (2007), species richness in benthic dinoflagellate assemblages is usually lower than that in planktonic ones. The common genera of benthic dinoflagellates include *Prorocentrum*, *Amphidinium*, *Gambierdiscus*, *Coolia* and *Ostreopsis*. According to Leaw et al. (2001), the importance and commonly found benthic and epiphytic dinoflagellate assemblage in coral reefs and seaweed beds of Malaysia is the genus *Ostreopsis*.

Benthic dinoflagellates comprise a high number of toxic or potentially toxic species that may produce toxin associated with human poisoning. *Gambierdiscus toxicus* is known to produce neurotoxins that associated with ciguatera fish poisoning (Leaw et al., 2011). Other species such as *Prorocentrum lima*, *P. concavum*, *Ostreopsis siamensis* and *O. ovata* have been reported to involve in ciguatera fish poisoning based on distribution, toxicity to mice and the presence of a fat soluble toxic fraction (Vila et al., 2001). The genera *Ostreopsis* and *Prorocentrum* isolated from Greek coastal waters also have been reported to be toxic (Katerina et al., 2009).

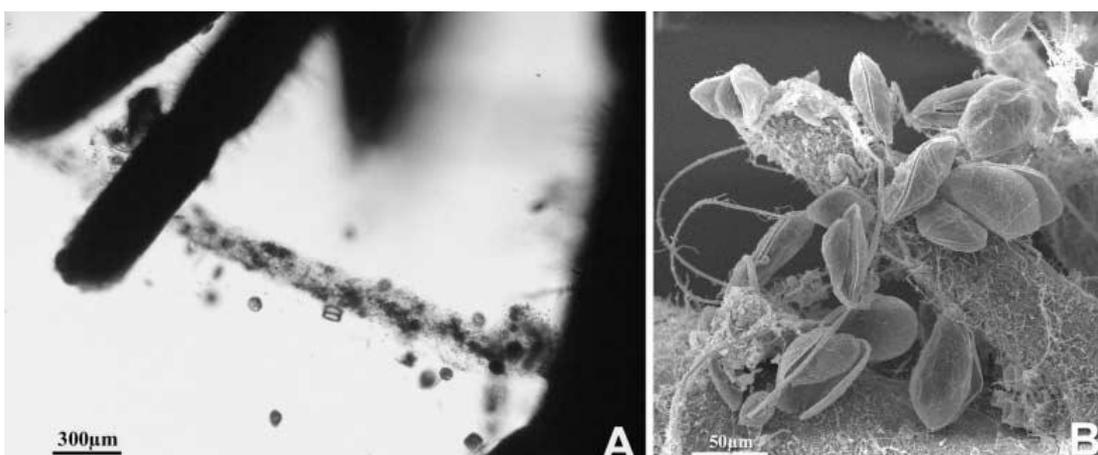


Figure 2.1: Mucilaginous matrix of epiphytic dinoflagellates on a macroalga observed under (A) light microscope and (B) scanning electron microscope (SEM) (Source: Vila et al., 2001).

2.2 Fish poisoning

2.2.1 Ciguatera fish poisoning

Benthic dinoflagellates are capable of producing bioactive compound (biotoxin) which causes human intoxication called ciguatera fish poisoning. Ciguatera is a particular type of seafood poisoning caused by eating tropical coral reef fish carrying ciguatera toxin. The best known harmful benthic dinoflagellate is *Gambierdiscus toxicus*, the primary producer of the ciguatoxins (CTX). Other toxigenic benthic dinoflagellates have been found to coexist with *Gambierdiscus* include *Amphidinium carterae*, *Coolia monotis*, *Prorocentrum concavum*, *P. mexicanum*, *P. lima*, *Ostreopsis lenticularis*, *O. siamensis*, *O. ovata*, and *O. heptagona* (Steidinger, 1993).

The mechanism for the transfer of toxin from benthic dinoflagellates into consumers is via herbivory. The herbivorous fish grazes on macrophytic algae that contain potentially high diversity of microalgae particularly benthic dinoflagellates. The toxins produced by the microalgae will accumulate in fish, larger fish and finally cause food poisoning in people eating them. The transfer of toxins into the benthic food web could increase if there is massive growth of toxic, benthic dinoflagellates. Consumption of ciguatoxic herbivorous and carnivorous fish will cause intoxication 1 to 12 hours after ingestions, including sensory disturbances such as paresthesias, arthralgia diarrhea and chills (Swift and Swift, 1993).

