Morphological and Molecular Characterization of Potential Toxic Benthic Dinoflagellate, *Gambierdiscus belizeanus* (Dinophyceae) from Malaysia

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(20188)

This project is submitted in partial fulfilment of the requirement of the degree of
Bachelor of Science with Honours
(Resource Biotechnology)

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Resource Biotechnology Programme
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Faculty of Resource Science and Technology
Universiti Malaysia Sarawak
2010

ACKNOWLEDGEMENTS

Thanks to God that I am able to finish this final year report smoothly. This project would not have been possible or successful without the valuable contributions and help from my lovely supervisor, Dr Leaw Chui Pin. I am heartily thankful to her whose give a fully encouragement, guidance and support from the initial to the final levels which enable me to develop the understanding of this project. Also I would like to thank Dr Lim Po Teen as my co-supervisor for the help and inspiration that he extended. My deepest appreciation goes to the seniors in the Exotoxicology Laboratory and IBEC Molecular Laboratory who assisted me in handling the apparatus and machines.

My deepest gratitude is due to my parents for their understanding with my altitude and their financial and emotional supports. Lastly, I offer my regards and blessings to all of those who support me in any during the completion of this final year project.

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ABSTRACT

Species in the genus *Gambierdiscus,* particularly *Gambierdiscus toxicus* have been known to produce a potent neurotoxin, ciguatoxin that cause ciguatera fish poisoning (CFP), the most common illness that associated with fish consumption worldwide. The dinoflagellate is commonly found in the coral reefs of ciguatera endemic area. In this study, a detailed morphological investigation by using Scanning Electron Microscopy (SEM) was conducted to characterize the *Gambierdiscus* species found in the Malaysian waters. A strain of *Gambierdiscus* sp. from Kota Kinabalu, Sabah was cultured and maintained in SWII medium at 26^{o}C under 12:12 hr light:dark photocycle. The SEM observation of the strain showed that the cells are areolated, ellipsoid in apical view and compressed anterioposteriorly. The plate formula observed was Po, 3´, 7´´, 5´´´, 1p and 2´´´´. Dimensions of the cells were 56.7 µm to 59.4 µm in depth and 57.8 µm to 64.4 µm in width. Detailed examination of important morphological features used to distinguish *Gambierdiscus* morphospecies such as thecal surface morphology, shape of apical pore plate (Po) and 1p plate as well as the morphometric information obtained in this study revealed similar morphological characteristics of *G. belizeanus*. Thus, the strain was identified as *G. belizeanus*. This represents the first report on the occurrence of *G. belizeanus* in the Pacific region.

Keywords*:* ciguatoxin; Ciguatera Fish Poisoning; dinoflagellates; *Gambierdiscus*; morphospecies; *Gambierdiscus belizeanus*;

ABSTRAK

Spesies dalam genus *Gambierdiscus,* terutamanya *Gambierdiscus toxicus* telah diketahui menghasilkan neurotoksin, ciguatoksin yang menyebabkan keracunan ikan siguatera (CFP), keracuanan yang paling biasa dikaitkan dengan pemakanan ikan sedunia. Dinoflagelat ini biasanya ditemui di terumbu karang di kawasan endemik siguatera. Dalam kajian ini, kajian mofologikal terperinci dengan menggunakan mikroskopi elektron imbasan (SEM) telah dijalankan untuk pencirian morfologi spesies *Gambierdiscus* yang terdapat di perairan Malaysia. Satu strain *Gambierdiscus* sp. dari Kota Kinabalu, Sabah telah dikultur dalam media SWII pada 26°C di bawah kitaran cahaya gelap 12:12 jam. Pemerhatian sampel melalui SEM menunjukkan sel-sel mempunyai permukaan yang areolated, elipsoid dalam pandangan apeks dan mampat secara anterioposterior. Formula plat diperhatikan ialah Po, 3´, 7´´, 5´´´, 1p dan 2´´´´. Dimensi sel-sel ialah 56.7-59.4 µm panjang dan 57.8-64.4 µm lebar. Pemeriksaan terperinci ciri-ciri mofologi penting yang digunakan untuk membezakan morfospesies *Gambierdiscus* seperti morfologi permukaan thecal, bentuk apeks liang plat (Po) dan plat 1p serta maklumat morphometric yang diperolehi dalam kajian ini menunjukkan sel dari Kota Kinabalu ini menunjukkan ciri-ciri morfologi yang serupa dengan *G. belizeanus*. Oleh yang demikian, strain ini telah dikenalpasti sebagai *G. belizeanus*. Ini mewakili laporan pertama kewujudan *G. belizeanus* di rantau Pasifik ini.

