MORPHOLOGY, MOLECULAR CHARACTERIZATION AND HABITAT PREFERENCE OF HARMFUL BENTHIC DINOFLAGELLATE *GAMBIERDISCUS* (DINOPHYCEAE) IN MALAYSIA

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ABSTRACT

Ciguatera fish poisoning (CFP) is a type of illness that causes human intoxication after consuming contaminated coral fishes. The neurotoxins, ciguatoxins, are produced by species of *Gambierdiscus*, one of the harmful benthic dinoflagellates. Identification of harmful dinoflagellate species is important to gain further insights into the global dispersion. In this study, morphological characteristics of species in the genus *Gambierdiscus* and their habitat preferences were investigated. Field sampling was undertaken at Pulau Rawa, Terengganu and Pulau Sibu, Johor. Single cells of *Gambierdiscus* were isolated and clonal cultures were established. Morphology of *Gambierdiscus* was examined by using light and scanning electron microscopes. Taxon sampling on the small subunit (SSU) and large subunit (LSU) ribosomal RNA genes (rDNAs) of *Gambierdiscus* was performed for phylogenetic reconstruction using branch swapping algorithm. Morphological character states were coded and mapped onto the molecular phylogenetic tree (MP tree) to investigate the character state evolution of *Gambierdiscus* species. Morphological information and the distribution of *Gambierdiscus* species were used to develop a comprehensive taxonomic database of *Gambierdiscus*, and a 3I web-based interactive identification key for species identification is presented. Based on the morphological observation and molecular characterization, *G. caribaeus* and *G. balechii* were identified in Pulau Rawa, Terengganu and a new ribotype designated as *Gambierdiscus* sp. type 7 was identified from Pulau Sibu, Johor. Field survey on the habitat preference of *Gambierdiscus* in Pulau Rawa was conducted; the results showed that *Gambierdiscus* abundance was positively correlated with the habitat of coral rubbles covered with turf algae as compared to other substrates. The epiphytic behavior of *Gambierdiscus* species towards macroalgae hosts were further examined in the laboratory setting. The results demonstrated that different species of *Gambierdiscus* preferred different macroalgal host and attachment of *Gambierdiscus* cells were also differed among various macroalgal hosts. This study provides an inventory of toxic *Gambierdiscus* species in Malaysian water, which is useful in identifying the hotspot of CFP in the region. Future study on the trophic transfer mechanism of the toxins in the marine food webs is needed to better understand the source and fate of these potent neurotoxins.

Keywords: Ciguatera fish poisoning, *Gambierdiscus*, benthic dinoflagellate

ABSTRAK

Keracunan ikan Ciguatera (CFP) adalah sejenis penyakit yang menyebabkan keracunan pada manusia selepas termakan ikan terumbu karang yang tercemar. Neurotoksin, ciguatoksin, dihasilkan oleh spesies *Gambierdiscus*, salah satu dinoflagelat bentik yang berbahaya. Pengecaman spesies dinoflagelat berbahaya adalah penting untuk menentukan maklumat lanjut taburan global. Dalam kajian ini, ciri-ciri morfologi spesies dalam genus *Gambierdiscus* dan habitat pilihan mereka telah dikaji. Kerja lapangan telah dijalankan di Pulau Rawa, Terengganu dan Pulau Sibu, Johor. Sel-sel tunggal *Gambierdiscus* telah dipencil dan dikultur. Morfologi *Gambierdiscus* telah diperiksa dengan menggunakan mikroskop cahaya dan pengimbasan electron. Persampelan takson jujukan pada subunit kecil (SSU) dan subunit besar (LSU) gen ribosomal RNA (rDNAs) *Gambierdiscus* telah dilakukan untuk pembinaan semula filogenetik menggunakan algoritma penukaran dahan. Keadaan ciri-ciri telah dikodkan dan dipetakan ke atas pokok filogenetik molekul (pokok MP) untuk mengkaji evolusi keadaan ciri morfologi spesies *Gambierdiscus*. Maklumat morfologi dan taburan spesies *Gambierdiscus* telah digunakan untuk menghasilakn pengkalan data taksonomi *Gambierdiscus* yang komprehensif, kekunci pengecaman spesies interaktif sesawang 3I untuk tujuan pengecaman dibangunkan. Berdasarkan pemerhatian morfologi dan pencirian molekul, *G. caribaeus* dan *G. balechii* telah dikenal pasti di Pulau Rawa, Terengganu dan ribotype baru yang ditetapkan sebagai *Gambierdiscus* sp. type 7 telah dikenal pasti dari Pulau Sibu, Johor. Tinjauan lapangan terhadap pilihan habitat *Gambierdiscus* di Pulau Rawa telah dijalankan; hasilan kajian menunjukkan bahawa kelimpahan *Gambierdiscus* berkolerasi positif dengan habitat terumbu karang mati yang ditumbuhi dengan rumput turf berbanding substrat lain. Tingkah laku epifit spesies *Gambierdiscus* terhadap perumah makroalga telah dikaji dengan lebih mendalam dengan pengesetan makmal. Hasilnya menunjukkan spesies *Gambierdiscus* yang berbeza telah memilih perumah makroalga yang berbeza dan perlekatan sel-sel *Gambierdiscus* juga berbeza diantara pelbagai perumah makroalga. Kajian ini menyediakan satu inventori spesies *Gambierdiscus* toksik di perairan Malaysia, yang mana boleh digunakan dalam mengenal pasti 'hotspot' CFP di rantau ini. Kajian di masa hadapan mengenai mekanisme pemindahan toksin dalam trofik jaringan makanan laut diperlukan untuk lebih memahami sumber dan nasib toksin saraf yang merbahaya ini.

Kata kunci: Keracunan ikan ciguatera, *Gambierdiscus*, dinoflagelat bentik

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LIST OF SYMBOLS AND ABBREVIATIONS

BHAB	:	Benthic harmful agal blooms
CFP	:	Ciguatera fish poisoning
IF	:	Imamura-Fukuyo
LM	:	Light microscope
SEM	:	Scanning electron microscope
CPD	:	Critical point drying
LSU	:	Large subunit
SSU	:	Small subunit
rDNA	:	Ribosomal deoxyribonucleic acid
AIC	:	Akaike information criterion
MP	:	Maximum parsimony
ML	:	Maximum lilekood
Po	:	Apical pore plate
PCR	:	Polymerase chain reaction

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CHAPTER 1: GENERAL INTRODUCTION

Dinoflagellates are microalgae that are associated with the production of marine biotoxin that may cause harmful effect to wildlife and humans (Camacho et al., 2007). High diversity of benthic dinoflagellates, with 24 species representing nine genera had been reported in Malaysia water (Mohammad-Noor et al., 2007). Benthic dinoflagellates prefer warm and shallow sea area and attached to substrates like seaweeds, coral rubbles as well as on sand of the ocean. However, not all the species are potentially toxic producer. There are six major genera categorized as benthic harmful dinoflagellates, viz *Amphidinium* (Claparède & Lachmann, 1859), *Gambierdiscus* (Adachi & Fukuyo, 1979), *Ostreopsis, Prorocentrum* (Fukuyo, 1981), *Coolia* (Besada et al., 1982) and *Fukuyoa* (Gómez et al., 2015).

The most well-known human intoxication caused by benthic dinoflagellate is Ciguatera Fish Poisoning (CFP), where the responsible species are of the genus *Gambierdiscus* (Yasumoto et al., 1977; Adachi & Fukuyo, 1979; Leaw et al., 2010). It is a common disease associated with consumption of contaminated coral fish especially finfishes in the tropical and sub-tropical regions (Lehane & Lewis, 2000). The biotoxin produced by these species accumulated via food chain by the herbivorous and carnivorous fishes (Quod & Turquet, 1996). Ciguatoxin acts to increase the permeability of excitable membranes to sodium ions (Gillespie et al., 1986). CFP will show its symptom within a few hours after consuming the contaminated fish. The symptoms are gastrointestinal (e.g. abdominal cramp, diarrhea, vomiting), neurologic (e.g. paresthesia, blurred vision) and cardiovascular symptoms (e.g. bradycardia, hypotension) (Friedman et al., 2007). Palytoxin (PTXs) produced by the genus *Ostreopsis* have been suspected to play a role in ciguatera cases (Parsons et al., 2012). Just like CFP, PTXs causes human illness after the consumption of contaminated fishes, crabs, mussel and clams (Aligizaki et al., 2011).

The genus *Gambierdiscus* is easy to identify under the light microscope (LM) as they are disc-shaped and an anterio-posteriorly compressed. This genus exhibits a deep hollow sulcus and distinctive in its anterio-posteriorly compressed body shape with a circular narrow deep cingulum. It is called *Gambierdiscus* because of its shaped and first found at Gambier Island in the South Pacific. To date, there are 14 taxonomically accepted species of *Gambierdiscus*. They are *G. toxicus* Adachi & Fukuyo, *G. belizeanus* M.A.Faust, *G. polynesiensis* Chinain & M.A.Faust, *G. australes* Chinain & M.A.Faust, *G. pacificus* Chinain & M.A.Faust, *G. caribaeus* Vandersea, Litaker, M.A.Faust, Kibler, W.C.Holland & P.A.Tester, *G. carpenteri* Kibler, Litaker, M.A.Faust, W.C.Holland, Vandersea & P.A.Tester, *G. carolinianus* Litaker, Vandersea, M.A.Faust, Kibler, W.C.Holland & P.A.Tester, *G. excentricus* S.Fraga, *G. scabrosus* T.Nishimura, Shinya Sato & M.Adachi, *G. silvae* S.Fraga & F.Rodríguez, *G. balechii* S.Fraga, F.Rodríguez & I.Bravo, *G. lapillus* Kretzschmar, Hoppenranth & Murray, and *G. cheloniae* K.F.Smith, L.Rhodes & S.A.Murray.

Studies on benthic dinoflagellates have increased intensively in the recent years, mainly due to the expansion of CFP intoxications globally (Van Dolah, 2000; Wong et al., 2005; Litaker et al., 2010; Catania et al., 2017) Furthermore, they are very limited information on the bloom dynamic and their effects to other organisms and ecosystem. The species identification of the benthic dinoflagellate is very challenging as some species have similar morphological characteristics. There are impossible to distinguish under the normal light microscope. They are delineated based minutes differences of theca plate with assisting of advance microscopy. Therefore, identification of harmful dinoflagellate species is important to gain further insight into their global dispersion as well as to minimize the fisheries damage.

This study aims to characterize the species of *Gambierdiscus* distributed in Malaysian coral reefs for establishing the species documentation, and access the distribution of harmful species for identifying the hotspots of Ciguatera in the country. The specific objectives of this study are to:

i. Morphologically characterize the species of *Gambierdiscus* by advanced microscopy and molecular approach.
ii. Develop a web-based interactive key to species of harmful dinoflagellate *Gambierdiscus* species and its taxonomical database.
iii. Investigate the habitat preference of *Gambierdiscus* in Pulau Rawa, Terengganu.

Thesis structure

This thesis was prepared in an article form and consisted of four chapters; **Chapter 1** introduced the general background of this study on benthic harmful dinoflagellate, *Gambierdiscus*, which caused ciguatera fish poisoning. Besides, this chapter also discussed the importance of the identification of this species in Malaysian waters. **Chapter 2** includes literature reviews that provide scientific information on this study. In **Chapter 3**, characterization of the *Gambierdiscus* species in Pulau Rawa, Terengganu and Pulau Sibu, Johor by using morphological observation and molecular analysis. This chapter also introduced the development of the web-based interactive identification key to *Gambierdiscus* species. **Chapter 4** discussed more on habitat preference of *Gambierdiscus* species in Pulau Rawa, Terengganu. Last but not least, **Chapter 5** concluded the outcomes of this study as well as some recommendation of future study.

CHAPTER 2: LITERATURE REVIEW

2.1 Benthic Harmful Algae Blooms (BHABs)

Benthic dinoflagellate is one of the important organisms in the benthic ecosystems that act as a primary producers and symbionts in the marine benthic food web (Berdalet et al., 2012). These benthic dinoflagellates contribute to the major Benthic Harmful Algal Blooms (BHAB) as some of the genus in this group produce toxin that may lead to the fish poisoning. Major blooms of harmful benthic dinoflagellates can cause serious and environmental and human health problems. Benthic dinoflagellates lived in a wide range habitat as some of the species adjusted to the benthic, epiphytic, and planktonic life (Faust, 1996). This dinoflagellates also usually found in a warm shallow water attach to the macroalgae, turf algae, sea grasses, coral rubbles and sediments (Tester et al., 2014). Those genera that are mainly epiphytic on macroalgae are the ones that usually cause benthic harmful algae bloom (Fraga et al., 2012).

Genera of the benthic dinoflagellates comes with different morphologies which are small and flattened cells unlike planktonic dinoflagellates that sometimes possess striking extension morphologies like spines or horns (Hoppenrath et al., 2014). It is believed that this unusual morphology help these species to facilitate their movement in this kind of habitat. Besides, Fraga et al. (2012) also proposed that flattened surface of the benthic dinoflagellates helps them in increasing their nutrient uptake in oligo-trophic condition as the surface and volume ratio is higher than in spherical cell.

2.2 Ciguatera fish poisoning (CFP)

Ciguatera fish poisoning (CFP) is a circumtropical human illness owing to consumption of ciguatoxin-contaminated reef fishes (Withers, 1982; Berdalet et al., 2012). This common type of marine food poisoning has affected mainly residents at tropical and sub-tropical marine areas, such as countries of Caribbean Sea, France

Polynesia, Central Pacific Ocean and Indian Ocean (Nicholson & Lewis, 2006; Friedman et al., 2008; Fraga et al., 2016). It is estimated that Ciguatera has caused 25, 000 – 500, 000 poisoning cases per year (Parsons et al., 2012). According to Lehane and Lewis (2000), ciguatera fish poisoning is uncommon in Indonesia, whereas there is one reported case of CFP in Malaysia in 2010. In this case, 22 out of 36 from 5 families felt sick after consuming the red snapper bought from market where it is claimed to be imported from China (Nik Khairol Reza M.Y., 2011).

Gambiertoxin produce by the *Gambierdiscus* species go into the coral reef food web through the grazing by herbivores. This toxin will continue to accumulated and bio-transformed in the herbivore's body until those animal were eaten by the predators (Xu et al., 2014). Ciguatoxins acts by increasing the permeability of excitable membranes to sodium ions. CFP is associated with gastrointestinal symptom (e.g., vomiting, diarrhea, abdominal pain, nausea), cardiovascular symptom (e.g., hypotension, bradycardia) and neurologic symptom like paraesthesia, join and muscle pain, fatigue and reversal of hot and cold sensation (Friedman et al., 2008). All this clinical symptoms and signs are variable, where it depending on type and amount of toxin present in the body (Lehane & Lewis, 2000). This ciguatera fish poisoning can be treated with drug called pregabalin (Brett & Murnion, 2015) or mannitol if diagnosed within 72 hours (Friedman et al., 2008). Understanding more about the taxonomy, toxicity and ecology of this genus provided more knowledge and information about the bloom and their effects to other organisms and ecosystems as well as providing education and community outreach about CFP in our country.

2.3 The genus *Gambierdiscus*

Gambierdiscus species is well-known as a toxin producer that cause ciguatera fish poisoning, a non-bacterial illness associated with the consumption of fish that contaminated with ciguatoxin (Litaker et al., 2009). This species has a deep hollow suclcus and distinctive in its anterio-posteriorly compressed body shaped with a circular narrow deep cingulum (Adachi & Fukuyo, 1979). It is called *Gambierdiscus* because of its shaped and the place where it is found and described which is Gambier Island in the South Pacific.

Gambierdiscus toxicus is the first species of *Gambierdiscus* that has been described by Adachi and Fukuyo from Gambier Island, French Polynesia in May 1975 (Adachi & Fukuyo, 1979). *Gambierdiscus belizeanus* is the second *Gambierdiscus* species that has been described by Faust from the coastal water of Belize (Faust, 1995). After 4 years, Chinain et al. (1999) described three more species of *Gambierdsicus* from French Polynesia. There are *G. australes*, *G. pacificus* and *G. polynesiensis*. Next, Litaker et al. (2009) described three more new species of *Gambierdiscus* species; *G. caribaeus*, *G. carpenteri* and *G. carolinianus*. Following year, the inventory of *Gambierdiscus* species was continuously added where *G. excentricus* from Canary Island described by Fraga et al. (2011), *G. scabrosus* from Coastal area of Japan and *G. silvae* from Canary Island in 2014 by Nishimura et al. (2014) and Fraga and Rodriguez (2014), and recently *G. balechii* from Celebes Sea (SW Pacific Ocean) (Fraga et al., 2016), *G. cheloniae* from Rarotonga, Cook Island (Smith et al., 2016) and *G. lapillus* from Great Barrier Reef, Australia (Kretzschmar et al., 2016). All these *Gambierdiscus* are relatively easy to identify under the microscope because of its shaped. However, detail observation need to be done as they have similar morphological characteristics which is difficult to distinguish (Figure 2.1 and Figure 2.2). Despite that, it is important to note that not all species in this genus are toxic. These *Gambierdiscus* species can be found attach to the substrates like corals, seaweed as well as in sand (Faust, 1995; Morton & Faust, 1997; Parsons et al., 2011; Tester et al., 2014).