Early work in the Gambier Islands of French Polynesia has discovered that the guts of ciguatoxic (herbivorous) fish contained significant numbers of dinoflagellates that are known as *Gambierdiscus toxicus* (Adachi and Fukuyo, 1979). Numerous CTX detection methods were then introduced to study the etiology of CFP. The researchers named Legrand et al. (1998) has chemically characterized the ciguatoxins from a variety of fish species.

2.2.2 Paralytic shellfish poisoning

Some *Alexandrium* species are responsible for paralytic shellfish poisoning (PSP) by producing PSP toxins. The toxic substance is named saxitoxin (STX) which blocks the voltage gated sodium channels of nervous cells and inhibit nerve conduction which may lead to paralysis of the neuromuscular system in humans (Doucette et al., 1997). The symptoms include tickling sensation of lips, numbness of extremities, gastrointestinal problems and difficulty in breathing as summarized in Shimizu (2000). PSP occurs through the consumption of contaminated shellfish such as mussels, clams, oyster, scallop or other filter feeders.

The *Alexandrium* species which have the ability to produce STX are *A. caternella*, *A. tamiyavanichi*, *A. minutum*, and *A. tamarensis*. In Malaysia, the two major PSP events have been reported in Sebatu, Malacca (1991) and Tumpat, Kelantan (2001).

2.2.3 Diarrhetic shellfish poisoning

The first dinoflagellate to be implicated was *Dinophysis fortii* (in Japan), followed by *D. acuminata* (in Europe), *D. acuta*, *D. norvegica* (in Scandinavia), *D. mitra*, *D. rotundata* and the benthic dinoflagellate *Prorocentrum lima* (Hallegraeff, n.d.). The clinical symptoms of diarrhetic shellfish poisoning (DSP) may often be misleading for those caused by bacteria gastric infection. Unlike PSP, no human fatalities have ever been reported and patients usually recover within three days. In some severe cases, some of the polyether toxins (okadaic acid) may cause stomach tumour and produce chronic problem.

2.3 Techniques in morphological observations

The morphology of benthic species can be examined with scanning electron microscope (SEM) and epi-fluorescence microscope. Morphological identification of dinoflagellate species is based on wide divergence in morphology and size, as well as surface ornamentation (pores, areolae, spines, ridges etc.). Different dinoflagellate species show different flagella arrangement. If the paired flagella arise from the side, one is beating sideways around the cell, the other beating backwards. This arrangement is called dikont condition (Taylor, 1987). Desmokont type has two dissimilar flagella inserted apically (e.g. *Prorocentrum*) while dinokont type has two dissimilar flagella inserted ventrally (e.g. *Alexandrium*) (Faust and Gullede, 2002). In dinokont, the transverse flagellum lies within the cingulum while longitudinal flagellum lies in the sulcus and projecting posteriorly (Taylor, 1987).

Plate designation systems have been proposed to describe individual thecal plate. In Kofoidian system, the plates immediately above the cingulum are precingulars. Any plates occur between the apical and precingulars are designated as anterior intercalaries. On the other hand, the plates immediately below the cingulum, except those in the sulcus, are the postcingulars. Any plates in between the antapical pole and postcingular, other than those in sulcus, are termed posterior intercalaries (Taylor, 1987). Besides that, Kofoid's system also used the combination of superscript notation and the number of plates to produce a plate formula: apicals ('), anterior intercalaries (a), precingulars ("), postcingulars (""), posterior intercalaries (p), and antapicals (""") (Taylor, 1987). For example, Po, 4', 3a, 7", 3c, 6s, 5"", 2"" in the genus *Protoperidinium*. Query is used when the number is unknown.