Katakunci: ciguatoxin; keracunan ikan siguatera; dinoflagellates; *Gambierdiscus*; morfospesies; *Gambierdiscus belizeanus*.

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LIST OF ABBREVIATIONS

CFP	Ciguatera Fish Poisoning
DNA	Deoxyribonucleic acid
D1	Domain 1
D2	Domain 2
EtOH	Ethanol
HABs	Harmful Algal Blooms
LM	Light Microscopy
LSU	Large Subunit
PCR	Polymerase Chain Reaction
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
SEM	Scanning Electron Microscopy
SSU	Small Subunit
TBE	Tris-borate-EDTA
UV	Ultraviolet
OsO_4	Osmium tetroxide

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1.0 INTRODUCTION

Algae are important component in the primary productivities of marine ecosystems. Most of them are not harmful to others organisms. However, there are some species that produced potent toxins that can harm the sea animals and humans. People who come in contact with these toxic algae whether by consuming the shellfish contaminated by the algal-origin toxins or swimming in areas infested with harmful algae, he or she may experience neurological symptoms such as tingling fingers and toes as well as respiratory and gastrointestinal symptoms. Certain species of benthic dinoflagellates are known to produce potent neurotoxins that may involve in ciguatera fish poisoning (CFP). They are several types of benthic dinoflagellates such as *Gambierdiscus, Ostreopsis, Coolia, Prorocentrum* and *Amphidinium.*

Ciguatera fish poisoning (CFP) is a human illness that cause by eating finfish that is contaminated by toxins produced by the marine algae, *Gambierdiscus toxicus*. People who have ciguatera may experience nausea, vomiting and neurologic symptoms such as tingling fingers or toes. Ciguatera fish poisoning has no cure. This symptom basically goes in days or weeks but can be last for a year. This study is important because fish is one the important source of proteins in Malaysia. People will always looking for fish as their source of protein in their daily life.

In this study the morphological and molecular characteristics of potentially toxic benthic dinoflagellate, *Gambierdiscus* spp. found in Malaysian waters were investigated. A strain of *Gambierdiscus* from Sabah was cultured and the detailed morphological features of this strain were examined by using advanced scanning electron microscopy (SEM). In addition to that, molecular characterization of the strain was also conducted by amplification of the nuclear-encoded large subunit ribosomal RNA gene in the region of domain 1 and 2 to infer the phylogenetic relationships of *Gambierdiscus.*

2.0 LITERATURE REVIEW

2.1 Harmful Algal Blooms (HABs)

Algal blooms can be occurred in freshwater and also in marine environment. An algal bloom is a drastic elevation of the algal density of in the aquatic system where the clear water turn turbid, colored and covered with accumulation of floating masses at the surfaces. Blooms can be recognized with the appearance of water discoloration result of the high density of pigmented cells. Although there is no officially recognized threshold level, algae can be considered bloom with cells density range changed from hundreds to thousands of cells per milliliter. Besides, algal bloom concentration may reach millions of cells per milliliter.

Harmful algal bloom (HAB) is a dense aggregation of harmful phytoplankton, algae or cyanobacteria in an aquatic environment. HABs may cause negative impacts to others organisms such as mammals via the production of natural toxins, mechanical damage or by other means. In addition, an algal bloom can kill fish and others aquatic life due to hypoxia/anoxic conditions in the water. Moreover, HABs will produces potent toxins where can be associated with specific, harmful consequences of such blooms in regions where one type is common with another, for example fish kills in Florida, fish and shellfish kills in Japan and paralytic fish poisoning (PSP) of humans on the Altlantic and Pacific coasts of North America (Loeblich et al., 1986). There are more than 5,000

species of marine phytoplankton that exist in worldwide, and 2% are known to be harmful or toxic to environment and organisms (Richlen et al., 2008)

2.2 Characteristic of Dinoflagellates

Dinoflagellates are one of the groups of predominantly unicellular, eukaryotic, flagellated organisms that posses of photosynthetic and non photosynthetic members. Both of photosynthetic and non photosynthetic members are able to swim because they have many cell walls and both botanists and zoologists have been claim that they are algae and protozoa and both have produced classification schemes for dinoflagellates.