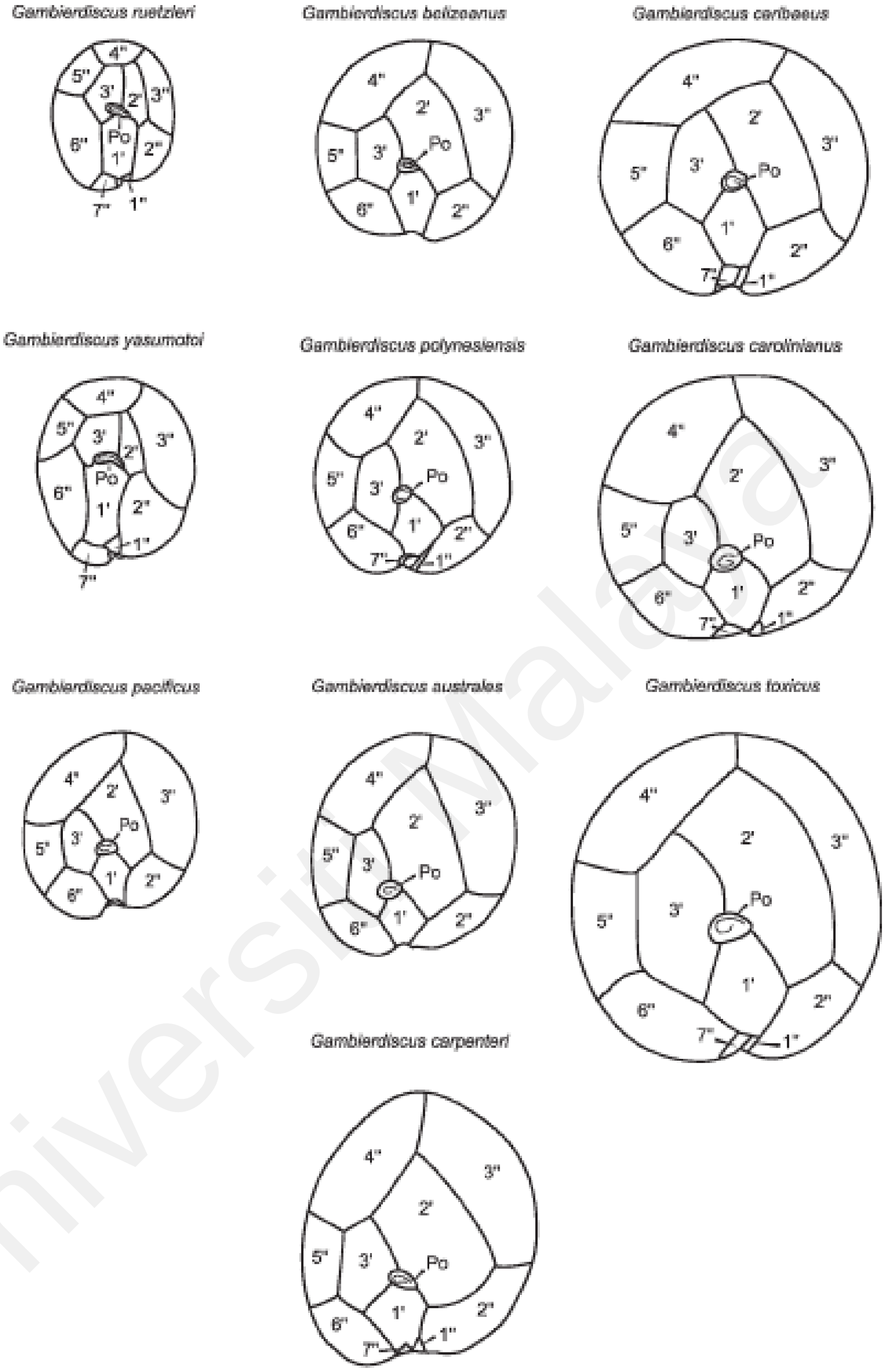


Figure 2.1: Comparative line drawing of epitheca for 10 *Gambierdiscus* species. (Litaker et al., 2009)

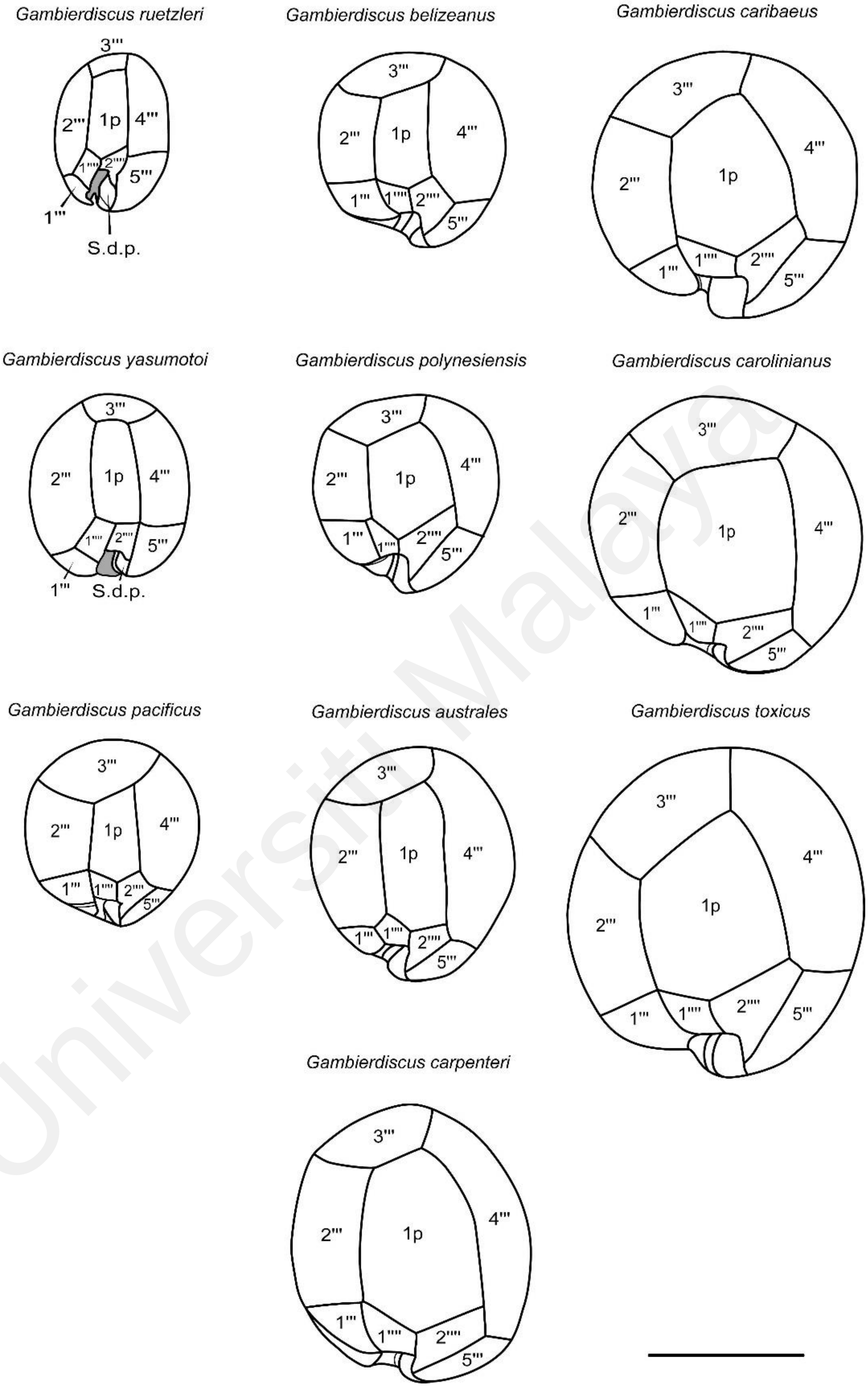


Figure 2.2: Comparative line drawing of hypotheca for 10 *Gambierdiscus* species. (Litaker et al., 2009)

CHAPTER 3: MORPHOLOGY AND MOLECULAR CHARACTERIZATION OF BENTHIC DINOFLAGELLATE *GAMBIERDISCUS* (DINOPHYCEAE) IN PULAU RAWA AND PULAU SIBU , WITH AN INTRODUCTION OF THE INTERACTIVE KEY TO SPECIES

3.1 Introduction

Harmful benthic dinoflagellates are microalgae that live on the surface of marine substrate like seaweed and coral rubbles where some are associated with the production of marine biotoxins. Some of the species in the genera *Gambierdiscus* (Adachi and Fukuyo, 1979), *Ostreopsis* (Fukuyo, 1981), *Coolia* (Besada et al., 1982), *Prorocentrum* (Fukuyo, 1981), and *Amphidinium* (Claparède & Lachmann, 1985) produce bioactive compounds (e.g. ciguatoxin, cooliatoxin, okadaic acid) that can cause harmful effects to fish, human and other wildlife as well as seafood toxicity (Camacho et al., 2007). However, the most well-known human intoxication due to the benthic dinoflagellates is known as ciguatera fish poisoning (CFP), where these toxins are produced by some potential harmful species of the *Gambierdiscus* (Friedman et al., 2007). It is a common fish poisoning especially in the tropical and subtropical regions (Lehane & Lewis, 2000), with the first case reported from Mauritius, Indian Ocean in 1973 (Quod & Turquet, 1996).

The biotoxin lipid soluble ciguatoxins and water soluble maitotoxins produced by the toxic *Gambierdiscus* species accumulate via food chains transfer from herbivorous to carnivorous fishes to higher tropic levels (Gillespie et al., 1986; Quod & Turquet, 1996). Early symptoms of CFP occur within hours of fish consumption, with gastrointestinal, neurologic and cardiovascular symptom observed (Friedman et al., 2007). CFP has become world health threat as the reef fish such as barracuda, grouper and snapper are increasingly exported for consumption (Lehane & Lewis, 2000).

Genus *Gambierdiscus* is relatively easy to identify under the light microscope (LM). Most of the species are disc-shaped and anterio-posteriorly compressed. The deep hollow sulcus and circular deep cingulum of these species are sometimes visible under the LM. The type species, *Gambierdiscus toxicus* Adachi *et* Fukuyo, 1979 was first described from Gambier Islands in the South Pacific Ocean. Up till now, 12 *Gambierdiscus* species that have been described, *viz. G. toxicus* (Adachi & Fukuyo, 1979), *G. belizeanus* (Faust, 1995), *G. australes*, *G. pacificus*, *G. polynesiensis* (Chinain et al., 1999), *G. caribaeus*, *G. carpenteri*, *G. carolinianus* (Litaker et al., 2009), *G. excentricus* (Fraga et al., 2011), *G. scabrosus* (Nishimura et al., 2014), *G. silvae* (Fraga & Rodríguez, 2014), *G. balechii* (Fraga et al., 2016), *G. lapillus* (Kretzschmar et al., 2016), and *G. cheloniae* (Smith et al., 2016). Recently, a new genus, *Fukuyoa* Gomez, Qiu, Lopes & Lin was erected to represents the globular species of *Gambierdiscus*. *G. ruetzleri* (Litaker et al., 2009) and *G. yasumotoi* (Holmes, 1998), former *Gambierdiscus* species which represents a globular shape instead of the typical anterio-posteriorly were transferred into the new genus together with newly described globular species, *Fukuyoa paulensis* (Gómez et al., 2015). Species identification in the genus is generally aided by the advanced scanning electron microscope (SEM), with detailed observations on the thecal architecture. Some of these species possess similar morphological characteristics which make it difficult to distinguish without the taxonomic expertise.

Over the years, research interests in benthic dinoflagellates have increased. This is partly due to the increasing information on the blooms and their impact to other organisms and ecosystems. Species identification is crucial, and requires experienced taxonomists and well-trained personnel in electron microscopy. In a previous work of Litaker et al. (2009), a dichotomous tree detailing the morphological characteristics of ten species of *Gambierdiscus* was introduced in order to differentiate the species. The aim of this study is to extend the usage of identification keys in an interactive manner to assist in identifying species of *Gambierdiscus*, where the morphology of *Gambierdiscus* species was characterized based on the previous descriptions as in the literature. Evolutionary lineage and character state evolution of these benthic dinoflagellates were determined and all morphological characters compiled was used to develop a comprehensive taxonomic database of the 12 valid species *Gambierdiscus* including a new ribotype, herein referred as *Gambierdiscus* sp. type 7. The database was then used to design a web-based interactive identification key for species identification.

3.2 Literature review

Species identification of the harmful benthic dinoflagellate is crucial as some of the taxa has caused serious problem to our ecosystem like fish kill event as well as human poisoning. Biologists have been fascinated by observing cells by using light microscope since the earliest examination of cellular structure (Stephens & Allan, 2003). Now, with the emergence of the new technologies like fluorescent labeling, microscope has become more sophisticated and useful tool for scientist in their research project. Therefore, observing features of dinoflagellate by staining the cell and observed under the microscope is one of the important techniques used in identifying the species.

Gambierdiscus can be identified based on its cell shape which is anterio-posteriorly compressed shape by using light microscope (LM).Cell size can be measured directly by using microscope software where the cell dimension is determined by measuring depth (D : dorso-ventral axis), width (W:lateral [right-left] axis) and ratio of D to W; for epitheca, length (L), width (W), ratio of L to W of apical pore plate (Po), number and diameter of pore in Po, ratio of 2′/1′ to 2″ /3″ plate suture length, and ration of 3″/2″ to 3″/4″ plate suture length; and for hypotheca, length (L: dorsal-ventral axis), width (W: lateral [right-left] axis) and ratio of L to W of 2″″ plate.

Besides, several morphologies like plate pattern, shape and size also can be used in *Gambierdiscus* species identification (Litaker et al., 2009). All these plate including the thecal surface can be viewed clearly under the scanning electron microscope (SEM). For example, *Gambierdiscus* species can be separated into two groups based on its 2′ plate shape (i.e. hatched or rectangular) as well as their thecal surface (i.e. reticulate-foveated or smooth). Apart from that, small plate like sulcal right posterior plate (S.d.p.), sulcal left posterior plate (S.s.p.) and sulcal left anterior plate (S.s.a.) which is necessary in *Gambierdiscus* species identification also can be observed under the SEM (Litaker et al., 2009; Nascimento et al., 2015). In addition, a modified Kofoid tabulation system (Kofoid, 1909), as described in Besada et al., (1982), is used to name the plates for comparison to other genera of *Gonyaulaclaes* based on plates homologies.

Species recognition based on morphological characteristics itself always present challenged in the discovery of new species or genus as well as in rearrangements of the dinfolagellates group. In order to overcome the weakness of identifying dinoflagellate species based on morphological characteristics only, molecular technique approach using the rDNA region was used. This genetic makeup can be used as one of the methods in differentiates the morphological resemblance between the species (Destombe et al., 1992). Thus, nowadays there are many DNA sequences that can be found in public database as the DNA sequencing technology has been developed into an advanced technology. This makes the DNA sequenced-based genotyping has become a promising tool for the identification of harmful benthic thecate dinoflagellates.

Identification is the process of identifying or finding taxon from specimen belongings that can be done by using conventional identification keys or interactive identification keys (Dallwitz et al., 2000). Before interactive identification keys were developed, researcher used a conventional identification key which is a phylogenetic tree that comes with characters at the internal nodes and taxon names at the terminal nodes to identify the taxon. Taxon was identified by following the branches from the root of the tree that corresponds to the characters showed in the taxon.

Therefore, in order to assist researchers to identify species or taxon in a faster and easier way, an interactive identification key by using computer program is introduced. Interactive identification key is an upgraded version of conventional identification key where all morphological characteristic of the taxon were compiled into the database. This interactive identification key works by removing taxa whose morphology does not match with the sample or specimens. (Dallwitz et al., 2000).

There are many types of interactive identification program that has been introduced such as Intkey, Lucid, (Dallwitz et al., 2002), 3I (Dmitriev, 2006), AlgaeBase (Guiry et al., 2014) and many more. As an example, 3i (Internet–accessible Interactive Identification) database (http://dmitriev.speciesfile.org/index.asp) is one of the program that created on-line interactive identification key, taxonomic database and virtual taxonomic revisions (Dmitriev, 2003 ; Dmitriev, 2006). This program also includes morphological measurements in numerical form, morphological illustration to explanatory images, bibliographical for user references, as well as the distributional data of the specimen. Despite that, this program also comes with many features like this program can be viewed in several different languages (Dmitriev, 2006).

Other than that, AlgaeBase (http://www.algaebase.org/) also known as one of the useful online databases used by many taxonomists working on a variety of genera and species of algae (Guiry et al., 2014). Information published in AlgaeBase is all related to the algae including marine, freshwater as well as terrestrial algae. Similar with some other interactive identification key programs, this online database also provide intraspecific names, images, distributional data, and citation from the original publications of all algae. With the existence of interactive identification key program, the identification of the taxon can be done online and used as the first step of recognition process.

3.3 Material and methods

3.3.1 Sample collections

The study was conducted at Pulau Rawa , Terengganu (5°57’44.45”N, 102°40’53.26”S) and Pulau Sibu, Johor (2°13’40”N, 104°03’38”E) (Figure 3.1). Plankton samples were collected by using artificial substrate sampling technique (Berdalet et al., 2012; Tester et al., 2014). Fiberglass screens were cut into a standardized pieces (10.2cm2 × 15.2cm2 in size) and connected to a small sub-surface buoy and weight (<200g) with monofilament fishing line. Live samples were brought back to the laboratory for single cell isolation by using micropipetting technique (Andersen & Kawachi, 2005). Samples were also preserved in acidic Lugol’s solution for quantitative analyses. Cell abundance was determined.

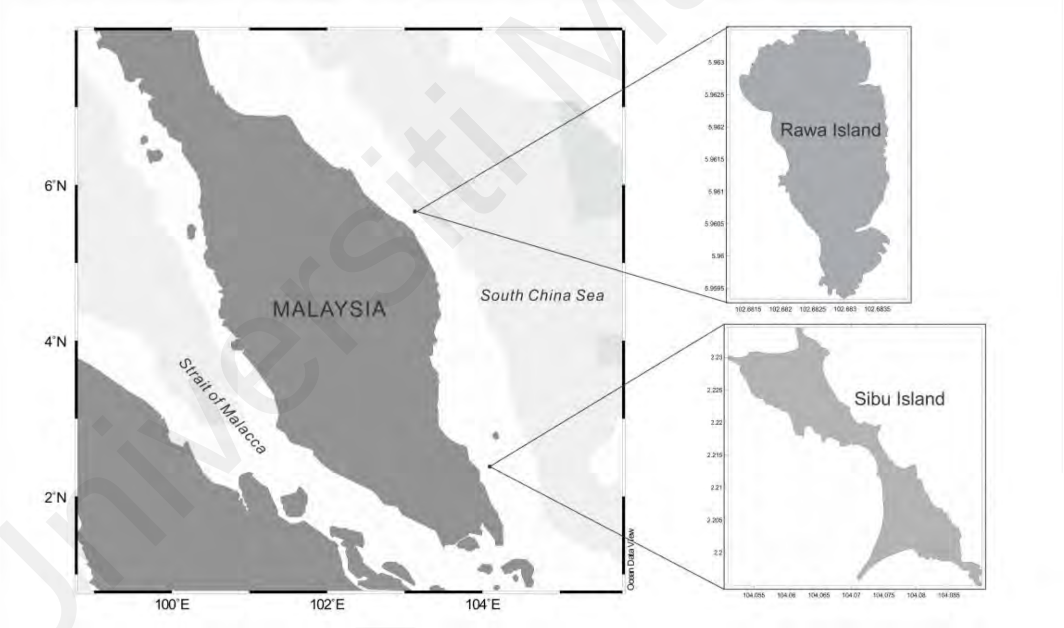


Figure 3.1: Map showing Pulau Rawa , Terengganu and Pulau Sibu , Johor as sampling site.

3.3.2 Culture establishments

Clonal cultures of benthic dinoflagellates were established by micropipetting technique (Hoshaw & Rosowki, 1973). Live samples were observed under a stereo microscope and transferred into a 24-wells plate containing ES-DK medium (Kokinos & Anderson, 1995), maintained at 25 ± 0.5 °C, 12:12 h light: dark photo cycle, with light intensity of 70 μmol photon $m^{-2}s^{-1}$. Algal cultures established in this study were deposited in the Harmful Algae culture Collection of Bachok Marine Research Station, Institute of Ocean and Earth Sciences, UM. Clonal cultures of *Gambierdiscus* species established in this study were listed in Table 3.1.

Table 3.1: Clonal culture of algae established in this study.

Species	Strain	Locality
Gambierdiscus balechii	T6PRGd03N	Pulau Rawa , Terengganu
Gambierdiscus balechii	T6PRGd07N	Pulau Rawa, Terengganu
Gambierdiscus balechii	T6PRGd12N	Pulau Rawa, Terengganu
Gambierdiscus caribaeus	T6PRGam28	Pulau Rawa, Terengganu
Gambierdiscus sp. type 7	GdPS03	Pulau Sibu, Johor
Gambierdiscus sp. type 7	GdPS04	Pulau Sibu, Johor
Gambierdiscus sp. type 7	GdPS05	Pulau Sibu, Johor

3.3.3 Morphological observations

The cultures were fixed in 4% glutaraldehyde and then examined with light microscope. Cells were washed with a drop of 5% sodium hypochlorite to remove the chlorophyll contents and then stained with Immamura-Fukuyo (IF) staining solution for plate identification. Scanning electron microscope (SEM) was performed as Leaw et al. (2010). The cells underwent six steps of SEM; cell fixation, dehydration intermedium substitution, critical point drying, mounting and gold coating. Cells were transferred into 50mL centrifuge tube and fixed with 4% glutaraldehyde for 1 hour. Cells were then rinsed with 0.1 M cacodylate buffer three times, and 1% osmium tetroxide solution was added for post fixed purposed. Cells were rinsed with ddH$_2$O three times to remove slats and fixatives, and then dehydrated in a graded series of ethyl alcohol (EtOH, 30-100%). Next, cells were transferred onto a polycarbonate (PC) membrane filter (0.2 µm pore size, Whatman, USA) by mild filtration using a vacuum manifold. The cells were treated with inter-medium substitution through the graded baths (75:25, 50:50. 25:75) of EtOH and amyl acetate in a glass beaker. Cells were then transferred into small beaker with 100% amyl acetate for 15 min followed by critical point drying (CPD) and stored in vacuum desiccators. After drying, cells were mounted on an aluminium stubs by using double stick carbon tape. Samples were coated with gold using JFC-1600 coater (JOEL, Japan).

Cells dimension were determined by measuring the depth (D: dorso-ventral axis), width (W: lateral [right-left] axis) and ratio of D to W; for epitheca, length (L), width (W), ratio of L to W of apical pore plate (Po), number and diameter of pore in Po, ratio of 2′/1′ to 2′/3″ plate suture length, and ration of 3″/2″ to 3″/4″ plate suture length; and for hypotheca, length (L: dorsal-ventral axis), width (W: lateral [right-left] axis) and ratio of L to W of 2′′′′ plate. The whole cell was also observed as well as the measurement of thecal pores was calculated (Penna et al., 2005; Leaw et al., 2011). All measurements were obtained through the observation of LM and SEM micrograph by using ImageJ 1.50b (Wayne Rasband, National Institute of Health, USA). A modified Kofoid tabulation system (Kofoid, 1909), as described in Besada et al (1982), was used in this study to name the plates for comparison to other genera of *Gonyaulaclaes* based on plate homologies.