2.4 Molecular Characterization

2.4.1 Ribosomal RNA genes

Ribosomal RNA is a non-coding ribonucleic acid that is not translated into a protein, but functions to decode messenger RNA (mRNA) into amino acids and to interact with transfer RNA (tRNA) during translation. Besides the enzymatic role in protein synthesis, ribosomal RNA has important applications in medicine and in evolutionary biology. In evolutionary biology, ribosomal RNA is considered the most conserved or the least variable gene in cells (Smit et al., 2007), while the proteins in ribosomes are more variable. Therefore, genes encoding rRNA (rDNA) are sequenced to identify an organism's taxonomic group, estimate related groups, and determine rates of species divergence (Ribosomal RNA, 2008). rRNA genes occur as multiple tandem repeats in the nuclear DNA precursor. Each repeat comprises of three coding regions, a single rRNA precursor, which is then cleaved to produce mature small subunit, 18S rRNA (SSU), mature 5.8S rRNA and the mature large subunit, 28S rRNA (LSU).

Most molecular phylogenetic studies of the in-group relationships of dinoflagellates have used rRNA, either partial sequences of the large-subunit rRNA gene (Lenaers et al., 1991; Zardoya et al., 1995) or the small-subunit rRNA gene (Gunderson et al., 1999; Saldarriaga et al., 2001). The study mostly targets the variation within the small and large subunit ribosomal RNA genes to identify the differential transcription and expression of genes throughout the life-cycle of dinoflagellates. This task will involve PCR which amplified the isolated DNA followed by sequencing of the PCR products to analyze any variation. This variation is important for phylogenetic analyses to determine its evolutionary history.

2.4.2 Phylogenetic reconstruction

Phylogenetic reconstruction is a way to restructure evolutionary history and involve the problem of classification. With the understanding of hereditary materials and molecular approach, it is assumed that molecular record of phylogeny is better to solve the problem of evolution and taxonomy than the one explained by morphology. Sequencing the genes of small subunit ribosomal RNA (rRNA) and partial large subunit rRNA can produce phylogenetic trees of the dinoflagellates containing many taxa. Maximum likelihood, maximum parsimony, and Bayesian methods are used to infer phylogenies from these genes.

Phylogenetic trees allow visually presentation of evolutionary relationship among a group of organisms. Node represents a taxonomic unit (species, populations, individuals). It can be an existing species or an ancestor. Clade is a group of two or more taxa or DNA sequences that have common ancestor and include all of their descendents (Systematic and molecular phylogenetics, 2004). On the other hand, branch defines the relationship between the taxa in term of descents and ancestry. The branch length indicates the number of changes that have occurred in the branch. Phylogenetic tree may be either rooted or unrooted. The root represents a common ancestor of all taxa. An unrooted tree only shows the relationship among species without identifying a common ancestor (Systematic and molecular phylogenetics, 2004).

Early phylogenetic studies showed the monophyly of dinoflagellates and disprove ideas that dinoflagellates are early branches of eukaryote tree (Dodge, 1965, 1966). In 1991, a new taxon, Alveolata was formed, encompassing ciliates, dinoflagellates, apicomplexans and their close relatives, protalveolates (Cavalier-Smith, 1991). Dinoflagellates are more closely related to the apicomplexans than to the ciliates (Fast et al., 2002).

2.4.3 DNA barcoding

There are a few limitations in morphology-based identification systems such as phenotypic plasticity and overlook cryptic morphological characters (Hebert et al., 2003). Besides, some morphological keys are often visible only for a particular life stage or gender, therefore many individuals cannot be identified (Hebert et al., 2003). These limitations propose that there is a need for new species recognition approach. Microgenomic identification systems allow discrimination of biological species through the analysis of genome sequences.

DNA barcode is widely used as molecular-based identification system that aims to identify biological species, and to group them to a given species (Casiraghi et al., 2010). It is based on the idea that the variability in the standard molecular marker(s) can be used to discriminate biological specimens (Casiraghi et al., 2010). When compared to a reference DNA sequences, any unique sequences that represent that specific species will be viewed as genetic barcodes. DNA barcoding can be used for molecular identification of already described species or for discovery of undescribed species (Casiraghi et al., 2010).