Other features that are common to dinoflagellates is that the presence of chlorophyll a, c_2 and c_1 (usually absent), the production of starch and oils as reserves, mitochondrial cristae with cellular cross-sections and a triple-membraned enveloped which surrounding chloroplast. There are others unique features of dinoflagellates such as rod-like, ejectile bodies where usually occurred in several phytoflagellates groups (Taylor, 1987).

The habitats in which dinoflagellates may be found are also very varied. Almost 90% of them are marine planktonic or benthic form, with the greatest diversity in tropical waters. Besides, dinoflagellates also can be found in polar waters, in sea ice and even in snow. Others groups such as diatoms or green algae are more successful in cold environment. The photosynthetic members are restricted to illuminated water because many of them can survive under very dim light conditions, whereas the heterotrophs can extend into non-illuminated depths and both in water and in sediments.

There are four genera of thecated species that is found in the warm tropical waters such as *Gambierdiscus, Ostreopsis, Coolia* and *Prorocentrum* (Adachi and Fukuyo., 1979; Fukuyo., 1981; Besada et al., 1982). Those thecated species belong to the toxic

dinoflagellates which are distributed world-wide in marine waters. Approximately 50 dinoflagellates species are known to produce toxin that will cause the death of marine animals and birds, toxicity in bivalves and seafood poisoning in humans (Steidinger., 1993).

2.3 Ciguatera Fish Poisoning (CFP)

Ciguatera fish poisoning (CFP) is a food borne illness that effect humans worldwide. CFP is caused by benthic microalgae species commonly known as *Gambierdiscus toxicus*. Humans get this illness by eating reef fishes which contained toxins from *G. toxicus* which is also known as ciguatoxins (CTXs).

CTXs are lipid soluble polyether compounds which consist of 13 to 14 rings fused by ether linkages into a most rigid ledder-like structure. Ciguatera is attributed to a lipid-soluble toxin produced by several genera of benthic-associated dinoflagellates, including those of *Ostreopsis* spp., *Prorocentrum* spp., and *Gambierdiscus* spp., especially *G. toxicus* (Bagnis and Yasumoto, 1977).

Ciguatoxins are relatively heat stable molecules that remain toxic although after cooking and exposed to the mild acidic and basic conditions. Ciguatoxins producing microalgae grow in association with macroalgae in coral reefs. The toxin is then transferred through the food web (Figure 2.1). The algae are then consumed by herbivorous fish, which are then consumed by carnivorous fish and last will be consumed by humans with each step concentrating the toxins.

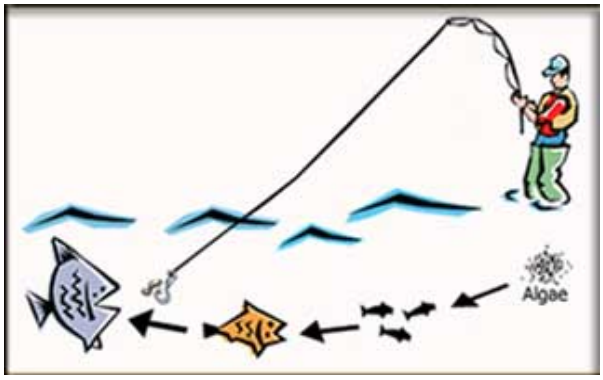

Figure 2.1: The food web that transfers the toxin to human.

Symptoms of CFP are gastrointestinal, neurological and cardiovascular symptoms. Basically, the gastrointestinal symptoms occurred first such as vomiting, diarrhea and abdominal pain. It is followed by neurological dysfunction including dizziness, anxiety, sweating, reversal of temperature sensation and a numbness. Paralysis and death have been documented, but symptoms are usually less severe although debilitating (Miller, 1991). There is no antidote but rapid treatment within 24 hours is reported to relieve some symptoms. However, the recovery time is different between individuals. It may take weeks, months or even years. The prevention of intoxication is depend upon complete abstinence from eating any tropical reef fish due to no practical way to routinely measure ciguatoxins in any seafood product prior to consumptions.