3.3.4 DNA extraction, gene amplification and sequencing

Genetic information of these benthic dinoflagellates was characterized by amplifying the nuclear-encoded ribosomal RNA genes (rDNAs), followed by DNA sequencing. The method adopted here is described in Leaw et al. (2010). The cells were harvested by transferring the exponential phase dinoflagellates culture into a 50 mL centrifuge tube with centrifugation at 2,800 ×g in 10 min. The supernatant was discarded and cell pellet was resuspend in 10× NET lysis buffer followed by 10% CTAB extraction. The mixture was incubate at 65 °C for 1 h and then extracted again by using 700µL of Chloroform: Isoamyl alcohol (C: I; 24:1) with centrifugation at 10,000 ×g, 4 °C for 10 min. The upper aqueous phase was then transferred into a new labelled microfuge tube and followed by adding 700µL of Phenol: Chloroform: Isoamyl alcohol (P: C: I; 25:24:21). The mixture was re-centrifuge for 10 min at 10,000×g. clear aqueous layer was transferred again into new microfuge tube and subsequently added 700µL of cold C: I (24:1) followed with centrifugation at 4°C, 10,000 ×g for 10 min. The transferred aqueous phase was then mixed inversely with 500µL of iced-cold EtOH and 25µL of 3 M sodium acetate (NaOAc). The sample was kept in the freezer for 3 hours at -20°C. Next, the sample was centrifuge at 13,000 ×g for 10 min at 4°C. The supernatant was removed and 700µL of cold 70% EtOH was added subsequently. The samples were spun again after gently inverse the tube. The DNA pellet was dried at room temperature after the supernatant has been removed. Then, DNA pellet was re-dissolved in 30µL of TE buffer and stored at -20°C. DNA yield and quality was checked by using gel electrophoresis and spectrophotometry.

Amplification of the D8-D10 region of large subunit (LSU) ribosomal RNA gene (rDNA) were carried out using a primer pair, FD8 and RB (Chinain et al., 1999) (Table 3.2). The whole region of small subunit (SSU) rDNA was amplified and sequenced using the primer Dino5′UF and 18ScomR1 (Zhang et al., 2005) (Table 3.2). PCR mixture contained of 1× KOD FX Neo buffer, 0.4 mM dNTPs, 0.5 U KOD FX Neo (TOYOBO, Japan), 2.5 mM of each primer and genomic DNA template. The amplification was performed as follow: denaturation at 94°C for 4 min, 25 cycles of 30s denaturing at 94°C, followed by annealing at 55°C for 55 min, 2 min elongation at 72°C and 10 min of final elongation at 72°C. Amplicons were electrophoresed in 1% agarose gel and run simultaneously with 1kb DNA ladder (GeneDireX, Taiwan). Gel was stained with SYBR Safe DNA stain (Invitrogen, USA) followed by visualization under blue light transilluminator. The amplicons were then purified by using UltraClean® PCR Clean-Up Kit (MoBio, QIAGEN, USA). DNA sequencing was carried out on an ABI 3700XL automated DNA sequencer (Applied Biosystems, USA) by a private sequencing laboratory (1st Base, Selangor, Malaysia). Both strand of the sample was sequenced.

Table 3.2: Primer pairs used in PCR reaction.

Primer	Sequence (5’–3’)	Direction
FD8	GGA TTG GCT CTG AGG GTT GGG	Forward
RB	GAT AGG AAG AGC CGA CAT CGA	Reverse
Dino5′UF	CAA CCT GGT GAT CCT GCC AGT	Forward
18ScomR1	CAC CTA CGG AAA CCT TGT TAC GAC	Reverse

3.3.5 Taxon sampling and phylogenetic reconstructions

Taxon sampling was carried out from the redundant nucleotide sequence data that is available in GenBank. The D8-D10 LSU rDNA and SSU rDNA sequenced obtained was compared with sequences deposited in GenBank nucleotide database (NCBI) using BLAST sequence similarity searches (National Center for Biotechnology Information). All sequences were then aligned and edited by using BioEdit Sequence Alignment

Editor, ver 7.0.9.0 (Hall, 1999) and ClustalX 2.0 (Thompson et al., 1997). Genus *Fukuyoa*; *Fukuyoa yasumotoi*, *Fukuyoa ruetzleri* and *Fukuyoa paulensis* were used to root the phylogenetic tree in this study. Maximum Parsimony (MP) and Maximum Likelihood (ML) analyses were carried out using PAUP* ver. 4.0b.10 (Swofford, 2003). MP tree were performed using heuristic search option with total of 1000 random additions, a branch swapping algorithm with three-bisection reconnection (TBR) and 1000 replications of tree bootstrapping. A total of 1000 random addition sequence replicates, heuristic search using TBR branch swapping and 500 replications of tree bootstrapping were used in ML analyses. Parameters for ML analyses was fixed using Akaike Information Criterion (AIC) based on the calculated based model as implemented in jModelTest 2.0 (Darriba et al., 2012). Bayesian analysis was carried out using MrBayes ver. 3.2.5 (Ronquist et al., 2012) with parameters that fixed based on the same best model in ML analyses, while the best fitted model for the SSU rDNA dataset was GTR+G. The posterior probabilities (PP) were estimated using four Markov chain Monte Carlo chains. Trees were performed at 3,000,000 generations per run, sampling every 100 tree and the PP was estimated with 25% burn-in.

3.3.6 Character coding, matrix construction and character state evolution

Morphological characteristics of *Gambierdiscus* were derived based on existing data from literature as well as data obtained in this study. Characters that are generally used in *Gambierdiscus* taxonomic classification were used in character coding. Character matrix were constructed using NEXUS data editor program ver. 0.5.0 (Page, 2001). All characters that include in this study were treated as unordered. The character state was mapped onto the MP gene tree by considering the parsimony ancestral state using Mesquite ver. 2.74 (Maddison & Maddison, 2010).

3.3.7 Development of web-based interactive identification key

An identification key of *Gambierdiscus* was illustrated based on the morphological phylogeny analysis of the 12 valid species of *Gambierdiscus*. The development of web-based interactive identification key was created using 3I (Internet-accessible Interactive Identification) Interactive Key and the Taxonomic Database Software Package. 13 morphological characters were compiled into the database where four of the morphological characters were numerical, based on morphological measurement.

3.4 Results

3.4.1 Clonal culture established

Several species of *Gambierdiscus* were successfully isolated from the samples that had been collected from Pulau Rawa. A total of 33 clonal cultures of *Gambierdiscus* species were established. Clonal cultures were maintained in the test tube containing ES-DK enriched medium with salinity of 33 psu, under 25 ± 0.5 °C and 12: 12 h light: dark photo cycle, with light intensity of 70 -100 µmol photon $m^{-2}s^{-1}$. All cultures were sub-cultured fortnightly to maintain the culture as the growth cycle of the strain was estimated to be around 14 days. Aseptic technique was applied on the whole process during the preparation of culture vessels, medium preparation, sub-culturing and inoculation to avoid contamination

3.4.2 Species identification

Cell of *Gambierdiscus* were observed under the LM and SEM where species identification was based on the cell size, cell shape, the architecture of thecal plates as well as the surface morphology and measurement. Three *Gambierdiscus* species, *G. caribaeus*, *G. balechii*, were recorded in Pulau Rawa and a new ribotype, herein referred as *Gambierdiscus* sp. type 7 was found in Pulau Sibu. All these three *Gambierdiscus* species were confirmed through their thecal plate tabulation and molecular characterization. All characteristic of *Gambierdiscus* obtained were compared with other species in the genus including the recently describes *Gambierdiscus* species; *G. balechii* (Fraga et al., 2016), *G. cheloniae* (Smith et al., 2016) and *G. lapillus* (Kretzschmar et al., 2016) (Table 3.3).

Table 3.3: Cell sizes and thecal morphometric of *G. balechii*, *G. caribaeus* and *Gambierdiscus*. sp. type 7 compared with other *Gambierdiscus* species. The number in parentheses represents the standard deviation. Species in bold are from this study; nd, not determined. *, measured from micrographs in literature

Species (References)	Cell size			Plate 2''''			Apical pore plate (Po)			2'/1'':2'/3''	Po pore	
	Depth	Width	L:W	Length	Width	L:W	Length	Width	L:W		Number	Diameter
G. balechii	59 (6.3)	60 (6.7)	0.69	30 (3.2)	18 (2.4)	1.72	5.6 (1.1)	4.1 (0.8)	1.4	0.64 (0.11)	28 (3.8)	0.27 (0.05)
G. caribaeus	76 (4.4)	74 (5.3)	1.03	nd	nd	nd	nd	nd	nd	nd	nd	nd
***Gambierdiscus* sp. type 7**	59 (4.9)	60 (5.7)	0.99	29 (3.7)	18 (1.6)	1.62	4.7 (1.0)	6.1 (0.9)	0.76	0.61 (0.09)	29 (2.1)	0.23 (0.08)
G. australes (Litaker et al., 2009)	86 (5.1)	77 (3.7)	0.61	54 (3.1)	27 (2.7)	2.00	7.1 (0.8)	6.1 (0.4)	1.16	0.69 (0.09)	31 (4.1)	0.45 (0.03)
G. belizeanus (Litaker et al., 2009)	63 (2.2)	58 (2.5)	0.80	22 (2.3)	12 (2.1)	1.83	5.38 (0.4)	3.9 (0.4)	1.38	0.64 (0.14)	19 (5.7)	0.35 (0.03)
G. caribaeus (Litaker et al., 2009)	77 (6.1)	79 (8.4)	0.73	43 (6.4)	34 (6.8)	1.26	8.3 (1.3)	5.3 (0.8)	1.59	0.91 (0.09)	37 (4.5)	0.34 (0.05)
G. carolinianus (Litaker et al., 2009)	73 (9.1)	80 (11)	0.59	51 (7.2)	31 (4.4)	1.65	7.8 (1.3)	5.7 (0.9)	1.37	0.63 (0.09)	37 (5.4)	0.30 (0.04)
G. carpenteri (Litaker et al., 2009)	75 (6.2)	66 (6.7)	0.67	45 (6.4)	28 (4.0)	1.60	8.1 (6.1)	4.9 (0.9)	1.65	0.93 (0.17)	42(4.6)	0.43 (0.01)
G. excentricus (Fraga et al., 2011)	97 (8)	83 (10)	0.45	55*	30*	1.83*	8.6*	6.5*	1.32*	nd	34*	nd

Table 3.3: Continued

Species (References)	Cell size			Plate 2''''			Apical pore plate (Po)			2'/1'':2'/3''	Po pore	
	Depth	Width	L:W	Length	Width	L:W	Length	Width	L:W		Number	Diameter
G. pacificus (Litaker et al., 2009)	70 (4.7)	63 (3.6)	0.71	36 (3.5)	14 (3.5)	2.57	5.2 (0.3)	4.1 (0.4)	1.27	0.36 (0.10)	30 (2.1)	nd
G. polynesiensis (Litaker et al., 2009)	70 (6.3)	71 (4.9)	0.73	44 (4.7)	32 (2.9)	1.38	7.2 (0.4)	6.4 (0.3)	1.12	nd	38 (3.4)	nd
G. scabrosus (Nishimura et al., 2014)	63 (5.4)	58 (5.5)	0.64	33 (0.6)	15 (2.0)	2.18	6.3 (0.6)	4.4 (0.6)	1.46	0.75 (0.12)	29 (3.7)	0.36 (0.03)
G. silvae (Fraga & Rodríguez, 2014)	69 (8)	64 (9)	0.72	43*	32*	1.34*	5.8*	4.8*	1.21*	nd	30*	nd
G. toxicus (Litaker et al., 2009)	93 (5.7)	83 (2.3)	0.65	56 (3.6)	37 (2.4)	1.51	9.4 (0.6)	6.3 (0.3)	1.49	nd	41 (3.3)	0.43 (0.04)
G. balechii (Fraga et al., 2016)	57 (0.3)	60 (0.3)	0.65	28*	14*	2*	5.3*	3.5*	1.51*	0.64 (0.14)	25*	nd
G. cheloniae (Smith et al., 2016)	60 (12.2)	63 (9.0)	0.52	30	16.2	1.85	7.0–7.9	4.4–5.6	1.4 −1.7	nd	30–32	0.37
G. lapillus (Kretzschmar et al., 2016)	41 (3.3)	39 (3.2)	nd	nd	nd	nd	5.2–6.1	3.6–3.7	nd	nd	28–34	nd

3.4.2.1 *Gambierdiscus caribaeus*

Cells of *Gambierdiscus* (strain T6PRGam28 and T6PRGam29) are 76 ± 4.4 µm in depth and 74 ± 5.3 µm in width, with the depth-to-width ratio of 1.03 (Table 3.3). The cells are round and anterio-posteriorly compressed, with reticulate-foveated thecal surface (Figure 3.2). The Plate 2′′′′ is broad, long and pentagonal in shape. The cells have symmetric Plate 3′′, and the apical pore plate (Po) is elliptical and fishhook-shaped. Plate 4′ is pentagonal shape, wedged and broad. Cells of strain T6PRGam29 possessed rectangular, long and asymmetric 2′ (Figure 3.2B and C).

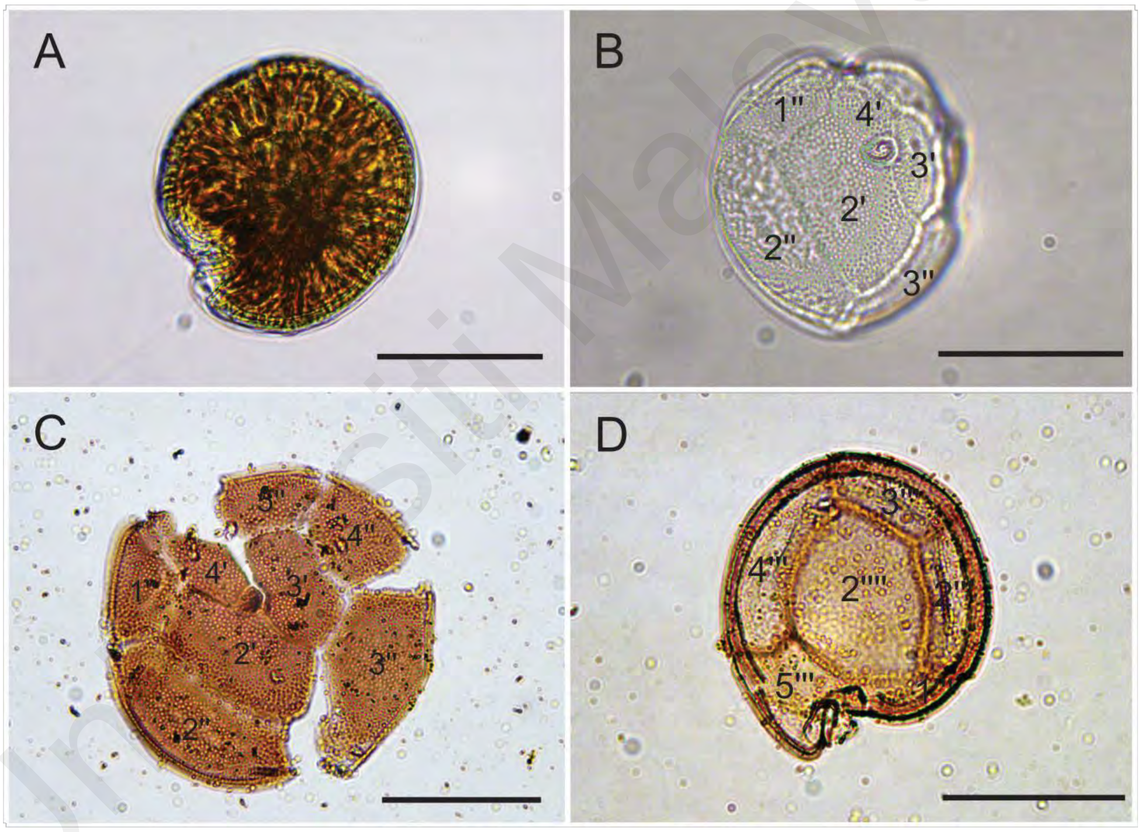


Figure 3.2: ***G. caribaeus*. (A) LM view of *G. caribaeus*. (B) Apical view of theca without staining. (C) Apical view of IF stained theca. (D) Antapical view of IF stained theca. Scale bars, 50 µm.**

3.4.2.2 *Gambierdiscus balechii*

Cells of *Gambierdiscus* (strain T6PRGd03N, T6PRGd07N and T6PRGd12N) are photosynthetic, anterio-posteriorly compressed with cell dimensions ranged from 59 ± 6.3 µm in depth and 60 ± 6.7 µm in width, with the depth-to-width ratio of 0.69 (Table 3.3). Besides, they were 41 ± 4.6 µm long (Table 3.3). Thecal surface of this *Gambierdiscus* cell is reticulate-foveated. Under LM, the cells are round and slightly intended in the ventral area, in both apical or antapical view (Figure 3.3C, D and E). Cells possessed broad, wedged, hexagonal Plate 4′ and hatched, long, asymmetric Plate 2′, with the ratio of suture 2′/1″ to 2′/3″ of 0.64 ± 0.11. Plate 3′ is asymmetrical (Figure 3.3B and C). The Po is oval and fishhook-shaped with 5.6 ± 1.1 µm length, 4.1 ± 0.8 µm, and L:W ratio of 1.4 (±0.2) that contained marginal pore of 28 ± 3.8 pores (Figure 3.3F). Plate 3″ of this cell is asymmetrical. In the hypotheca, Plate 2″″ is narrow, long and pentagonal in shape with mean length ranged between 30 ± 3.2 µm, width of 18 ± 2.4 µm, and length/width ratio of 1.72 (Figure 3.3D). The cingulum was narrow and deeply excavated, with descending one girdle width (Figure 3.3E). The sulcus was deep and funnel like, with the posterior area covered with a list near the margins of Sp and 5‴ (Figure 3.3D and E).