3.0 MATERIALS AND METHODS

3.1 Sample collection

Plankton samples were collected from Samariang, Sarawak (Figure 3.1) using a 20 µm-mesh plankton net. Sampling was carried out during high tide with salinity of ~30 psu. Field *in situ* data i.e. temperature, salinity and pH of the seawater was collected. Live samples were kept in 1 L bottles in ice pack and brought back to the laboratory for cell isolation. A portion of samples (1/3) were preserved in acidic Lugol's solution.

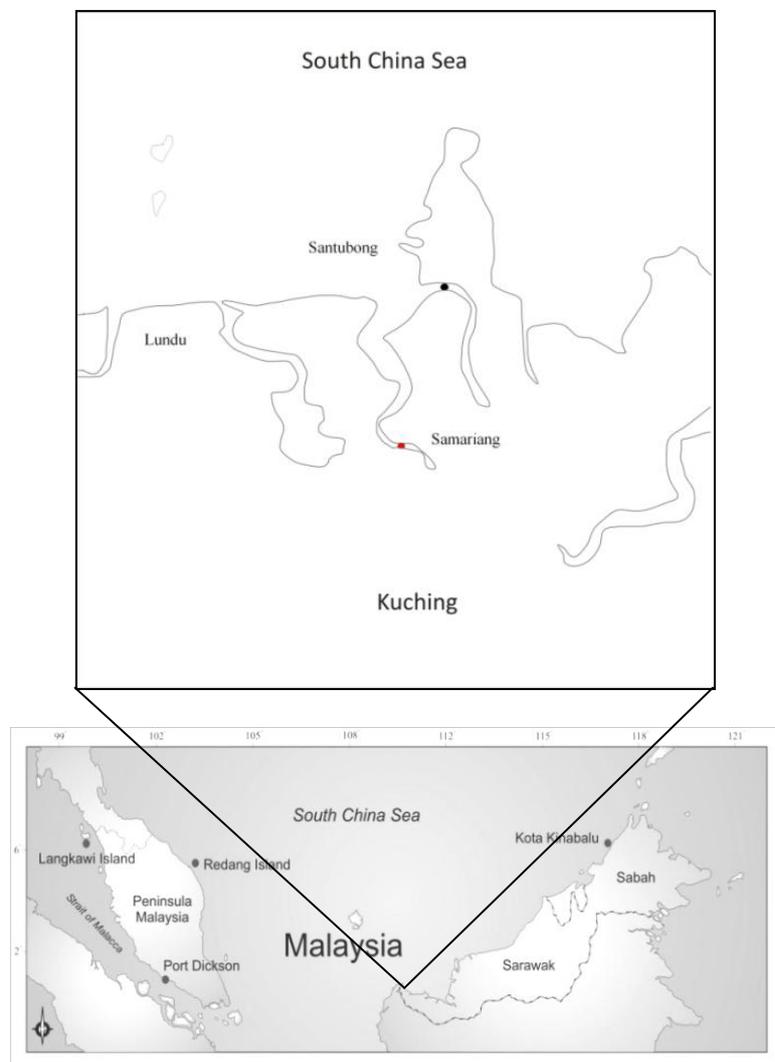


Figure 3.1: Map showing sampling location at Samariang, Kuching, Sarawak.

3.2 Cell isolation and cultures

Samples were sieved through a 20 µm mesh sieve and back-washed to a petri dish for cell isolation by micropipetting technique under an inverted light microscope (Olympus IX51). Clonal cultures were established in a 96-well format microtiter plate containing filtered-sterile seawater. Cultures established were grown in ES-DK medium (Kokinos and Anderson, 1995) at 25°C under a 12:12 h light:dark photoperiod in a Shelab temperature-light controlled growth chamber (Shelab, USA).

3.3 Species identification

Morphological observation was performed with epi-fluorescence microscopy. Identification of the species was based on overall cell shape and theca plate tabulation. For epi-fluorescence microscopy, fixed samples were stained with 1% Calcofluor white solution (Fluka, Japan), and nucleus was stained with SYTOX Green (Invitrogen, USA). Stained samples were viewed under an Olympus IX51 epifluorescence microscope (Olympus, Melville, USA) with UV filter sets. Images were captured with a cooled CCD camera (SIS Colorview F12, Germany).