2.4 Taxonomy of *Gambierdiscus* species: from morphology to phylogeny

The Benthic Dinoflagellate, *Gambierdiscus toxicus* is the causative agent of ciguatera which was found to produce and transmit ciguatoxin and maitotoxin to herbivorous fish (Yasumoto et. al., 1979). The species was originally described from material collected from the Gambier Islands in the Pacific Ocean (Adachi and Fuyuko, 1979). For the first time, *G. toxicus* was thought to be the only species of the genus *Gambierdiscus* until the recent description of *Gambierdiscus belizeanus* by Faust 1995. Unfortunately, the production of toxins or other secondary metabolites of *G. belizeanus* has not been reported because *G. belizeanus* was found in a sand dwelling community of benthic dinoflagellates (Faust, 1995).

In the coral reef areas, it was found that benthic dinoflagellates were richer in both numbers and species on epi-benthic layers than in the water. If the toxic metabolites of this benthic species are taken up by herbivorous fish, the toxin will be contributed to the manifestation of the complex symptoms of ciguatera. The actual occurrence of minor toxins in the viscera of herbivorous fish has been confirmed and the toxigenicity of several benthic species has also been demonstrated before (Yasumoto et. al., 1976: Nakajima et. al., 1981).

The morphological characters are the primary means in describing dinoflagellate species. Dinoflagellates morphological characters such as the shape and size of the apical pore plate (Po) and posterior intercalary plate provide a very useful and complex characteristic for species recognition. Species in the genus *Gambierdiscus* are anterio-posteriorly compressed in the ventral view and theca plate tabulations are observed in the apical or antapical view (Figure 2.2). The epitheca and hypotheca are not noticeably

different in size. A distinguishing feature is the shape and size of the apical pore complex (Faust, 1992). The cell surface is smooth with numerous deep and dense pores. Thecal plates are very thick. The thecate formula of the *Gambierdiscus* spp. is Po, 3′, 7′′, sulcus plate, 5′′′ and 2′′′′.

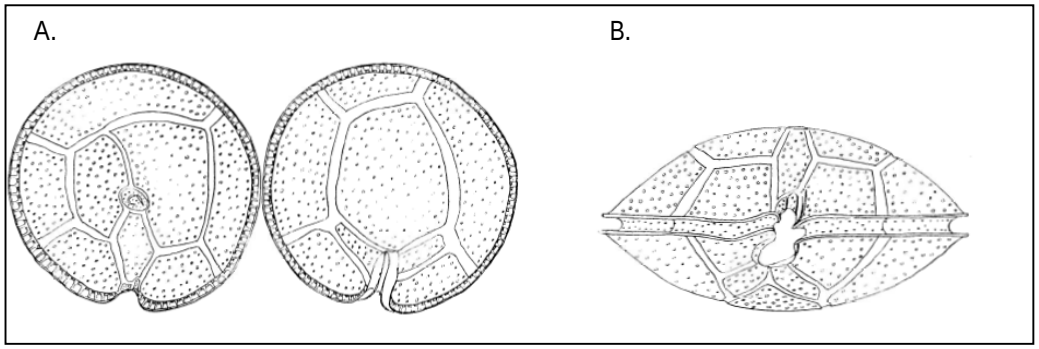

Figure 2.2: Ink drawing of *Gambierdiscus toxicus.* A. Theca plate tabulations, B. ventral view (Adachi and Fukuyo, 1979).