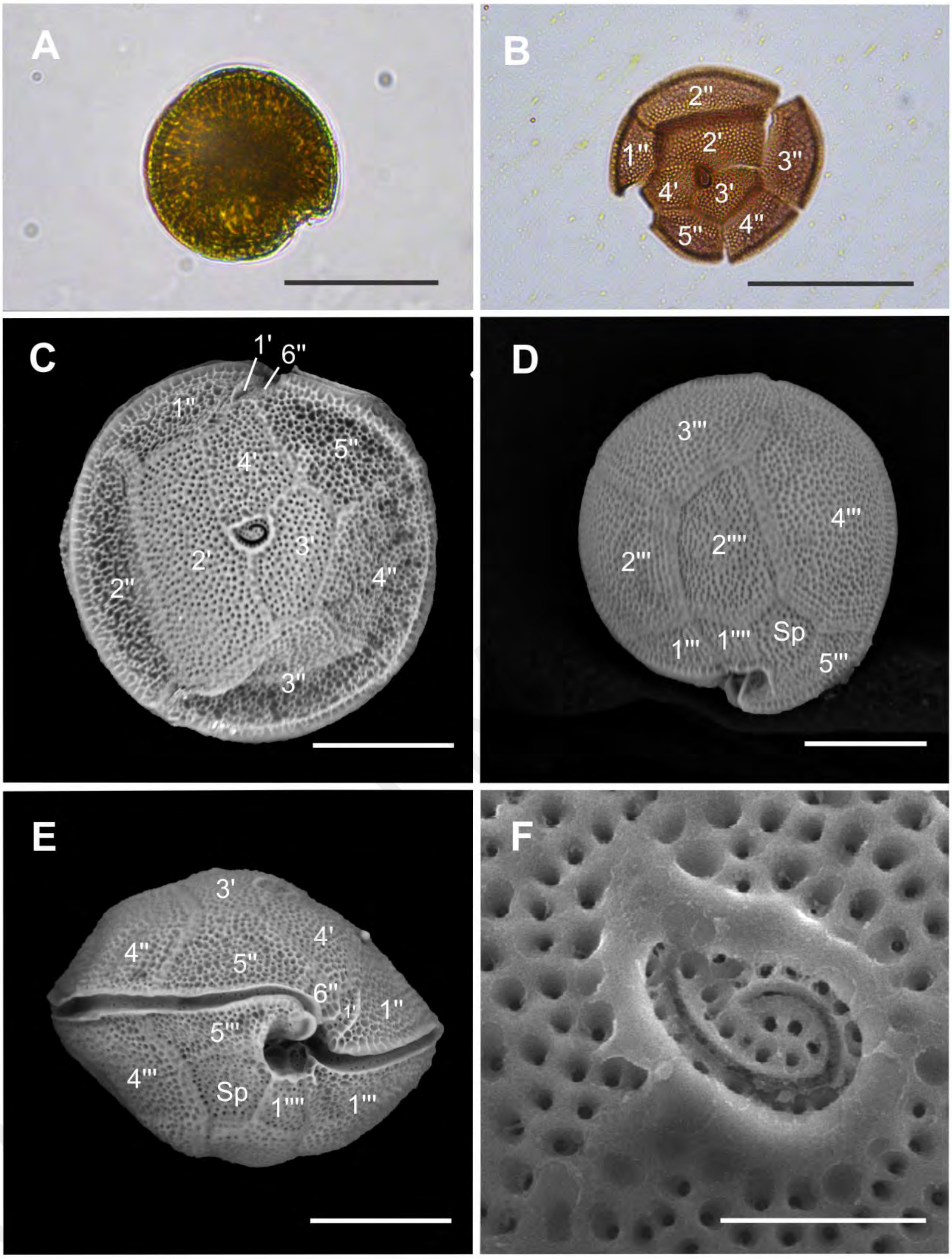


Figure 3.3: ***G. balechii*. (A) LM images (B) Apical view of IF stained theca plate. Scale bar: 50 µm. SEM, (C) Apical view, (D) Antapical view, (E) Ventral view; Scale bar: 20 µm, (F) Po plate; bar, 5 µm .**

3.4.2.3 *Gambierdiscus* sp. type 7

Cells of strain T6PRGd03N, T6PRGd07N and T6PRGd12N were photosynthetic and the thecal surface was reticulate-foveated. Cells were anterio-posteriorly compressed, depth 59 ± 4.9 µm, width 60 ± 5.7 µm and depth/width ratio of 0.99. Plate 2′ is hatched, long and asymmetric with 2′/1″ to 2′/3″ ratio range between 0.61 ± 0.09. The 4′ is broad, wedged, and pentagonal and the 3″ is asymmetric (Figure 3.4C and D). Apical pore plate is ellipsoid shaped with characteristic fishhook-shaped apical opening that had average length ranged between 4.7 ± 1.0 µm, width 6.1 ± 0.9 µm and length-to-width ratio of 0.76 (Figure 3.4G). The number of marginal pore of this *Gambierdiscus* was between 29 ± 2.1 pores with pore diameter range from 0.23 ± 0.08 µm. Average length of 2⁗ range between 29 ± 3.7 µm, width 18 ± 1.6 µm, length to width ratio of 1.62 with characteristics of broad, long and pentagonal shape.

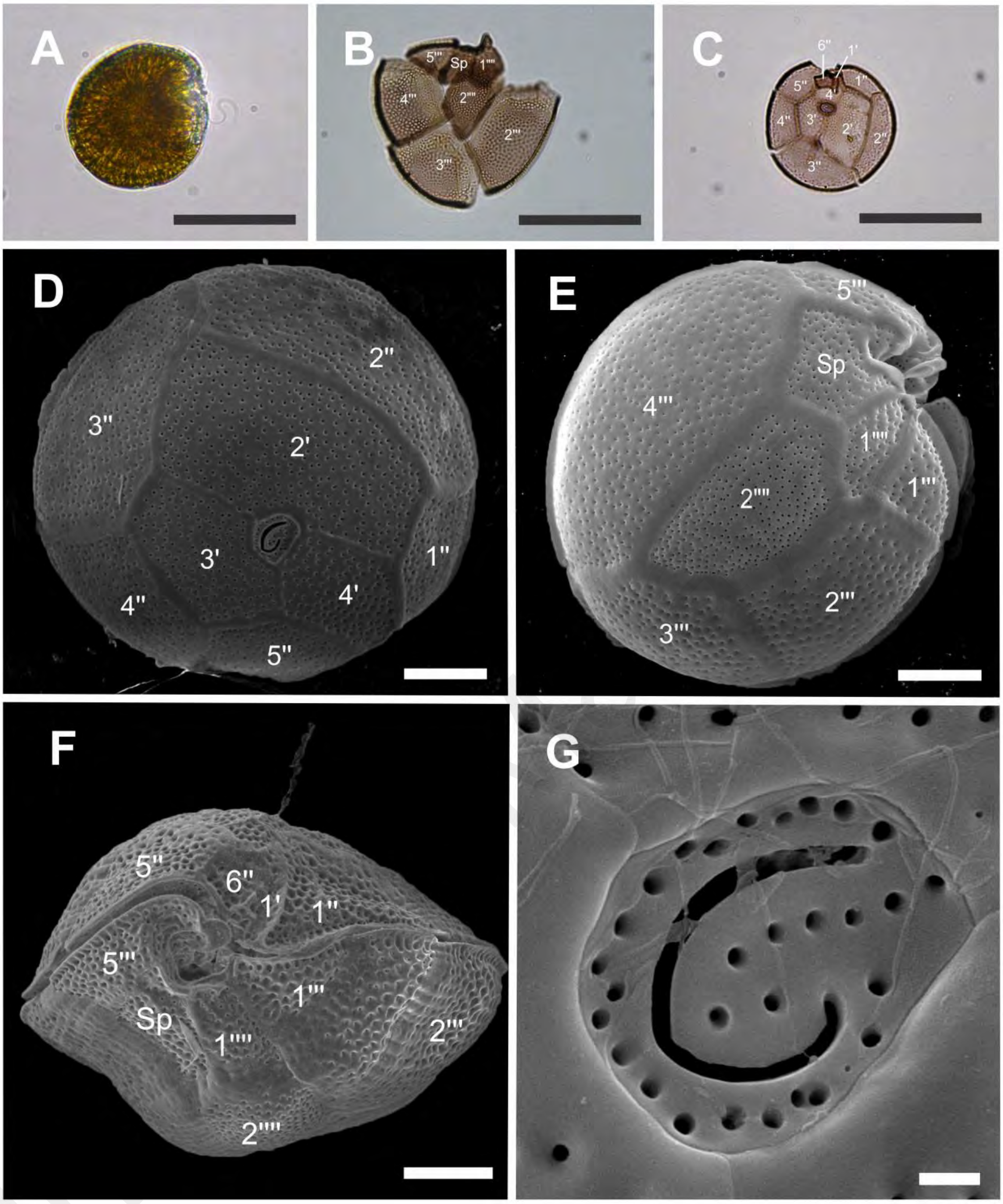


Figure 3.4: *Gambierdiscus* sp. type 7. (A) LM images, (B) Antapical view of IF stained thecae, (C) Apical view of IF stained thecae; Scale bar: 50 µm. SEM images (D) Apical view, (E) Antapical view, (F) Ventral view; Scale bar: 10 µm, (G) Po plate; bar, 1 µm.

3.4.3 Phylogenetic inference

DNA sequences obtained in this study were compared with DNA sequence deposited in the GenBank. Sequences were aligned with its closely related taxa and genetic relationships were examined using MP, ML and BI. D8-D10 LSU and SSU phylogenetic analyses showed that the tree was well resolved where the monophyletic clade of *Gambierdiscus* was clearly separated from outgroup, *Fukuyoa* spp. with a very strong bootstrap support (Figure 3.5 and 3.6).

Both trees had revealed three strongly support monophyletic lineages: Clade X (*G. carolinianus*, *G. polynesiensis*, *G. silvae*, *Gambierdiscus* sp. type 3 and 4); Clade Y (*G. caribaeus*, *G. carpenteri* and *Gambierdiscus* sp. type 2); and Clade Z, which form major group of *Gambierdiscus* consisting of *G. australes*, *G. excentricus*, *G. belizeanus*, *G. scabrosus*, *G. balechii*, *G. lapillus*, *G. pacificus*, *G. toxicus*, and two ribotype, *Gambierdiscus* sp. type 5 and ribotype 2 (Figure 3.5 and 3.6). LSU rDNA sequences of T6PRGd12N, T6PRGd07N and T6PRGd03N were identical to *Gambierdiscus* sp. type 6 which also known as *G. balechii* sequences that available in GenBank. Additionally, strain T6PRGam29 and T6PRGam28 were clustered together with *G. caribaeus* as a separate group, sister to *G. carpenteri*. Meanwhile, sequence of *Gambierdiscus* strains GdPS03, GdPS04, and GdPS05 was clearly separated and formed a sister group with one of the *Gambierdiscus* ribotype, *Gambierdiscus* sp. type 5, with a strong bootstrap value (Figure 3.5). Besides, SSU phylogeny analysis also showed that *Gambierdiscus* strain GdPS04 was clade out from its sister group (*G. lapillus*, *G. balechii* and *Gambierdiscus* sp. type 5) (Figure 3.6). This indicates that *Gambierdiscus* strain GdPS03, GdPS04, and GdPS05 might be a new ribotype in *Gambierdiscus* genus, herein referred as *Gambierdiscus* sp. type 7.

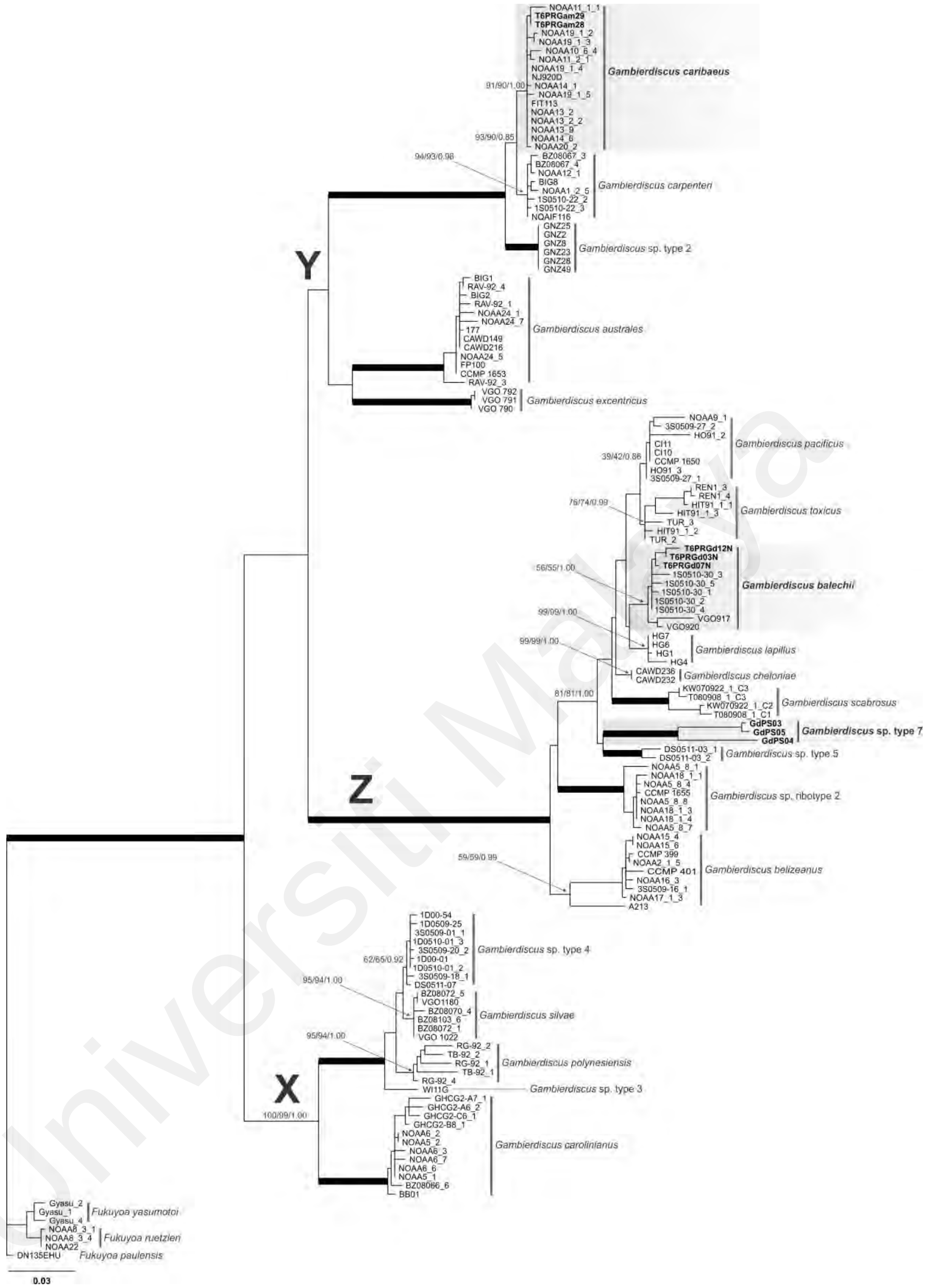


Figure 3.5: LSU (D8-D10) rDNA based phylogeny of *Gambierdiscus* species with *Fukuyoa* genus as an outgroup. Sequences obtained in this study are bold-type. Thick lines indicate MP/ML boostraps of 100% and PP at 1.00. Clade X-Z represents different monophyletic groups.

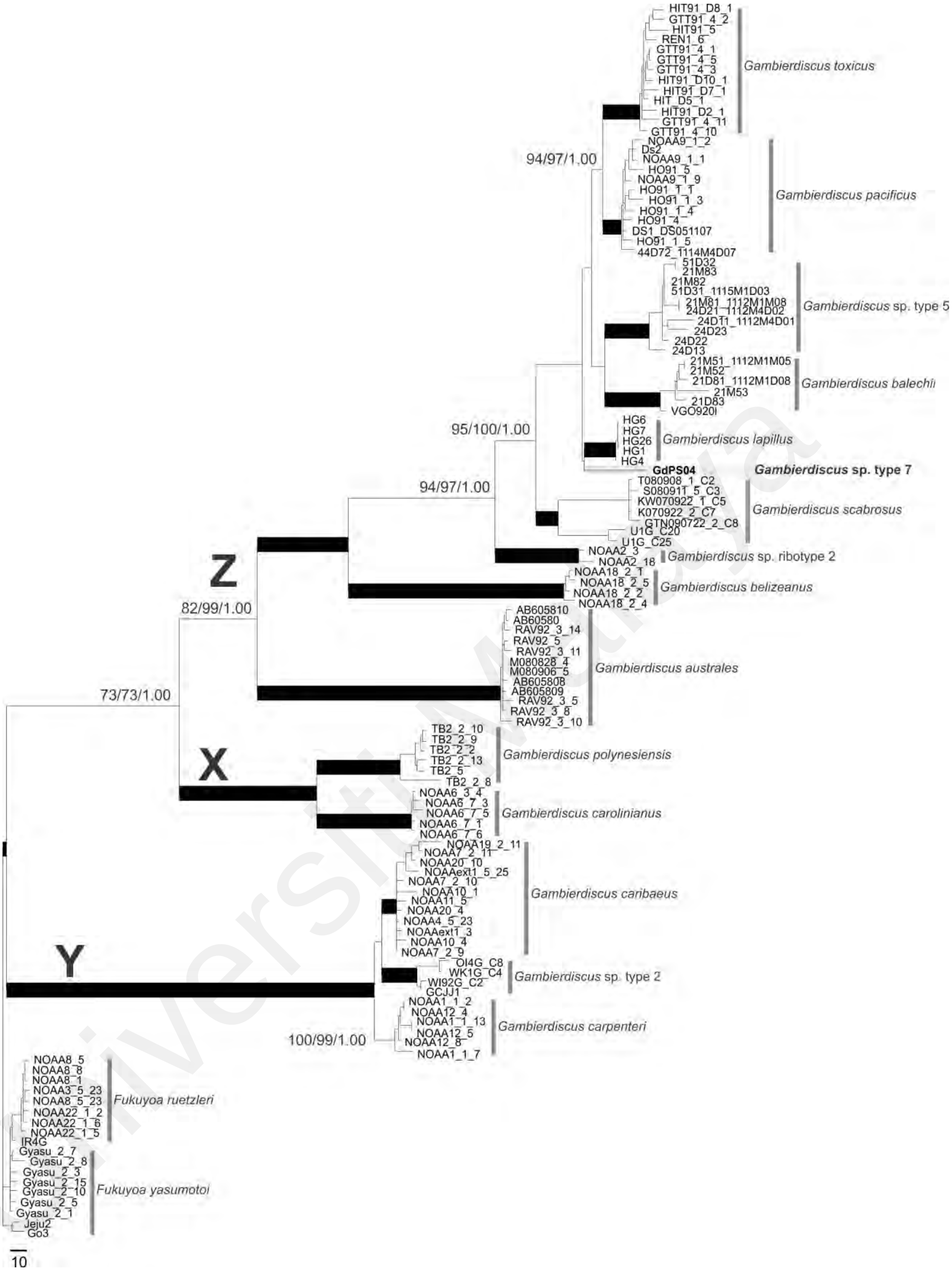


Figure 3.6: ML tree based on SSU rDNA dataset of Gambierdiscus species. Thick lines indicate MP/ML boostraps of 100% and PP at 1.00. Taxa in bold indicate sequence obtained in this study. Clades X-Z represents different monophyletic group.

3.4.4 Character state evolution

All morphological characteristics of species in this genus were derived from the literature description of Adachi and Fukuyo (1979), Faust (1995), Holmes (1998), Chinain et al. (1999), Litaker et al. (2009), Leaw et al. (2011), Fraga et al. (2011), Nishimura et al. (2014), Fraga and Rodriguez (2014), Gomez et al. (2015) and Fraga et al. (2016). A total of 13 morphological characters were found and numerically coded for 13 strains of *Gambierdiscus* spp. and 3 strains of *Fukuyoa* spp. (Table 3.4). All morphological traits of *Gambierdiscus* spp and *Fukuyoa* spp. were analyzed and matrices constructed (Table 3.5.)

Table 3.4: Morphological characters of *Gambierdiscus* analysed and their possible character states.

	Character	Character states	Description
A	Cell shape	0	Globular
		1	Reticulated-foveated
B	Cell length	0	Length of 30 – 40 µm
		1	Length of 40 – 50 µm
		2	Length of 50 – 60 µm
		3	Length of 60 – 70 µm
C	Cell width	0	Width of 30 – 50 µm
		1	Width of 50 – 70 µm
		2	Width of 70 – 90 µm
D	Cell depth	0	Depth of 40 – 60 µm
		1	Depth of 60 – 80 µm
		2	Depth of 80 – 100 µm
E	Po marginal pores abundance	0	10 – 20
		1	20 – 30
		2	30 – 40
		3	40 – 50
F	Po plate	0	Ellipsoid, fishhook shaped/comma-shaped
		1	Elongated, narrow fishhook-shaped
		2	Oval, fishhook-shaped

Table 3.4: Continued

Character		Character states	Description
G	4’ plate	0	Broad, wedged, hexagonal
		1	Broad, wedged, pentagonal
		2	Narrow, wedged, pentagonal
		3	Narrow, wedged, hexagonal
H	2’ plate	0	Hatched, long, asymmetrical
		1	Rectangular, long, symmetrical
I	3” plate	0	Symmetric
		1	Asymmetric
J	2”” plate	0	Broad, long, pentagonal
		1	Narrow, long, pentagonal
K	Cingulum	0	Narrow & deeply excavated
		1	Lipped
		2	Descendent
		3	Equivocal
L	Sulcus	0	Deeply concaved
		1	Deep
		2	Broad
		3	Short
		4	Hollow
		5	Equivocal
M	Thecal surface	0	Heavily-areolated
		1	Smooth

Table 3.5: Distribution of the character states among *Gambierdiscus* and *Fukuyoa* species for the 13 characters used in the character state evolution. Symbol '?' represents missing data, '+' equivalent to, '/' equivalent to or, and '-' as inapplicable.