3.4 DNA extraction, PCR amplification and rDNA sequencing

Cultures were harvested at mid-exponential growth phase by centrifugation at 2,800 rpm for 10 min. The cell pellet was then suspended in distilled water, followed by cetyltrimethylammonium bromide (CTAB) extraction. Cells were lysed by adding equal volume of 2× CTAB lysis buffer containing 50 mM CTAB, 14 mM NaCl, 10 mM Tris-base, 20 mM EDTA, and 1% mercaptoethanol. The mixture was re-extracted with chloroform: isoamyl alcohol (C:I, 24:1) and subsequently phenol:chloroform:isoamyl alcohol (P:C:I, 25:24:1). Two vol. of absolute ethanol and 1/10 vol. of 3M sodium acetate

were added to precipitate the DNA, followed by centrifugation at 13000 rpm for 10 min. DNA pellet was rinsed with 70% cold ethanol and then dissolved in 30 μ l TE buffer (10 mM Tris-HCl, pH 7.4 and 1 mM EDTA, pH 8.0). Genomic DNA samples were kept overnight at 4°C to totally dissolve the DNA before stored at -20°C for further analysis.

The D1/D3 region of nuclear LSU (28S) rDNA gene and the internal transcribed spacer region (ITS1-5.8S-ITS2) were amplified by polymerase chain reaction (PCR) with primer pairs D1R and D3Ca (Scholin et al., 1993) and ITS1F and ITS1R (Leaw et al., 2001), respectively (Table 3.1). The amplification condition is as stated in Appendix A.

Table 3.1: List of primer for PCR amplification of LSU rDNA

Primer	Sequence (5'-3')	Direction	Target region	Reference
D1R	ACCCGCTGAATTTAAGCATA	Forward	D1-D3 LSU	Scholin et al., 1993
D3Ca	CTTGGTCCGTGTTTCAAGA	Reverse	D1-D3 LSU	Scholin et al., 1993
ITS1F	TCGTAACAAGGTTTCCGTAGGTG	Forward	ITS	Leaw et al., 2001
ITS1R	ATATGCTTAAGTTCAGCGGG	Reverse	ITS	Leaw et al., 2001

PCR products were subjected to further purification prior to DNA sequencing by First Base private sequencing laboratory, using an ABI 377 automated sequencer (PE Applied Biosystems, Foster City, CA, USA). Sequencing was performed in both strands.

3.5 Sequence analysis and taxon sampling

Sequences were evaluated by using the Basic Local Alignment Search Tool (BLAST) program (Altschul et al., 1990) and analyzed by ABI sequence Scanner ver. 1.0 (ABI Biosystem, USA). Ambiguous bases were determined by comparing both forward and reverse strands of each strain and among sequences from the strains. The reverse strands were reversed complemented by using BioEdit version 6.0.7 (Hall, 1999) and pairwise-

aligned with the forward strands using Clustal-X (Thompson et al., 1997). These were followed by assignment according to the IUPAC nucleotide code.

Taxon sampling was achieved by blasting in the nucleotide database, GenBank in conjunction with the National Center of Biotechnology Institute (NCBI) to acquire related sequences. The selected sequences were saved as FASTA format for advance analysis.

3.6 Phylogenetic analyses

Phylogenetic analyses was carried out by using Phylogenetic Analysis Using Parsimony (PAUP) ver. 4.0b10 (Swofford, 2000) with maximum likelihood (ML) and maximum parsimony (MP) algorithms used to estimate the phylogeny.

For ML analysis, Modeltest ver. 3.06 (Posada and Crandall 1998; 2001) with α value of 0.01 was used to identify the hierarchical likelihood ratio so that the best model of evolution can be determined. The evolutionary model selected for the sequences data set and ML parameters was used to reconstruct the ML tree using PAUP. The sequences divergences were calculated by heuristic search with tree bisection-reconnection (TBR) branch swapping and 10 random additions.

Maximum parsimony (MP) was performed by heuristic search of 1000 random addition and TBR branch swapping. Bootstrapping (Felsenstein, 1985) was performed on both ML and MP analyses with 1000 replication ($P < 0.001$) to determine the confidence limits of tree topology using TBR branch swapping algorithm.