The sulcus of the *Gambierdiscus* spp. is very short and most parts except posterior plate are deeply concave like a hollow. The sulcus is composed of eight plates. They are the sulcal right anterior plate (S.a.r), the sulcal left anterior plate (S.a.l), the sulcal right (S.r), the sulcal left (S.l), the transitional (t) plate, the sulcal posterior plate (S.p), the sulcal medium anterior plate (S.m.a) and the sulcal medium posterior plate (S.m.p) (Adachi and Fukuyo., 1978). The S.p plate comprises of five sided and relatively large. Most of this part is out of the sulcal hollow. It develops to the right along the postcingular 6′′′. The S.r plate fits to the concave of the 6′′′, which is constitutes the right posterior wall of the sulcus. The upper half of the S.r plate is ridged in the same manner of postcingulars. In addition, the transitional (t) plate is boat-shaped, where it is located at the extension of the cingulum and enters deeply into the sulcus. The left posterior wall of the sulcus is occupied by S.l plate which is located between the S.m.p plate and posterior

intercalary plate. The round S.m.a plate is located at the bottom and its anterior margin contacts with the S.a.r plate and S.a.l. plate. They are subrectangular and situate between the precingular 1´´ and the S.m.a plate (Figure 2.3).

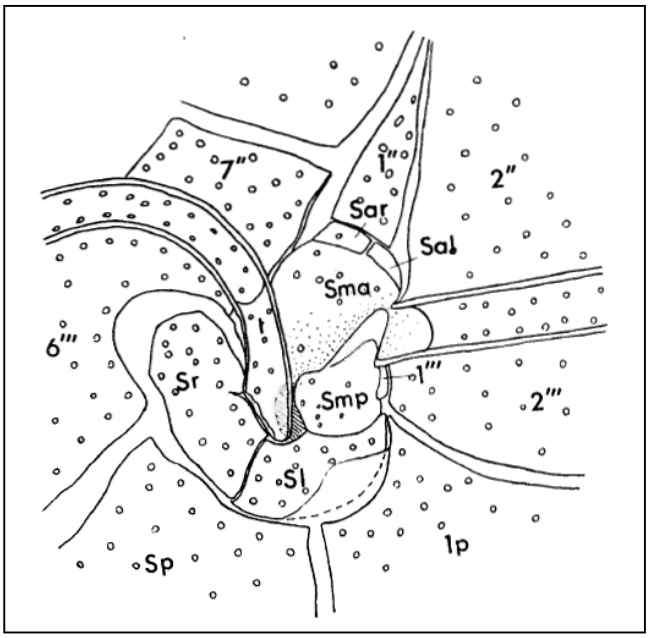

Figure 2.3: Line drawing of *Gambierdiscus toxicus*. The ventral view of sulcal plate pattern (Adachi and Fukuyo, 1978).

In the recent studies morphological approach tends to combine with molecular analysis. For example, in the genus of *Alexandrium*, the morphological features used to classify the species level of the genus showed ambiguity in their taxonomy and molecular approach has been introduced to solve the cryptic species of the genus (Scholin and Anderson, 1994). The molecular techniques involving DNA analyses have provided a rich set of data for various dinoflagellate species (Chinain et. al., 1997). In addition, the morphological approach gives particular emphasis to phenotypic characters, consistently cited the advantage of DNA analyses is that they measure changes in the genome rather than the phenotype (Chinain et. al., 1999). Nucleic acid sequencing has been proven to be

a very useful tool in systematic and phylogenetic studies in many dinoflagellate species (Scholin and Anderson, 1994).

Studies of *Gambierdiscus* clones based on comparisons of small subunit (SSU) and large subunit (LSU) rRNA genes (Figure 2.4). Based on previous study, Babinchak et al. (1994) directly examined the genetic diversity by using restriction fragment length polymorphism (RFLP) of the LSU ribosomal DNA gene, which showed that substantial genetic variability does indeed exist among distributed strains, while RFLP of the SSU rDNA recovered a different ribotypes within populations of *G. toxicus*. A comprehensive examination of genetic variation among globally distributed strains using DNA sequencing is still lacking. Furthermore, the phylogenetic of the *Gambierdiscus* morphospecies are not fully understood.