Species	Characters and character states												
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
G. australes	1	0	1	1	2	0	2	1	1	1	?	?	1
G. balechii	1	?	0/1/2	0/1	1	2	0	0	1	1	2	?	0
G. belizeanus	1	1	1	1	1	0	3	0	0	1	?	1/3	0
G. caribaeus	1	2/3	2	2	3	0	1	1	0	0	0	1	1
G. carolinianus	1	2	2	1	3	0	1	0	1	0	1	1	1
G. carpenteri	1	2	2	2	3	0	1	1	1	0	0	1	1
G. cheloniae	1	0	1/2	0/1	2	2	1	0	1	0	0	?	0
G. excentricus	1	0	2	2	2	2	2	1	1	1	2	1/4	1
G. lapillus	1	?	0	0	1/2	2	0	0	1	1	0	1	0
G. pacificus	1	2	1	0	2	0	2	1	0	1	?	?	1
G. polynesiensis	1	1	1	1	3	0	1	0	1	0	?	?	1
G. scabrosus	1	0	1	1	1	0	3	1	1	1	0	1	0
G. silvae	1	1	2	2	2	2	0	0	1	0	0	3	1
G. toxicus	1	2	2	2	3	0	1	0	1	1	0	0	1
Gambierdiscus. sp. type 7	1	1	1	0/1	1	0	1	0	1	1	0	1	0
F. ruetzleri	0	2	0	0	3	1	2	0	0	1	?	1	1
F. yasumotoi	0	3	1	0	1	2	3	2	1	1+3	?	2	1
F. paulensis	0	2/3	0	0	2	0	1	1	1	0	2	1/2	1

All morphological characteristics of *Gambierdiscus* and *Fukuyoa* have been well described and some of the morphological features are taxonomic informative and useful in identifying and discriminating species of *Gambierdiscus* and *Fukuyoa*. Among the characters scored and the characters mapped on the tree, the most consistent morphological characters appear to be cell shapes (Figure 3.7, character A) and the thecal surface (Figure 3.7, character M). Character A supported the two major clades, with the states of anterio-posteriorly compressed and globular morphology distributed in G1 and G2, respectively (Figure 3.7).

F. ruetzleri and *F. yasumotoi* share the same cell shape but could be easily distinguish based on cell size; where *F. ruetzleri* is smaller than *F. yasumotoi*, while *F. paulensis* is intermediate in size between *F. ruetzleri* and *F. yasumotoi* (Figure 3.7, character B). Besides, these three *Fukuyoa* species can also be differentiate based on the number of marginal pore in the apical pore plate (Po) plate as *F. paulensis* possessed marginal pores between 30 – 40 pores, *F. yasumotoi* contained 20 – 30 pores and *F. ruetzleri* around 40 – 50 pores (Figure 3.7, character E).

In distinguishing species among the anterio-posteriorly compressed species, they can be differentiated based on their thecal surface where only *G. cheloniae*, *G. lapillus*, *G. balechii*, *G. scabrosus*, *G. belizeanus* and *Gambierdiscus.* sp. type 7 possessed reticulate-foveated thecal surface (Figure 3.7, character M). Most taxa in G1 and G2 are characterized by the number of marginal pores between 40 – 50 pores, except *G. pacificus* that contained 30 – 40 pores (Figure 3.7, character E). *Gambierdiscus caribaeus* was morphologically similar to *G. carpenteri* where 11 out of 13 described morphological characters appeared to be identical, except cell length (Figure 3.7, character B) and the symmetrical feature of 3″ plate (Figure 3.7, character I).

Apart from that, our character state analysis revealed that the state of ellipsoid, fishhook-shaped or comma-shaped of Po is common among the species of

Gambierdiscus with the exception of *G. excentricus*, *G. silvae* and *G. balechii* that have oval, fishhook-shaped Po (Figure 3.7, character F). *G. silvae*, *G. balechii*, *G. scabrosus* and *G. belizeanus* have the wedged, hexagonal shape of 4′. However, these four species can be differentiate by the size of the 4′ plate where *G. silvae* and *G. balechii* have a broad 4′ plate while the other two species has a narrow 4′ plate (Figure 3.7, character G). For the characters of cingulum and sulcus, most species were equivocal, but several were coded as missing data as no information is available (Figure 3.7, character K and L). The diagnostic characters and their states that used in differentiating the species of *Gambierdiscus* and *Fukuyoa* species are illustrated in Figure 3.8.

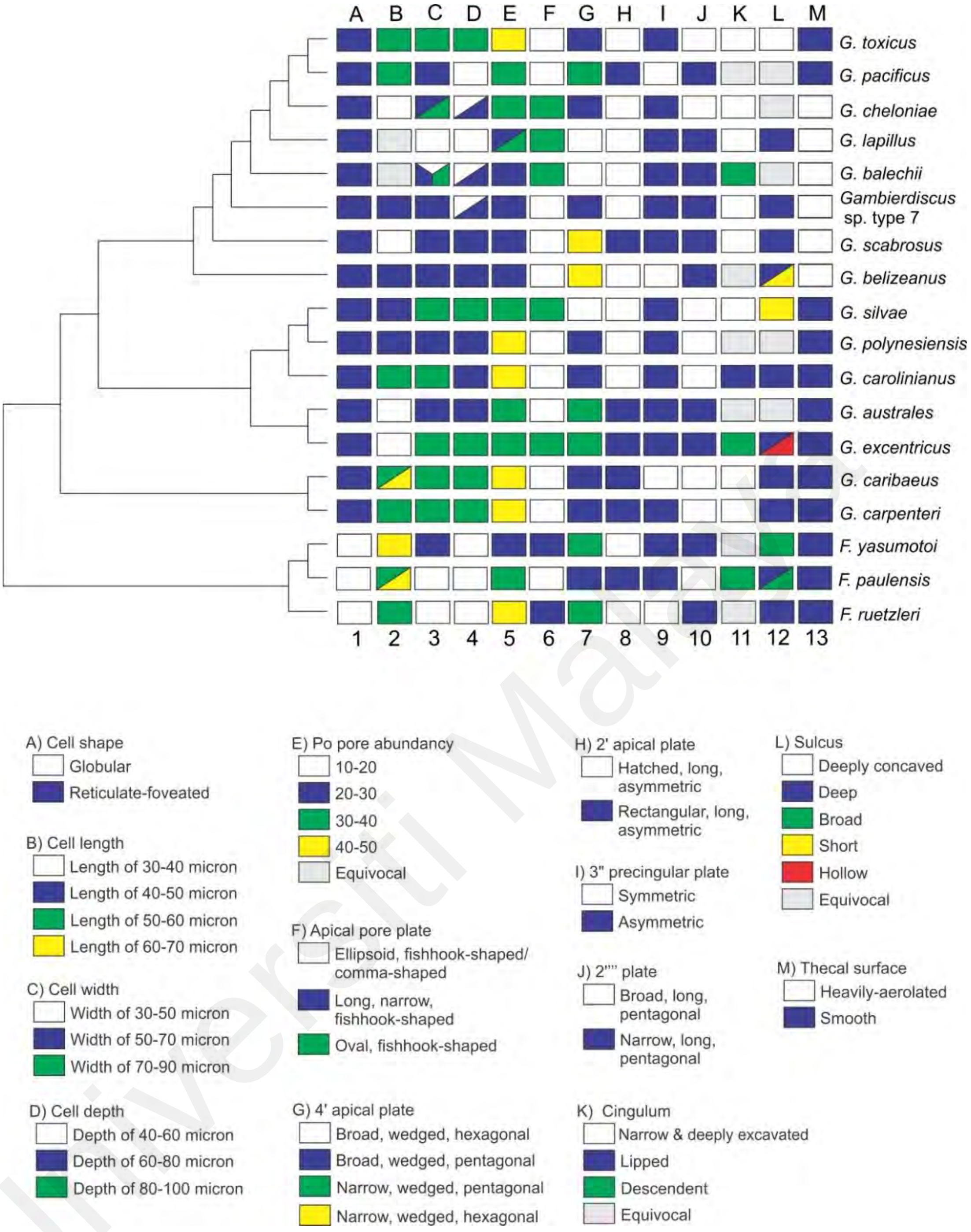


Figure 3.7: Mapping of character states of each species of *Gambierdiscus* and *Fukuyoa* with their 13 morphological characters

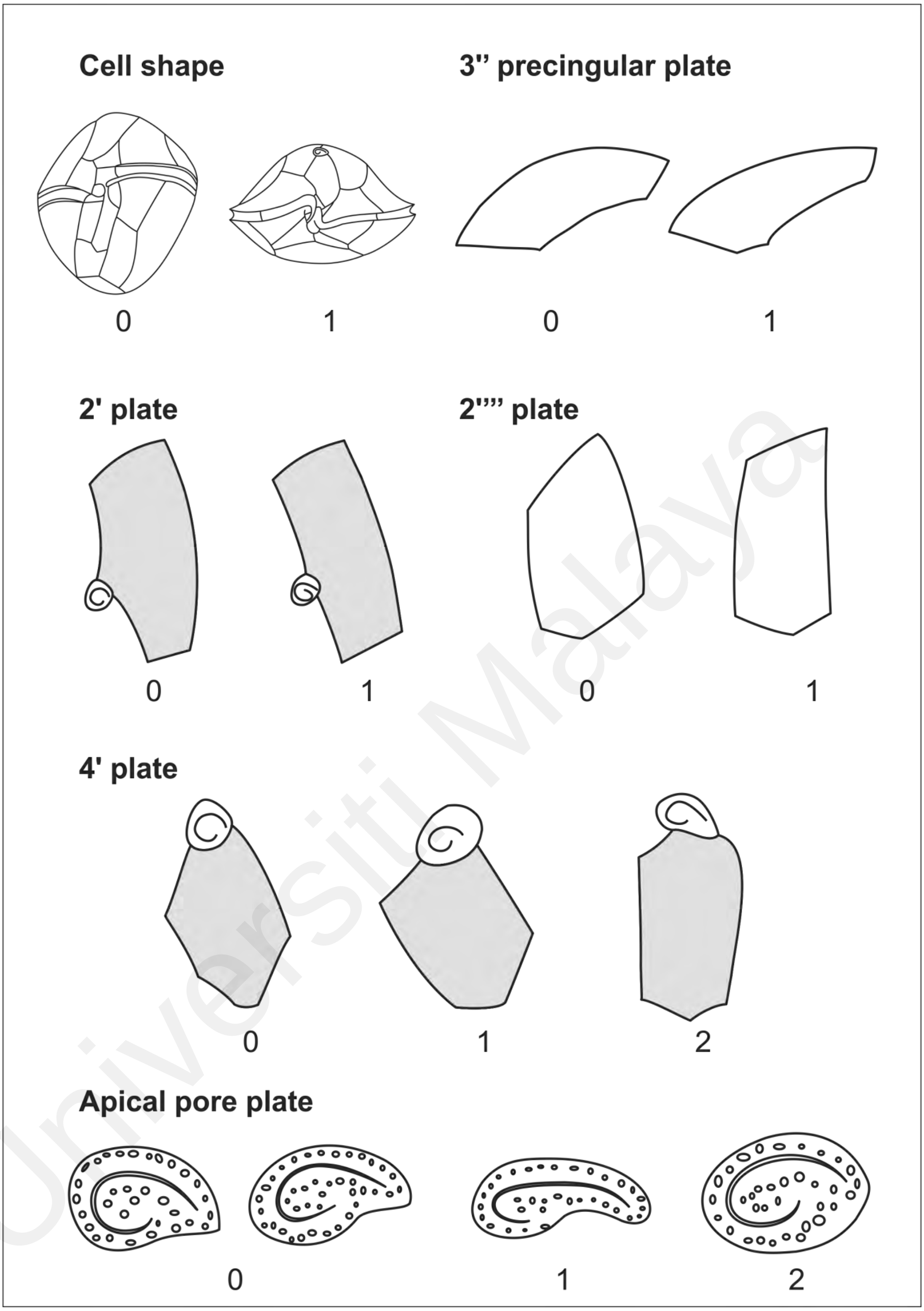


Figure 3.8: A schematic drawing of some morphological characters. Number below indicates the character states

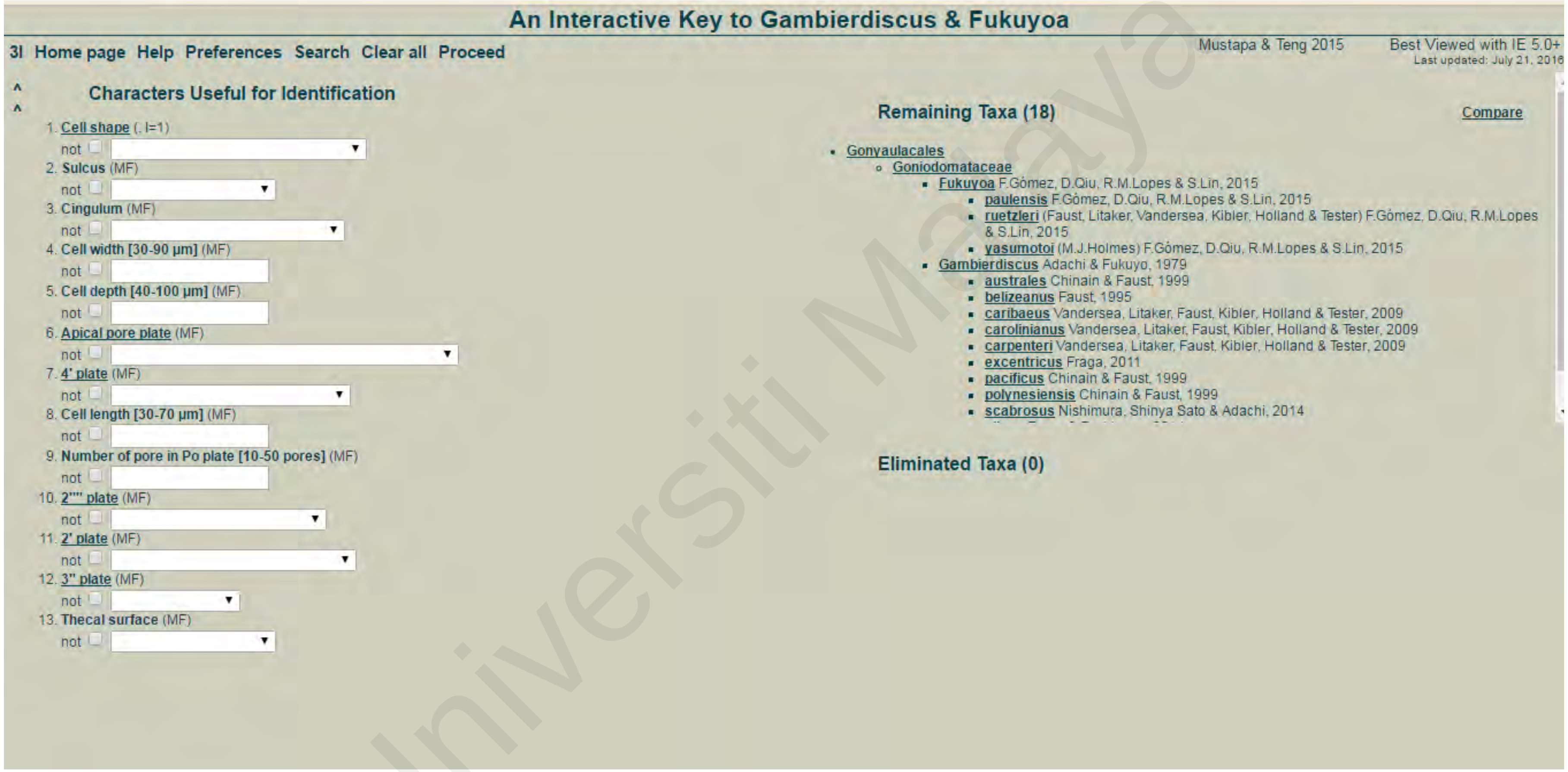
3.4.5 3i web-based interactive identification key

A total of 13 described species of *Gambierdiscus* and three described species of *Fukuyoa* were compiled in the 3i taxonomic database (Figure 3.9). The 3i key was based on the character state matrix that input in Microsoft Access 2010 (Microsoft Inc.). Here, the "character form" shows the list of the characters, where each character linked to a set of character states. Thus, 13 morphological characters are included; some are numerical characters which are based on the morphometric measurements.

The web-based interface of the key is accessible via http://dmitriev.speciesfile.org/key.asp?key=Bacillariales&lng=En&i=1&keyN=1. Species identification started by choosing a state of any character from the drop-down box. The ranges of valid values were shown in a square bracket for the characters with numerical values. This database also provides global distribution of the species and references.

After the <Proceed> button is pressed, the list of taxa that fit the search criteria was updated and displayed in the web-based interactive identification key of *Gambierdiscus* and *Fukuyoa* species website. The identification of the species may begin after user input the data obtained from the morphometric measurements. Character states were chosen based on the states that had been keyed in and the numerical character was input in the range form. The <not> small box under the character was for the missing character information where the character automatically will be eliminated from the search after ticking it. Schematic drawing of the morphological characters linked to the each of the morphological characters to assists users for the identification (Figure 3.10A).

Non-target species results were shown in the eliminated taxa section while the results of the species were shown in the remaining taxa section. The detail result of the target species were link with diagnosis, distribution, description, studied material, and reference of the species. Accurate GPS location was marked with blue dot showed in the distribution part were also linked with the reference (Figure 3.10B). Besides that, the <Compare> button was used to make comparison between two or more species of *Gambierdiscus* and *Fukuyoa* or comparison between genus *Gambierdiscus* and *Fukuyoa* (Figure 3.10C). <Preferences button> can be used if the user wants to modify the interface and the set of character displayed in website (Figure 3.10D).