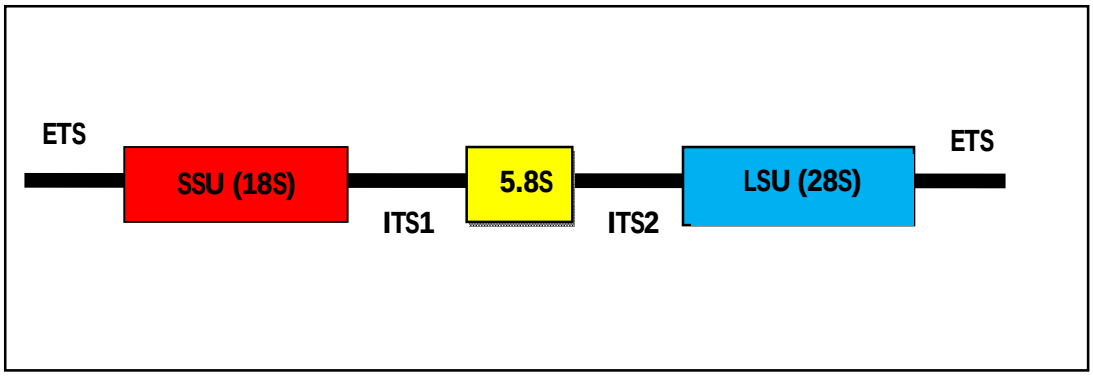

Figure 2.4: A single repeat of ribosomal RNA genes precursor showing the positions of 5.8S rDNA, the large subunit (LSU) and small subunit (SSU) of rRNA genes, the internal transcript spacer (ITS1 and ITS2) and external transcript spacer (ETS).

Combination of morphological and molecular analyses accounts for both phenotypic and genotypic characters will provide a robust taxonomic classification of the genus. Furthermore, detailed examination of the morphological variability within a phylogenetic framework could distinguish phenotypically plastid characters and between ancestral and derived characters used in morphological classifications. Therefore combined morphology-molecular approach provides a reliable phylogenetic characterization of this important species.

3.0 MATERIALS AND METHODS

3.1 Algal Cultures

3.1.1 Preparation of Culture Vessels

Proper care need to be employed when cleaning test tube to remove carbonate deposits and others materials that attached to the test tubes. Test tubes were soaked in 10% dilute acid hydrochloride (HCl) for overnight to remove diluted carbonate deposits, followed by washing in phosphate free detergent and scrubbed with a brush to dislodged solid algal remains. Test tubes were rinsed with distilled water several times to avoid residual amounts of detergent that will affect the growth of culture.

3.1.2 Sea Water Medium (SWII)

Natural sea water was used as a medium base with a salinity of 30 psu. One liter of natural sea water was added in the beaker. Salinity of natural sea water was checked by using reflectometer. Salinity of seawater will be adjusted to 30 psu by adding natural salt or distilled water. Medium stock solutions were added into the beaker that contained natural sea water. The stock solutions were listed as below:

Table 3.1: Stock Solution used in preparing SW II medium

Stock Solution	Volume	Final Concentration
KNO_3 [7.2×10^{-1} mol/L]	1 mL	7.2×10^{-4} mol/L
KH_2PO_4 [3.31×10^{-2} mol/L]	1mL	3.31×10^{-5} mol/L
Na_2-glycero.PO_4 [3.33×10^{-2} mol/L]	1mL	3.33×10^{-5} mol/L
Vitamin mix:	1mL	
• P vitamin B_{12}(cyanocobalamin)		4.43×10^{-10} mol/L
• P Biotin		4.1×10^{-9} mol/L
• P Thiamine-HCl		3×10^{-7} mol/L
Fe-EDTA	1mL	1.19×10^{-6} mol/L
Tris-HCl (pH 7.8)	5mL	4.13×10^{-3} mol/L

Sea water and medium stock solutions were filtered through a 0.22 µm membrane filter. The pH of the medium was adjusted to pH 7.8-8.0. Sterilization of the medium was accomplished by autoclaving at 121°C for 30 minutes. After autoclaved, the media were cool down t room temperature for about 24 hours to allow gasses diffused into the liquid. Then, 20mL of the medium was transferred into each test tube.

3.1.3 Algal Clonal Culture

One strain of *Gambierdiscus* sp. (GdSA03) from Kota Kinabalu, Sabah was obtained from the harmful algal culture collection in Ecotoxicology laboratory of UNIMAS. About 10mL of the strain was poured into new test tubes. These works were carried out in the laminar flow hood to avoid contamination. A Bunsen burner and 70% EtOH (for surface

cleaning) were needed on bench top to avoid cultures contaminated. Flames used on culture tube and on lips of media tubes before transferred into the test tubes.