An Interactive Key to Gambierdiscus & Fukuyoa

Mustapa & Teng 2015

Best Viewed with IE 5.0+
Last updated: July 21, 2016

Characters Useful for Identification

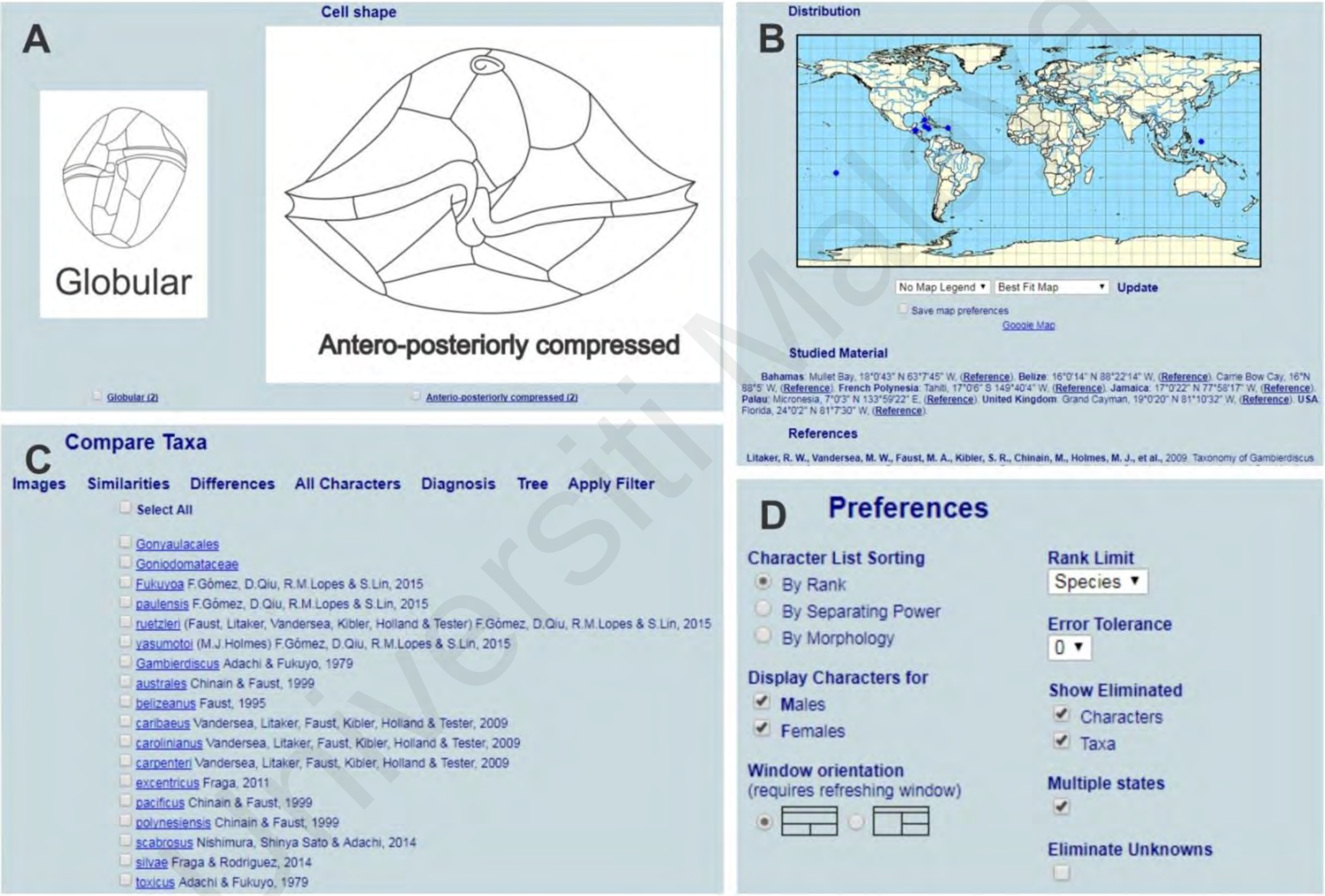
1. Cell shape (, l=1)
not ☐
2. Sulcus (MF)
not ☐
3. Cingulum (MF)
not ☐
4. Cell width [30-90 µm] (MF)
not ☐
5. Cell depth [40-100 µm] (MF)
not ☐
6. Apical pore plate (MF)
not ☐
7. 4' plate (MF)
not ☐
8. Cell length [30-70 µm] (MF)
not ☐
9. Number of pore in Po plate [10-50 pores] (MF)
not ☐
10. 2'''' plate (MF)
not ☐
11. 2' plate (MF)
not ☐
12. 3'' plate (MF)
not ☐
13. Thecal surface (MF)
not ☐

Remaining Taxa (18)

- Gonyaulacales
  - Goniodomataceae
    - Fukuyoa F.Gómez, D.Qiu, R.M.Lopes & S.Lin, 2015
      - **paulensis** F.Gómez, D.Qiu, R.M.Lopes & S.Lin, 2015
      - **ruetzleri** (Faust, Litaker, Vandersea, Kibler, Holland & Tester) F.Gómez, D.Qiu, R.M.Lopes & S.Lin, 2015
      - **yasumotoi** (M.J.Holmes) F.Gómez, D.Qiu, R.M.Lopes & S.Lin, 2015
    - Gambierdiscus Adachi & Fukuyo, 1979
      - **australes** Chinain & Faust, 1999
      - **belizeanus** Faust, 1995
      - **caribaeus** Vandersea, Litaker, Faust, Kibler, Holland & Tester, 2009
      - **carolinianus** Vandersea, Litaker, Faust, Kibler, Holland & Tester, 2009
      - **carpenteri** Vandersea, Litaker, Faust, Kibler, Holland & Tester, 2009
      - **excentricus** Fraga, 2011
      - **pacificus** Chinain & Faust, 1999
      - **polynesiensis** Chinain & Faust, 1999
      - **scabrosus** Nishimura, Shinya Sato & Adachi, 2014

Eliminated Taxa (0)

Figure 3.9: The web interface of 3i key to the species of *Gambierdiscus* and *Fukuyoa*.



Studied Material

Bahamas: Mullet Bay, 18°0'43" N 63°7'45" W, (Reference). **Belize**: 16°0'14" N 88°22'14" W, (Reference). Carrie Bow Cay, 16°N 88°5' W, (Reference). **French Polynesia**: Tahiti, 17°0'6" S 149°40'4" W, (Reference). **Jamaica**: 17°0'22" N 77°58'17" W, (Reference). **Palau**: Micronesia, 7°0'3" N 133°59'22" E, (Reference). **United Kingdom**: Grand Cayman, 19°0'20" N 81°10'32" W, (Reference). **USA** Florida, 24°0'2" N 81°7'30" W, (Reference).

References

Litaker, R. W., Vandersea, M. W., Faust, M. A., Kibler, S. R., Chinain, M., Holmes, M. J., et al., 2009. Taxonomy of Gambierdiscus

☐ Select All

☐ Gonyaulacales
☐ Goniodomataceae
☐ Fukuyoa F.Gómez, D.Qiu, R.M.Lopes & S.Lin, 2015
☐ paulensis F.Gómez, D.Qiu, R.M.Lopes & S.Lin, 2015
☐ ruetzleri (Faust, Litaker, Vandersea, Kibler, Holland & Tester) F.Gómez, D.Qiu, R.M.Lopes & S.Lin, 2015
☐ yasumotoi (M.J.Holmes) F.Gómez, D.Qiu, R.M.Lopes & S.Lin, 2015
☐ Gambierdiscus Adachi & Fukuyo, 1979
☐ australes Chinain & Faust, 1999
☐ belizeanus Faust, 1995
☐ caribaeus Vandersea, Litaker, Faust, Kibler, Holland & Tester, 2009
☐ carolinianus Vandersea, Litaker, Faust, Kibler, Holland & Tester, 2009
☐ carpenteri Vandersea, Litaker, Faust, Kibler, Holland & Tester, 2009
☐ excentricus Fraga, 2011
☐ pacificus Chinain & Faust, 1999
☐ polynesiensis Chinain & Faust, 1999
☐ scabrosus Nishimura, Shinya Sato & Adachi, 2014
☐ silvae Fraga & Rodriguez, 2014
☐ toxicus Adachi & Fukuyo, 1979

Character List Sorting
● By Rank
○ By Separating Power
○ By Morphology

Display Characters for
☑ Males
☑ Females

Window orientation
(requires refreshing window)
● ○

Rank Limit
Species ▾

Error Tolerance
0 ▾

Show Eliminated
☑ Characters
☑ Taxa

Multiple states
☑

Eliminate Unknowns
☐

Figure 3.10: Auto-generated functions that available in the 3i key to species of *Gambierdiscus* and *Fukuyoa*.

3.5 Discussion

Clonal cultures of *Gambierdiscus* from Pulau Rawa and Sibu were successfully established in this study. All aseptic technique had been applied during the whole process of establishment of culture such as preparation of culture vessel, medium preparation and sub-culturing. The dinoflagellates were successfully isolated but only 33 clonal cultures were successfully established into cultures. Some of them were dead after several days of isolation because of the contamination of culture plate. The cultures were overgrown by bacteria which can contaminate cultures and died off easily during the whole process. Besides, growth of dinoflagellate is strongly related to the seawater temperature, salinity, nutrient concentrations as well as the presence of the epiphytic bacteria (Chateau-Degat et al., 2005). Therefore, in order to maintain the viability of the cultures, routine of subculturing of the clonal cultures of *Gambierdiscus* was needed.

To date, genus *Gambierdiscus* consists of 14 described species. All morphological characteristics and molecular data of the species in this genus have been well described in the previous studies (Adachi & Fukuyo, 1979; Chinain et al., 1999; Litaker et al., 2009; Fraga et al., 2011; Leaw et al., 2011; Fraga & Rodríguez, 2014; Kretzschmar et al., 2016; Smith et al., 2016). *Gambierdiscus* species were identified or characterized based on their cell size and shape, architecture of thecal plates as well as cell surface morphology. This is because some of the plates could be identified without ambiguity as the measurement of the plates served as informative information in distinguishes the *Gambierdiscus* species (Litaker et al., 2009; Fraga et al., 2011).

A total of two species of *Gambierdiscus* were identified from Pulau Rawa and one species from Pulau Sibu. Cell size of *G. caribaeus* strain T6PRGam28 and T6PRGam29 was in the range of 76 ± 4.4 µm in depth and 74 ± 5.3 µm, which agree with size of *G. caribaeus*, *G. carolinianus* and *G. carpenteri* decribed by Litaker et al. (2009). *G.*

caribaeus has a wedged and hexagonal shape plate 4′, a symmetrical 3″ and a long 2⁗. Both plate 4′ and 2⁗ are broad. The apical pore plate is ellipsoid, fishhook or comma-shaped, as *G. carpenteri* and *G. carolinianus*. In general, *G. caribaeus* was very similar with *G. carpenteri* but differ from it as *G. caribaeus* has hexagonal shape of 4′ while *G. carpenteri* has pentagonal shape of 4′. Both *G. carolinianus* and *G. caribaeus* possessed long and asymmetrical 2′, but hatched shape of 2′ in *G. carolinianus* while rectangular shape in *G. caribaeus*.

Gambierdiscus strain T6PRGd03N, T6PRGd07N and T6PRGd12N had a reticulate-foveated thecal surface unlike *G. pacificus*, *G. toxicus*, *G. australes*, and *G. excentricus* that has smooth thecal surface (Litaker et al., 2009; Fraga et al., 2016). The cells resembled *G. balechii*, *G. belizeanus*, *G. pacificus*, *G. scabrosus*, *G. cheloniae*, *G. lapillus* and *Gambierdiscus* sp. type 5 when observed under LM and SEM. All three strains were believed to be *G. balechii* because of its 2′, 4′ and 3″ plate. *G. balechii* has an asymmetrical plate 3″ same as *G. lapillus* and *G. scabrosus* but differ from *G. belizeanus* as it has a symmetrical 3″ (Litaker et al., 2009; Fraga et al., 2016; Kretzschmar et al., 2016). *Gambierdiscus. balechii* can be discriminated from *G. scabrosus* by the shape of 2′ which is hatched shape in *G. balechii* and rectangular shaped in *G. scabrosus* (Kretzschmar et al., 2016). Although *G. balechii* has similar Po pore abundancy with *G. lapillus*, but they can be differentiated by their size because cell dimension *of G. lapillus* is smaller compare to *G. balechii* (Table 3.3). Besides, 4′ plate of *G. balechii* is broad, wedged and hexagonal, unlike *G. scabrosus* and *G. belizeanus* that has narrow and wedged 4′.

A new ribotype of *Gambierdiscus* strain GdPS03, GdPS04 and GdPS05, referred as *Gambierdiscus* sp. type 7 was discovered in this study. Previous study also reported 5 ribotype of *Gambierdiscus*; *Gambierdiscus* sp. ribotype 2, *Gambierdiscus* sp. type 2, *Gambierdiscus* sp. type 3, *Gambierdiscus* sp. type 4 and *Gambierdiscus* sp. type 5 from

Atlantic and Pacific Ocean region, but not yet described (Litaker et al., 2010; Nishimura et al., 2013; Xu et al., 2014). Cell size of *Gambierdiscus* sp. type 7 was 59 ± 4.9 µm in depth, 60 ± 5.7 µm with depth/ width ratio of 0.99, similar as *G. cheloniae*, *G. balechii*, *G. scabrosus* and *G. belizeanus* (Table 3.3). According to the original description of *G. balechii*, its apical pore plate is oval and had fishhook-shaped slit while in *Gambierdiscus* sp. type 7 is usually ellipsoid with fishhook-shaped or comma-shaped slit opening (Fraga et al., 2016). *Gambierdiscus* sp. type 7 has a deep sulcus. Sulcal plates in *Gambierdiscus* type 7 was not being able to observed and determined as the attempt to dissect the plate was failed. Sulcal plates usually twisted and sunken into the cell which make it difficult to observed clearly (Nascimento et al., 2015). Until now, only a few *Gambierdiscus* species had described the number and structure of the sulcal plates. However, its different for genus *Alexandrium* because this species had distinct feature of sulcal plate which make it became one of the morphological taxonomic informative feature in identifying the species (Balech, 1995). In the hypotheca, the hatched shape plate 2′ of *Gambierdiscus* sp. type 7 also same as *G. cheloniae*, *G. balechii*, *G. scabrosus* and *G. belizeanus.* However, ratio of the 2′/1″ to 2′/3″ suture length of *Gambierdiscus* sp. type 7 was in the range of 0.61 ± 0.09 which differ from *G. scabrosus* that has range of 0.75 ± 0.12 and rectangular plate 2′. This morphological characters is unique and proved to be one of the morphological characteristics that can be used in delineating *Gambierdiscus* species (Litaker et al., 2009; Fraga et al., 2011).

The D8-D10 LSU rDNA phylogeny relationship between the *Gambierdiscus* species in this study were similar with the previous studies where the monophyletic clade of genus *Gambierdiscus* was clearly separated from the outgroup (Litaker et al., 2009; Litaker et al., 2010; Fraga et al., 2011; Nishimura et al., 2013; Fraga & Rodríguez, 2014; Xu et al., 2014; Nascimento et al., 2015). Both former *G. yasumotoi* and *G. ruetzleri* were include in the outgroup as these two species were transferred into the new genus *Fukuyoa* (Gómez et al., 2015). This gene is useful in delineating *Gambierdiscus* species (e.g., Chinain et al., 1999, Litaker et al., 2009) as the domain D8-D10 appeared to be more conserved and provide potential information in evaluating the taxonomy analysis of closely related species (Lenaers et al., 1991). However, SSU phylogeny gave more information in discriminating species than LSU, although the resulted topologies for both genetic markers were similar (Litaker et al., 2009; Fraga et al., 2011). This is because of the SSU gene domain evolved more slowly compare to the LSU gene which considered to be more informative in discriminating species at deeper branch (Litaker et al., 2009). Genetic data that include in analysis for two strains of *G. caribaeus* (T6PRGam28 and T6PRGam29) and three strains of *G. balechii* (T6PRGd03N, T6PRGd07N and T6PRGd12N) were clade together with original *G. caribaeus* and *G. balechii*, respectively. The long branching within three strains of *Gambierdiscus* sp. type 7 (GdPS03, GdPS04 and GdPS05) might be due to the appearance of pseudogene that caused variation in the genome of same species (Litaker et al., 2007). Nevertheless, *Gambierdiscus* sp. type 7 was clearly separated from its sister clade and might be appear to be a new species. However, further analysis need to be done in order to confirm the novelty of *Gambierdiscus* sp. type 7.

In order to distinguish various *Gambierdiscus* species, Litaker et al., (2009) introduced a dichotomous tree detailing the morphological characteristics of ten species of *Gambierdiscus*. As the species identification is crucial with the addition of new *Gambierdiscus* species recently, a hypothetical evolutionary tree based on the morphometric, shape and thecal structure was taken in this study. Character state evolution analysis had a showed a significant phylogenetic and taxonomic values in the characters studied (Mustapa et al., 2015). The globular morphology of *F. ruetzleri*, *F. yasumotoi* and *F. paulensis* has been acknowledged as a plesiomorphic trait, and forms a transition point and derived to the synamorphic anterio-posteriorly compressed trait.

Consistent with the previous study by Litaker et al. (2009) that the most obvious characteristics that can be used in distinguishing species in this genus is cell shape, before the two globular species of *Gambierdiscus* were transferred into a new genus. The development of the anterio-posteriorly compressed shaped of genera *Gambierdiscus* is believed due to the migration from planktonic to the benthic habitat as the shape and size of the species become more bigger as well as the compression of the anterior-posterior cell shape (Gómez et al., 2015). This support that genus *Fukuyoa* and *Gambierdiscus* shared a common ancestor as both possessed almost similar cell shape, but evolving during the time in order to allow it to adapt to its environment and help it survived.

Apart from that, character state evolution tree also revealed the inconsistency of the morphology of the sulcal plate among the *Gambierdsicus* species. This is because some of them were coded as missing data as there is no description in the literature. According to the Nascimento et al., (2015), there is not much studied were done on the sulcal region of *Gambierdiscus* species as the sulcus is twisted and forming a deep tight funnel which is difficult to observe and represent graphically under the microscope. Some of the previous studied also has different elucidation about this sulcal region. For example, numbers of sulcal plates were described as either 7 or 8 according to the studies of Adachi & Fukuyo (1979), Holmes (1998), Loeblich & Indelicato (1986) and Litaker et al., (2009). This is because of some of the study did not include S.p. plate as a part of the sulcus plate as this plate situated outside of the sulcus region and some of the plate was small and hard to detect.

Investigating the phylogenetic relevance of dinoflagellate morphological characters by mapping the morphological traits on the phylogenetic trees had provides an evolutionary insights into the relationships between the taxa, and the evolutionary shift of important morphological traits within the lineage (Leaw et al., 2005; Hoppenrath, 2017). In our character state analysis, the two fishhook- and comma-shape of apical pore plate were considered as similar. The characteristics of Po have been previously described as either fishhook-shaped or comma-shaped by various researchers even for the same species of *Gambierdiscus*. Therefore, in the character state coding, the state were assign based on shape of Po, viz. ellipsoid, elongated and oval.

The 3I web-based identification key to the species of *Gambierdiscus* and *Fukuyoa* was developed in order to assist taxonomist in species identification based on their morphological observation under the microscope. 3I has variety features that make it different with other web online interactive identification key. As mentioned, this database facilitate multi-entrance polytomous keys that allow users to enter more number of characters, character states and taxa (Dmitriev, 2006). Most importantly, the taxa can be arranged or ranked by user according to its hierarchic levels as well as including their nomenclature names. Illustrative images of the morphological characters were linked to the morphological characters in order to help user for identification of the species (Figure 3.10A). Some of the previous researcher described the characters by using different term even for the same species. Thus, in order to give user a clear view, morphological characteristics illustration was upload. This has given another advantage to 3I compare to other database that lack of morphology illustrations or images. Apart from that, 3I database can do comparison either to find differences or similarities of morphological characteristics between species and genus (Figure 3.10C). The data matrices that have been keyed in can be exported and used for phylogenetic analysis as well as generate a phenetic tree online. (Dmitriev, 2006). All this function could not be find in other database program like LucID and AlgaBase.

In contrast with the conventional identification keys, interactive identification key does not restricted the character use which allow user to include all characters of the taxon that can be used for identification purpose. Besides, character that is not clear or confirmed as well as its value (e.g. morphometric measurement of plate) can also be deleted or changed during the identification process. This program also is able to indicate any data error that has been made by user during the stage of the identification. Thus, it is important for every user to verify the accuracy of the identification with the original description or illustration of the taxon as an error when the assigning the character state by user, may lead to the wrong identification (Dallwitz et al., 2002).

3.6 Conclusions

The morphological observation of *G. caribaeus* and *G. balechii* from Pulau Rawa were in agreement with the previous taxonomic descriptions. Further analysis on *Gambierdiscus* sp. type 7 from Pulau Sibu is needed in order to verify as a new ribotype of *Gambierdiscus*.

The web interactive key to species of *Gambierdiscus* and *Fukuyoa* has provided a taxonomic database to assist species identification and can be used as one of the identification tool in field studies. The 3i interactive key and database of *Gambierdiscus* will be updated periodically.

CHAPTER 4: HABITAT PREFERENCE OF BENTHIC DINOFLAGELLATE *GAMBIERDISCUS* (DINOPHYCEAE) IN PULAU RAWA

4.1 Introduction

Ciguatera fish poisoning (CFP) is the common disease which is associated with the fish consumption in the tropical and subtropical region such as coral reef fish like barracuda, grouper and snapper (Lehane & Lewis, 2000; Friedman et al., 2007). It is estimated that ciguatera affecting ~50 000 people annually worldwide and currently there is no reliable, no clinically validated treatments and no quick test that can detect ciguatoxins after consumption of contaminated fish (Lewis & Vetter, 2016). People who suffered from CFP will have both gastrointestinal and neurological symptoms, like nausea, vomiting, diarrhea, headaches, muscle ache and reversal of hot and cold sensation. Some of the symptoms can persist for days to months or years after the exposure (Friedman et al., 2007; Friedman et al., 2008).

In recent years, research on benthic dinoflagellates has spread as the knowledge on benthic dinoflagellates is limited. This is because the species has similar morphological characteristics which make it difficult to distinguish under the light microscope and yet not all species are toxic. Although some of this species can be identified based on the plate patterns like 2′, 3″ and 2″″, the variability is very small and requires identification by molecular tools (Fraga et al., 2016). Thus, more studies are required to identify the species and determine the toxicity of the benthic dinoflagellate species that are found in the country.