Cultures were then kept in the culture box which the temperature and day length were controlled. Clonal cultures establish were routinely grown in SW II medium (Iwasaki, 1979) at 26°C under 12:12 hour light: dark cycle.

3.2 Morphological Observation

Detailed morphological observation of *Gambierdiscus* species was carried out using advanced scanning electron microscopy (SEM). Two different approaches of SEM preparation were tested for better resolution in SEM observation. The first method was adopted from Faust et al. (1996) and the second was described by Litaker et al. (2009).

3.2.1 Faust Methods of SEM preparation

For primary fixation, cells in suspension were transferred into 50 mL centrifuge tube and an equal volume of glutaraldehyde fixative was added. Next, the cell suspension incubated for one hour at room temperature. After that the suspension was spun at 1000g for 5 minute. Discarded fixative and washed with 0.1 M cacodylate buffer. In the second fixative, cells were then post-fixed in 1% O_sO_4 in 0.1 M cacodylate buffer. Cells were then incubated again at room temperature for one hour. Next, spun at 1000g for 5 minute and discarded and washed with 0.1 M cacodylate buffer once again.

Before dehydration steps, cells were transferred into polycarbonate (PC) membrane in mild filtration by using vacuum. The membrane then covered with a new

PC membrane where the shiny sides were facing each other. Later on, the Whatman Filter Paper used as an envelope. Then the samples were dehydrated by placing in beakers that filled with graded concentration of ethanol (50%, 70%, 80%, 90%, 95%, and 100%) for 15 minute each and followed by substitution through graded bath (75:25, 50:50, and 25:75) of ethanol and amyl acetate. Then, samples were transferred into beaker that filled with 100% amyl acetate for 15 minute. Lastly, the specimen was loaded onto Critical Point Drying (CPD) chamber.

3.2.2 Litaker methods of SEM preparation

Cells harvested from cultured cells. Approximately 700µL of cells were mixed with 20% of Triton X-100 which made in phosphate-buffered saline, PBS (pH 7.4) at 1:1 ratio in a 1.5 µL centrifuge tube. Next, the tube was placed in an ultrasonic cleaner for 20 minutes to remove cell surface debris. After that, glutaraldehyde (50% solution) was added to make a final concentration of 2% to preserve cells. Cells were then stored and settled overnight at 4 °C.

Later on, the supernatant from settled cells were removed and the remaining pellet was transferred onto a polycarbonate filter and the cells were rinsed with deionized water (dH_2O) for 7 times. The cells were dehydrated in a graded series of ethanol concentrations (Table 3.2). Then the cells were carried out by substitution through graded baths of EtOH and amyl acetate (Table 3.3). Lastly, the cells were loaded onto critical point drying (CPD) chamber.

Table 3.2: Graded series of ethanol concentration

EtOH conc. (%)	Time (min)
30	15
50	15

70	15
80	15
90	15
95	15
100	15

Table 3.3: Graded bath of EtOH and amyl acetate

EtOH (%)	Amyl acetate (%)	Time (min)
75	25	15
50	50	15
25	75	15
0	100	15

3.2.3 Critical Point Drying (CPD)

The power of Critical Point Drying (CPD) was on and the "Display Control" pressed to ensure all LED lights for different conditions were in a good condition. The stirrer of the CPD chamber was inserted in the sample chamber. Then, the specimen was placed and stubbed in the chamber. Amyl acetate was added about to cover the sample (half full) and the chamber was closed tight. Next, the "Cooling" button pressed and the temperature gradually dropped until the pre-set temperature reached 10°C.

After that, the "Medium In" button pressed to allow the liquid CO_2 filled the chamber. Later on, the "Medium Out" button was pressed to stop the liquid CO_2 when the liquid CO_2 level reached 2-3 mm from the top of the chamber. The "Stirrer" button was on and the "Medium Out" button was pressed when the intermedium was released above than half. These steps were repeated to 8 times. Then, the "Stirrer" and "Cooling" buttons were stopped. The "Heating" button pressed and the temperature gradually increased until the pre-set temperature reached 40°C. When the temperature reached at 40°C, the gas in the chamber released by pressed the "Gas Out" and adjusted the knob to control the gas