Genus *Gambierdiscus* is the main source and cause of CFP. Cells of *Gambierdiscus* can be identified with its anterio-posteriorly compressed body shape with a circular narrow deep cingulum as well as a deep hollow sulcus. To date, there are 14 *Gambierdiscus* species that has been described, *G toxicus* (Adachi & Fukuyo, 1979), *G. belizeanus* (Faust, 1995), *G. australes*, *G. pacificus*, *G. polynesiensis* (Chinain et al., 1999), *G. caribaeus*, *G. carpenteri, G. carolinianus* (Litaker et al., 2009), *G. excentricus* (Fraga et al., 2011), *G. scabrosus* (Nishimura et al., 2014), *G. silvae* (Fraga & Rodríguez, 2014), *G. balechii* (Fraga et al., 2016), *G. lapillus* (Kretzschmar et al., 2016) and *G. cheloniae* (Smith et al., 2016)

The abundance of *Gambierdiscus* in Pulau Rawa, Terengganu was determined in this study. Field survey on habitat preferences for *Gambierdiscus* was also investigated. This was followed by habitat preferences experiment that was conducted in the laboratory on *G. balechii* and *G. caribaeus* and *Gambierdiscus* sp. type 7, a new ribotype species to examine the preference of *Gambierdiscus* towards different macroalgal hosts.

4.2 Literature Review

Gambierdiscus was name based on its shape and placed where it is found which is Gambier Island, French Polynesia (Adachi & Fukuyo, 1979). *Gambierdiscus* species were distributed circumtropically as they are found in the Atlantic, Pacific and Indian Ocean regions. As this toxin benthic dinoflagellate was distributed globally, the ciguatera fish poisoning cases also seems to expand. Ciguatera outbreak appeared to be increasing due to the demand of the fish trade and consumptions as well as disruption of coral reef caused by climate change and international tourism (Gingold et al., 2014; Chan, 2015).

Apart from that, information on the distribution of this species is still lacking because of the discriminating and discovery of a new species in *Gambierdiscus* genus. *G. scabrosus*, *Gambierdiscus* sp. type 2, 3, 4 and 5 have been reported from Pacific region where *G. scabrosus*, *Gambierdiscus* sp. type 2 and 3 are from Japan while *Gambierdiscus* sp. type 4 and 5 are from Marakei, Kiribati (Nishimura et al., 2013; Nishimura et al., 2014; Xu et al., 2016). Recently, three new *Gamberdiscus* species were described from the Pacific Ocean, *G. balechii* from Manado, Indonesia, *G.*

cheloniae from Cook Island and *G. lapillus* from Heron Island, Australia. So far, only *G. carolinianus*, *G. excentricus*, *G. silvae* and *Gambierdiscus* sp. ribotype 2 have been reported from Atlantic Ocean and the rest of described *Gambierdiscus* species were reported to be found from various island in both Pacific and Atlantic Ocean. Although there is no CFP cases outbreak in Arabian Sea, *G. toxicus*, *G. belizeanus*, *G. polynesiensis* and *G. australes* were reportedly found in the coastal water of Pakistan (Munir et al., 2011).

Gambierdiscus can be found attach on the seaweed, seagrass and corals, as well as in sediments and subtidal areas (Berdalet et al., 2012; Tester et al., 2014; Yong et al., 2018; Mustapa et al., 2019). *Gambierdiscus* species usually attach itself to the host by mucus thread that they formed, detach and swim around the host when there is sudden disturbance (Nakahara et al., 1996). This species prefer to grow in the shallow water with lower light intensity that has surrounding temperature around 21 °C to 31 °C, salinity range of 28 – 35 PSU, and with the presence of substrate like macroalgae for their habitat (Litaker et al., 2010; Parsons et al., 2012). Apart from that, previous studies also reported that this species has their own preferences for a certain algal host (Grzebyk et al., 1994; Cruz-Rivera & Villareal, 2006). Class of algae, surface area and structure of the algae, stimulatory compound or presence of chemical extract from the algae are the factors that influenced the habitat preference of *Gambierdiscus* towards its host (Carlson et al., 1984; Bomber et al., 1989a; Bomber et al., 1989b; Parsons & Preskitt, 2007).

Bleached corals grown with turf algae and macroalgae have been known to serve as better substrates for *Gambierdiscus* species compared to living corals (Hallegraeff, 2010). Grzebyk et al. (1994) detected high abundance of *Gambierdiscus* on dead corals. Dead coral surfaces usually colonized by turf algae which is composed of small, filamentous and fast-growing red algae (e.g., *Polysiphonia* sp., *Centoceras* sp., *Ceramium* sp.), green (e.g., *Cladophora* sp., *Chaetomorpha* sp.) and brown algae (e.g., *Giffordia* sp.), along with cyanobacteria, diatoms and coralline algae (Kendrick, 1991; Littler & Littler, 2013; Connell et al., 2014). It is likely that *Gambierdiscus* prefers turf algae as many studies found high abundance of *Gambierdiscus* on turf algae (reviewed in Cruz-Rivera and Villareal, 2006). However, the preference of *Gambierdiscus* species towards macroalgae is varied in different region. For example, *Gambierdiscus* species was found on *A. spicifiera* (Rhodophyceae) in Belize (Morton & Faust, 1997; Delgado et al., 2006). In contrast, this species was found on *Dictyota dicotoma*, *Padina* sp. and *Ulva lactuta* (Phaeophyceae) (Ballantine et al., 1988; Bomber et al., 1989a; Cruz-Rivera & Villareal, 2006). But Yasumoto et al. (1979) did not found any *Gambierdiscus* on *Ulva lactuta* in French Polynesia, as well as Parsons and Presskitt (2007) in Hawaii. The above examples had demonstrated that there are differences in algal host preferences for *Gambierdiscus* species in different region.

Previous studies claimed that extract elucidated from the macroalgae helps in favoring the growth of the *Gambierdiscus* , although some of them did not examined the algal extracts (Withers, 1981; Carlson et al., 1984; Grzebyk et al., 1994; Parsons et al., 2011; Rains & Parsons, 2015). It is believed that some of the host had produced compound extracts that might inhibit the growth of the species. This can be seen from the study by Parsons et al. (2011) in Hawaii where *Gambierdiscus* cell avoided contact from red algae; *Proteria hornemannii* and green algae; *Bryopsis* sp. whereas they grew and attach well on *Jania* and *Chaetomorpha*.

4.3 Material and Methods

4.3.1 Field survey on *Gambierdiscus* substrate preference

Field survey on *Gambierdiscus* substrate preference was carried out at Pulau Rawa, Terengganu (Chapter 3). This island has high diversity of coral reefs as well as other tropical marine resources, where the fringing reef is covered with various types of hard corals and soft corals at deeper water. However, there was some part of the reef area that were severely damaged and allowed macrophytes to cover the coral rubbles there. This has become a good spot for the habitat of BHAB communities. Sampling was undertaken monthly from April 2015 until January 2016. A non-destructive sampling method was adopted in this study (Berdalet et al., 2012; Tester et al., 2014). Artificial substrate sampling devices utilizing fiberglass window screens (Chapter 3) were deployed under the water for 24 hours by snorkeling or SCUBA diving (depth of ~3-10m) (Figure 4.1). The screens were collected after 24 hours and brought back to lab for further process.

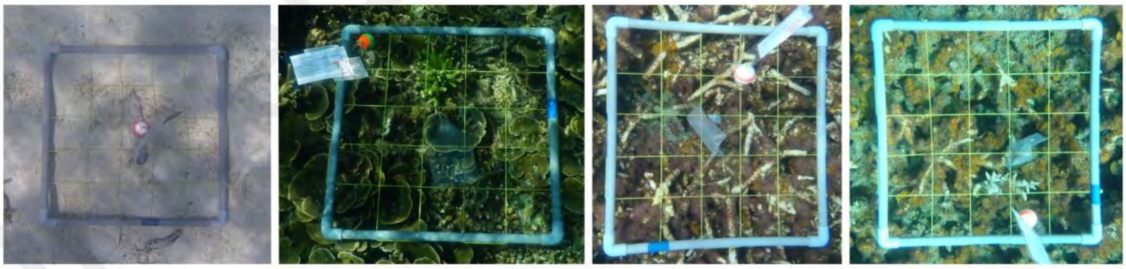


Figure 4.1: Artificial substrate deployed underwater.

Screens were shaken vigorously to detach the epiphytic cells. Samples were sieved through two sizes of sieves with 250 µm and 20 µm mesh. The cells retained in a 20 µm mesh sieve were back-washed into a 50mL falcon tube. Samples were preserved in acidic Lugol's solution for cell enumeration. Cell count was conducted in triplicate using a Sedgewick Rafter slide under a Leica DM750 light microscope (Leica Microsystem) at 100× magnification.

In order to establish *Gambierdiscus* abundance and substrate variability relationship, data from the field survey were condensed into two matrices which are *Gambierdiscus* abundance per site and substratum variables. Substratum types were determined by photoquadrat method where the image of the photoquadrat were reviewed and analyzed by using ImageJ 1.50b (National Institute of Health, USA; http://imagej.nih.gov/ij/), and the bottom coverage was then estimated as percent cover The 9 substratum variables that examined in this study were hard corals, bleached corals, sand, rubbles with turf algae, rubbles covered with sands, green fleshy algae, brown fleshy algae, red filamentous algae and other (e.g. clams and rocks). These data were then analyzed with canonical correspondence analysis (CCA) under 1000 permutations using PAST 3.08 (Hammer et al., 2001).

4.3.2 Experimental set-up for substrate preference of *Gambierdiscus*

In order to monitor the growth and behavior response like attachment attributes of *Gambierdiscus* towards the host, an experiment was conducted as described in Rains and Parsons (2015). This experiment was performed with four treatments of macroalgae. All macroalgae were selected either based on their common presence in Rawa Island or macroalgae that are known to associated with *Gambierdiscus* population based on the previous studies.

The macroalgae were identified to genus level based on its morphology and description from the previous studies (Kendrick, 1991; Littler & Littler, 2013; Connell et al., 2014). They are micro-filamentous turf algae comprised of coralline algae, filamentous red algae, *Polysiphonia* sp., *Ceramium* sp., and filamentous green algae, *Cladophora* sp.; *Laurencia*, corticated red algae (Rhodophyta); *Padina*; foliose macro-blade brown algae (Phaeophyta) and *Dictyota*, foliose micro-blade brown algae

(Phaeophyta) (Figure 4.2). *G. balechii* and *G. caribaeus* were identified from Pulau Rawa and *Gambierdiscus* sp. type 7 was from Pulau Sibu as detailed in Chapter 3.

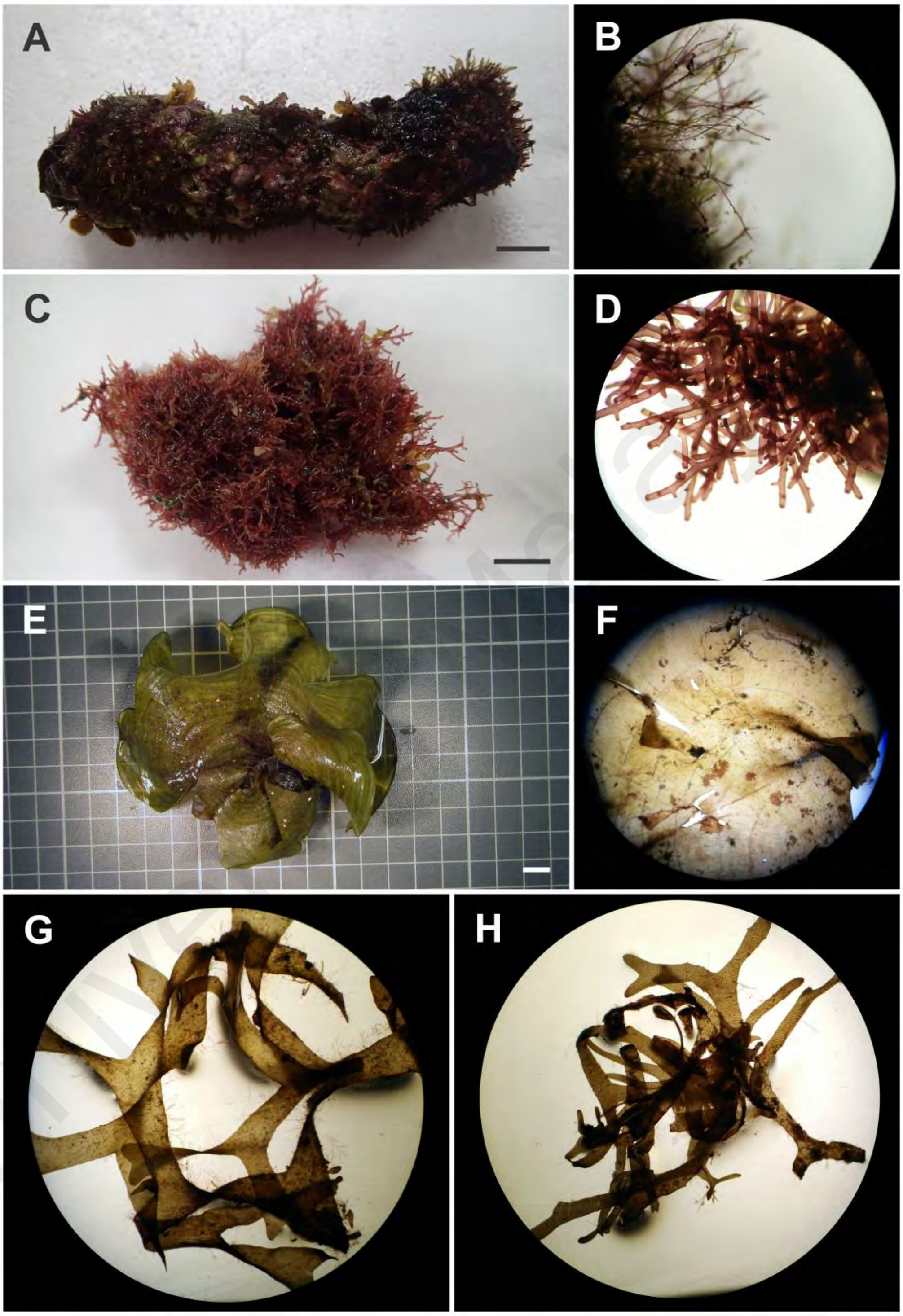


Figure 4.2: Macroalgae host that commonly found in Pulau Rawa and known to be associated with *Gambierdiscus* populations. (A-B) Turf algae; scale bar: 1 cm. (C-D) *Laurencia* sp.; scale bar: 1 cm. (E-F) *Padina* sp.; scale bar: 1 cm. (G-H) *Dictyota* sp.

Macroalgae fragments were collected by scuba diving and brought back to the lab for further process. Macroalgae thalli were shake vigorously, rinsed with filter seawater for at least five times, and sonicated in filter seawater to remove epiphytes. Each macroalga were cut into small fragments, weighted (approximately 0.04 g wet weight) and placed individually into separate wells of 12-wells culture plate that contained 6 mL of ambient filter seawater. The macroalgae were acclimated for a day and further examined microscopically to confirm no epiphyte attached. 10µl of *Gambierdiscus* cells were added to each well of a 12-well culture plate containing 6 ml of ESDK medium. The initial cell density in each well was determined in Day-1 by direct cell count. Macroalgal fragments were then placed individually into the separate wells. The culture plates were then sealed with Parafilm™ and maintained at 25 ± 0.5 °C under 12:12 h light: dark photo cycle. On day 2, 2 mL of ESDK medium was added. The experiment was conducted in triplicate, with four macroalgal hosts, four strains of *Gambierdiscus*; a control treatment for each *Gambierdiscus* strain was conducted in the same culture conditions but without algal substrates (4 algae + 1 control × 4 *Gambierdiscus* strains × triplicates = 60 wells). Water changes were done carefully with minimum cell loss by slowly removing 3 ml of water using micropipettes under the stereo microscope, and replaced back with 3 ml ESDK-enriched medium. Water changes were performed 1 – 2 times weekly on different days of cell counts to ensure the changes did not disturb the cells (Rains & Parsons, 2015). Cells were counted daily for the first 14 days followed by counting every two days thereafter. Cell enumeration was conducted by using an Olympus SZX10 stereo-microscope (Olympus, Japan) at 40× magnification.

Cells were counted both alive and unattached, or alive and attached, or in contact with the host, in each treatment on Day 10, 20 and 32. The relative abundances were determined to examine the differences of attachment attributes of each *Gambierdiscus* strains/species in each treatment. Specific growth rate, μ (day^{-1}), was calculated over the exponential growth phase using the following equation (Guillard 1973):

$$\mu = \frac{lnN_1 - lnN_0}{t_1 - t_0}$$

where N_0 and N_1 are the cell numbers at time t_0 and t_1, respectively.

Growth rates were calculated from the cell count data for each replicate obtained throughout the experiment (n = 3) and the mean growth rates of each treatment were computed. The normality of the data was first analyzed with Shapiro-Wilk test to assess the statistical significance in cell yields and growth rates within *Gambierdiscus* strains in all treatments. By depending on the data normality, one-way analysis of variance (ANOVA) with Turkey's pairwise comparison (normality) or non-parametric Kruskal Wallis with Dunn's multiple comparisons (non-normality) was performed. The coefficient of variation (CV) between conspecific strains was calculated for the intraspecific comparison. All data were statistically analyzed and presented in PAST 3.25 (Hammer et al., 2001)and GraphPad Prism 5.03 (GraphPad Software, Inc., USA).

4.4 Results

4.4.1 Natural substrate preference of *Gambierdiscus* species

A total of 106 artificial substrates sampling device has been deployed at various bottom microhabitats with different natural substrates. Based on the field survey in this study, *Gambierdiscus* abundances in Pulau Rawa, Perhentian Marine Park, Terengganu, varied spatio- and temporally; with maximum cell abundance reached up to 1200 cells/100 cm^2. The CCA ordination revealed the relationship between *Gambierdiscus* abundances and the benthic natural substrate variability, with the eigenvalues of 61.47% and 24.13%, for axis 1 and 2 respectively. The results showed that *Gambierdiscus* abundance was positively correlated with the natural benthic substrates of turf algae and hard corals as compared to other substrata (Figure 4.3). *Gambierdiscus* cells were less likely to be found in substratum types of sands and sand-covered rubbles. Cells of *Gambierdiscus* were also found attached to red-filamentous algae, bleached corals and brown fleshy macroalgae, however, the cell abundance was lower compared to those on the substratum of turf algae.

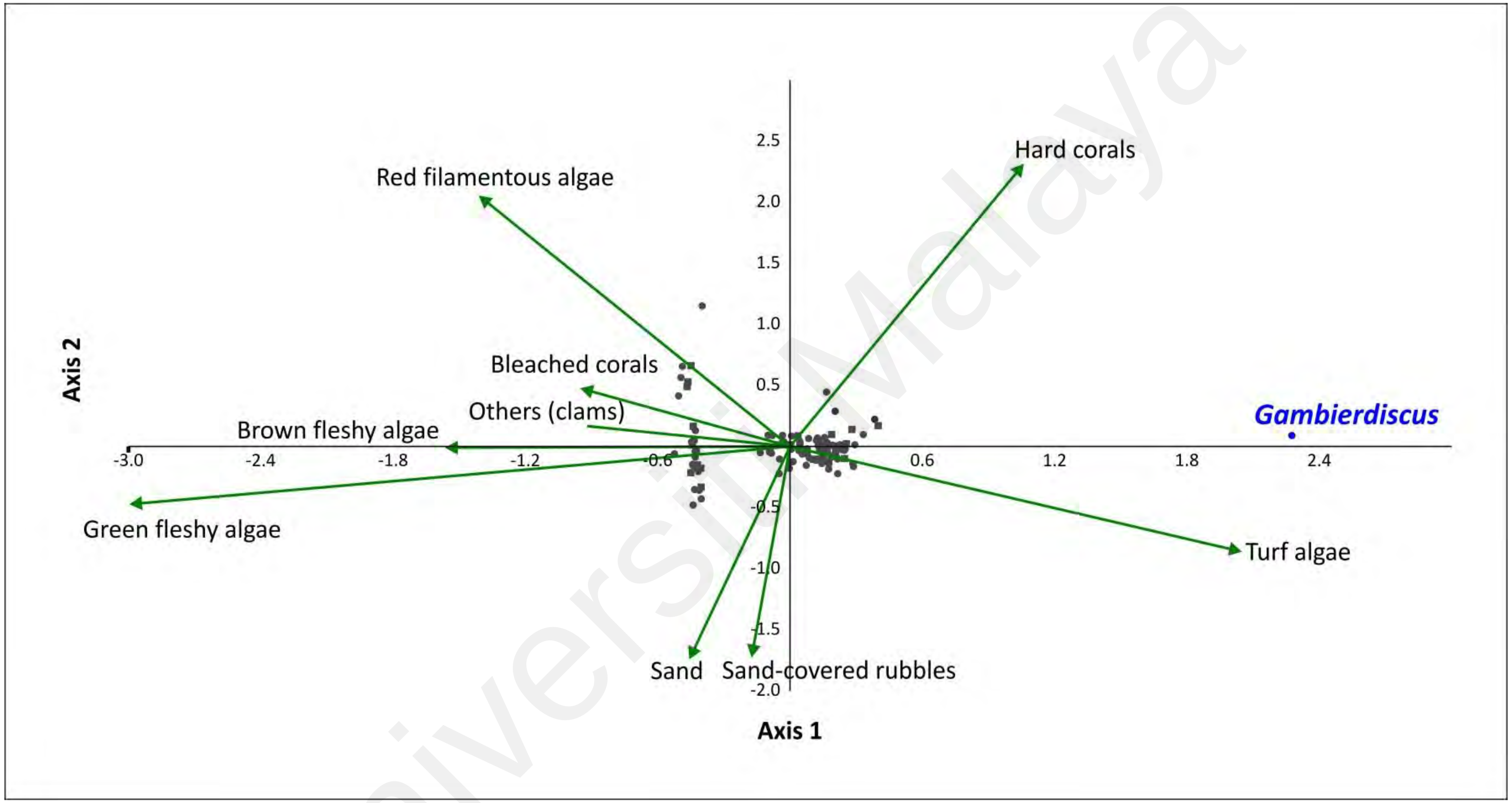


Figure 4.3: CCA showing the relationship between *Gambierdiscus* cell abundance in Pulau Rawa and the substrate variables (arrows). Black dots represent each artificial substrate samples where *Gambierdiscus* cell abundances were collected (site scores). Blue dot represents the species score, which is the *Gambiediscus* abundance.

4.4.2 Examination on substrate preference of *Gambierdiscus* in a laboratory setup

All four strains of *Gambierdiscus* species showed typical growth curves characterized by an exponential phase from Day 0 to Day 20, stationary phase from Day 20 to Day 30 and late phase starting from Day 30 (Figure 4.4). Overall, most of the *Gambierdiscus* strain in this experiment grew gradually with time. For the control treatments, the maximum cell yield were occurred on day 32-36, with the maximum yield of 464 ± 22 cells/well observed in *G. balechii* strain T6PRGd03N (Figure 4.4). Compared to the control treatment, the *Gambierdiscus* cells yield in all host treatment reached maximum on day 17-20 (Figure 4.4), but with maximum cell yields varied among treatments (Figure 4.5). The maximum growth rate in the control treatment was observed in *G. balechii* strains T6PRGd03N, while the lowest was in *Gambierdiscus* sp. type 7 GdPS03, where the mean growth rates ranged from 0.08 ± 0.01 day^{-1} to 0.16 ± 0.02 day^{-1} (Figure 4.5). Meanwhile, the growth rates of all four strains were increase rapidly in the presence of macroalgae host tested (Figure 4.4). However, there is no significant host preference for the macroalgae host tested in between-group comparisons on the growth response of *Gambierdiscus* in different host treatment (Kruskal-Wallis Dunn' comparison, $p > 0.05$) (Figure 4.5).

For *G. balechii*, the growth rates were higher in almost all host treatment as compared in the control (Figure 4.4). However, the results were not consistent between the two conspecific strains (CV values = 14 – 58%). For instance, strain T6PRGd03N grew better in *Dictyota* and *Padina* treatments (0.31 ±0.05 and 0.34 ±0.09 day^{-1}, respectively) versus controls (0.16 ± 0.02 day^{-1}; one-way ANOVA Tukey's; $p < 0.01$) (Figure 4.5). Although the growth rates of T6PRGd03N was higher in host treatments, the results showed no significant difference in the selective preference of the strains towards any macrolagal host tested (one-way ANOVA Tukey's, $p > 0.08$). Meanwhile, growth rates of T6PRGd07N were higher in turf, *Laurencia* and *Dictyota* treatments (0.22 – 0.25 day^{-1}) versus controls (0.12 ± 0.01 day^{-1}), except *Padina* treatment (one-way ANOVA Tukey's, p = 0.0216 – 0.0087). In term of cell yields, there were no significant differences among the treatments (Kruskal-Wallis, T6PRGd03N, *p* = 0.4641; T6PRGd07N, *p* = 0.1739), although maximum cell yields observed in T6PRGd03N among the host treatments were twice lower than those of control (Figure 4.4 and 4.5), whereas T6PRGd07N exhibited higher maximum cell yields in some host treatments when compared with controls (Figure 4.4 and 4.5).

For *G. caribaeus*, there was significant difference in growth variations among the treatments (Kruskal-Wallis test, *p* < 0.0001). The maximum cell yields of *G. caribaeus* was higher in turf and *Laurencia* treatments (80 – 100 cells/well), which consistent with the controls, unlike in the foliose algal treatments that only yielded 23 – 30 cells/well (Figure 4.5). Besides, started from the exponential phase, cell deformity was observed in this strain.

Additionally, this experiment also included *Gambierdiscus* species strain from other locality. As a result, the growth rates of *Gambierdiscus* sp. type 7 strain GdPS03 was higher in all treatments versus controls (one-way ANOVA Tukey's, *p* = 0.0228 – 0.0002), except *Padina* treatments Figure 4.4 and 4.5). Higher growth rates were observed in *Laurencia* and *Dictyota* treatments (0.23 ± 0.04 and 0.23 ± 0.01 day^{-1}, respectively. However, the growth of *Gambierdiscus* sp. type 7 cells was retarded after day-20 but not in controls (Figure 4.4).

Gambierdiscus behavior (attachment) in the presence of different macroalgal host was observed in this study. Most of the *Gambierdiscus* cells attached to the host in the beginning of the experiments and detached at the later time (Figure 4.6). From the observation, it showed that all *Gambierdiscus* strains had higher percentage of attachment which up to 100% of cell attached towards turf algae, except *G. caribaeus*.

Similarly, most of the *Gambierdiscus* cells were also observe attached to the algal thalli of *Laurencia*, but no cells of *G. caribaeus* were found attached to *Laurencia* in the early stage of the experiment (Figure 4.6). Meanwhile for *G. balechii*, there is significant different (two-way ANOVA, p = 0.0098) in host attachment of *G. balechii* strain T6PRGd07N towards different macroalgae host, but not significant in strain T6PRGd03N. The percentage of attachment of *G. balechii* strain T6PRGd07N was higher in turf algae treatment, while strain T6PRGd03N was higher in *Dictyota* treatment (Figure 4.6). Differently, the attachment of *Gambierdiscus* sp. type 7 GdPS03 was highest in turf algae and *Dictyota* treatment, while *G. caribaeus* T6PRGam29 showed higher host attachment to *Dictyota* in the early stage of the experiment (Figure 4.6). There was no significant correlation (Spearman r = 0.3015, p = 0.0373) between attachment and the growth rate over the *Gambierdiscus* strains tested.

For the changes in wet weight of host algae, *Padina* gained the biomass and *Laurencia* lost the biomass over the course of the experiment; turf algae, *Dictyota* gained or lost the biomass in the presence of different *Gambierdiscus* species (Appendix C).

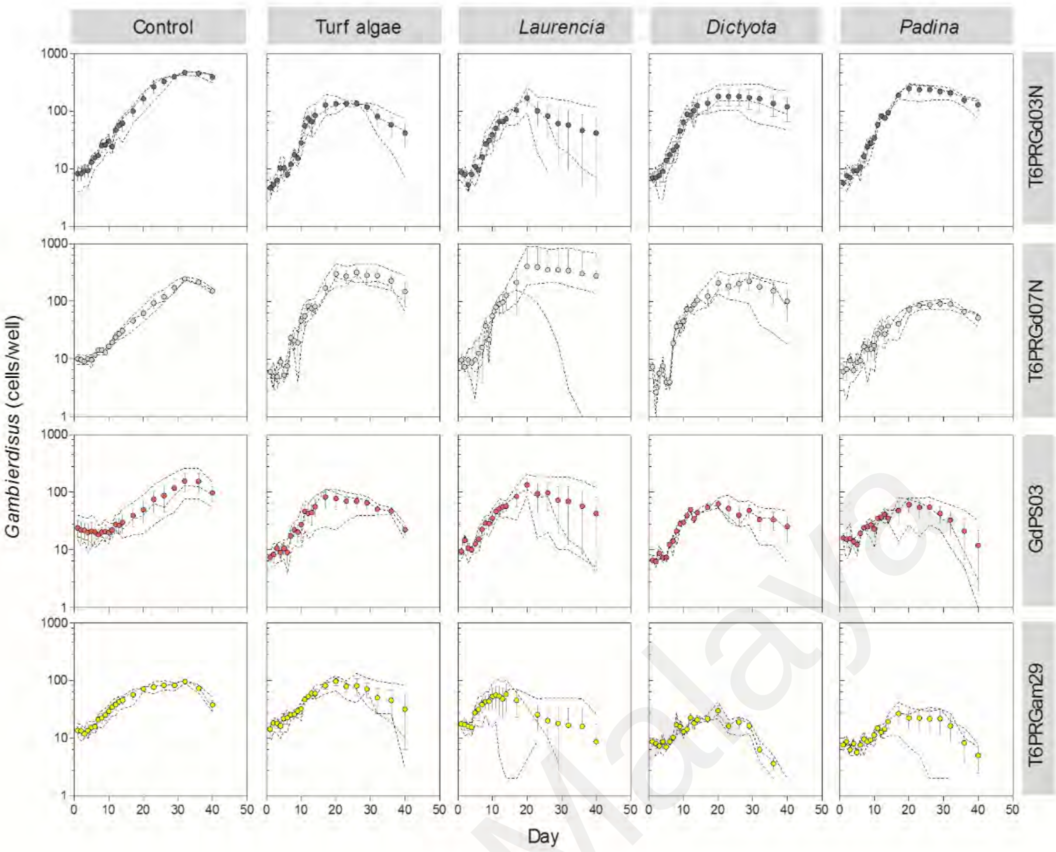


Figure 4.4: Growth of *Gambierdiscus* species across controls and host treatments throughout the 40 days studied period. T6PRGd03N and T6PRGd07N, *G. balechii*; GdPS03, *Gambierdiscus* sp. type 7; and T6PRGam29, *G. caribaeus*. Dots represent means with standard error bars, each replicate is represented by connecting dash line.

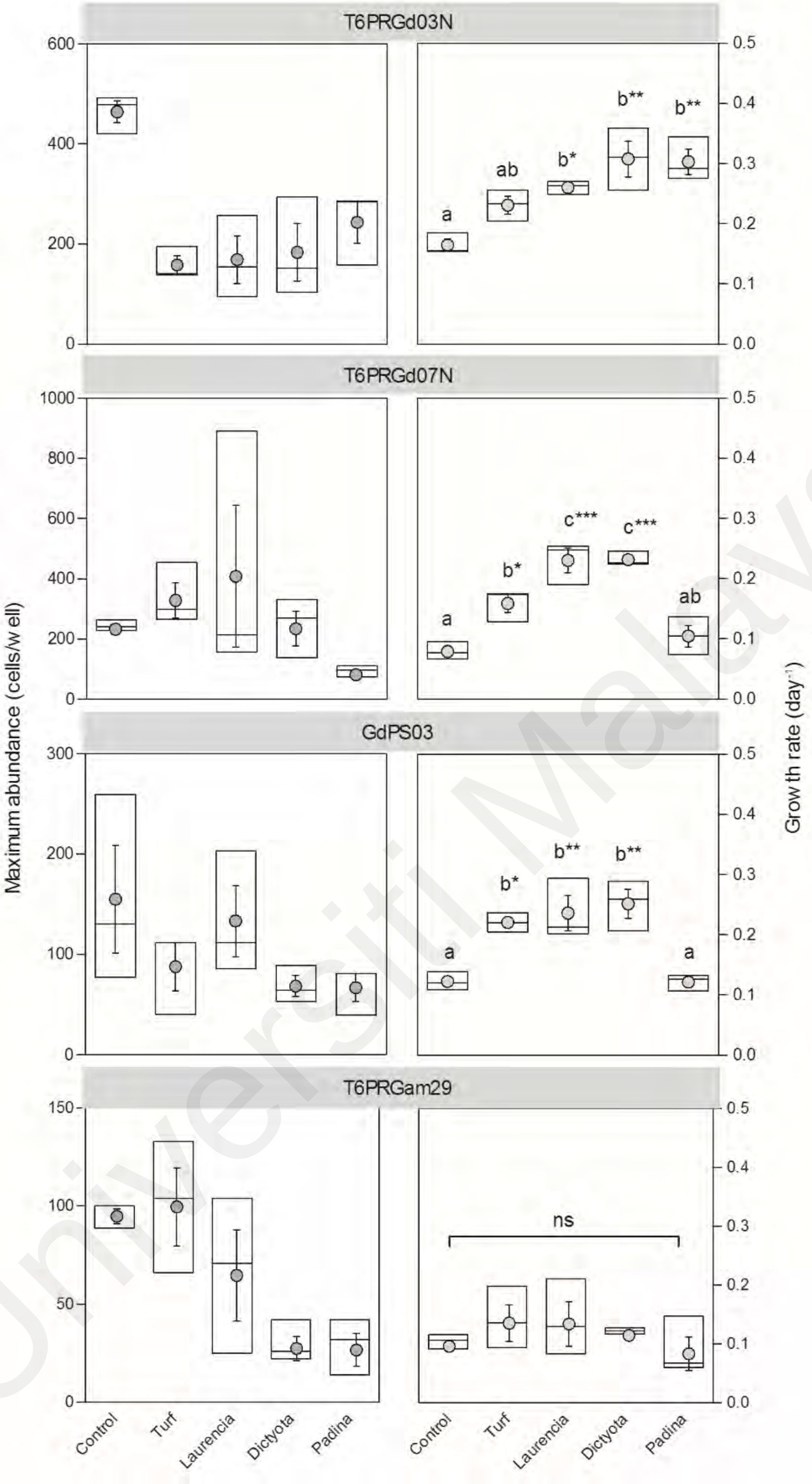


Figure 4.5: Maximum abundances and growth rates of *Gambierdiscus* species in different macroalgal host treatments. Dots represent means with standard error bars, rectangles display values of minimum, median, and maximum. Letters denote significant differences among treatments (post hoc Tukey's test, *p < 0.05, **p < 0.01, ***p < 0.001).

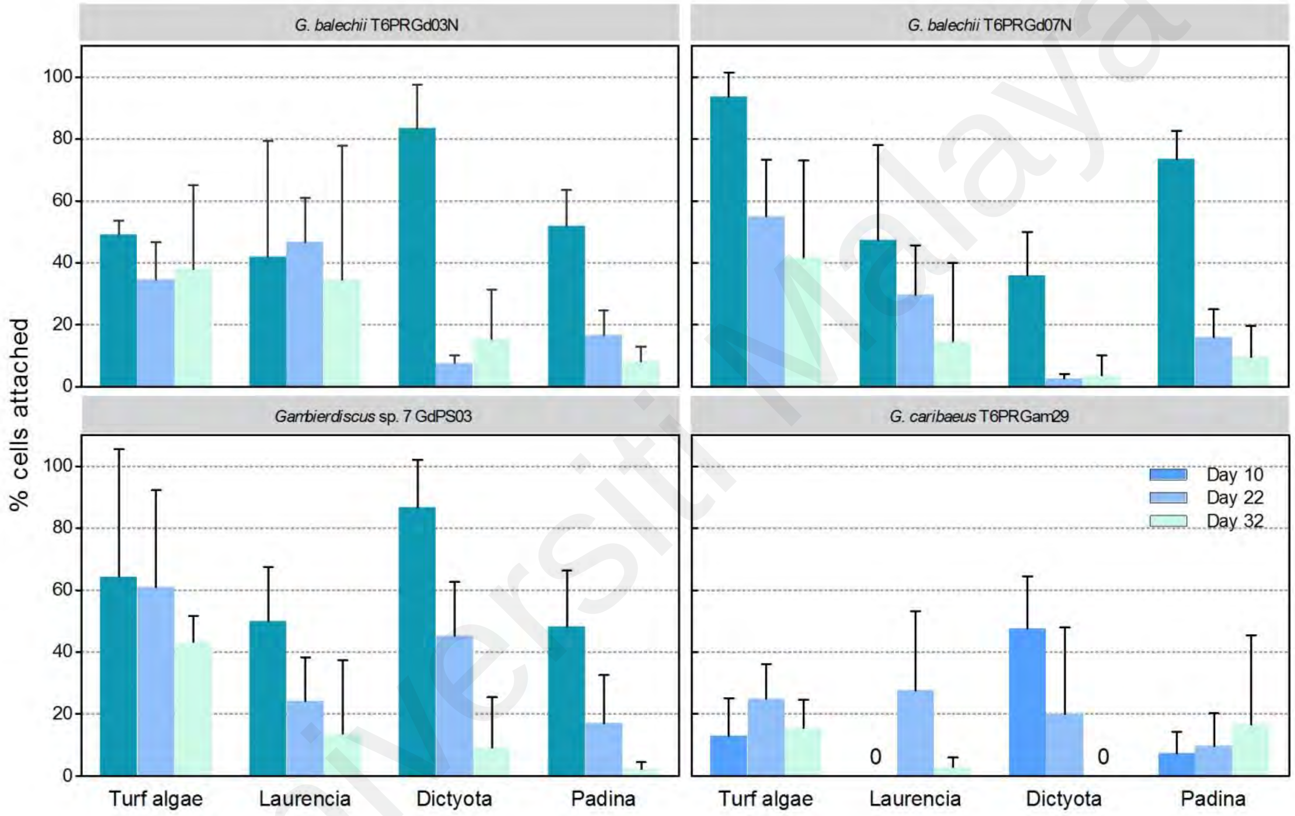


Figure 4.6: Percentage of *Gambierdiscus* cells attached to different macroalgal hosts on day-10, 20 and 32 of the study period.

4.5 Discussion

This study documented several species of *Gambierdiscus* in Malaysian waters (Chapter 3); *G. balechii* and *G. caribaeus* from Pulau Rawa, Terengganu, and a new ribotype of *Gambierdiscus* referred as *Gambierdiscus* sp. type 7 from Pulau Sibu, Johor. Besides, *G. pacificus* and *G. belizeanus* were also found in Malaysia (Mohammad-Noor et al., 2007; Leaw et al., 2011). All *Gambierdiscus* species are isolated from macroalgae (e.g. coral rubble, red filamentous, brown and green algae) and found associated with other benthic dinoflagellates; *Ostreopsis* spp., *Prorocentrum* spp., *Coolia* spp., and *Amphidinium* spp.

The interaction between *Gambierdiscus* is different towards different host either within strain or species (Grzebyk et al., 1994; Parsons et al., 2011; Rains & Parsons, 2015). Reviews from literature indicated that there were factors that influenced the *Gambierdiscus* preferences such as classes of algae (Yasumoto et al., 1979; Bomber et al., 1989b), stimulatory compounds (Carlson et al., 1984; Carlson & Tindall, 1985), surface area of the algae (Bomber et al., 1989b) as well as the presence of chemicals in the algae (Bomber et al., 1989b). This explained why the species and strains of *Gambierdiscus* tested in this study showed a varied epiphytic behaviors and tendency of preference towards different macroalgal host.

The results of the presents study showed the abundance of *Gambierdiscus* was higher in some turf algae or *Laurencia* treatments compared with other macroalgal host (Figure 4.5). Similarly, previous study by Yasumoto et al. (1979), Bomber et al. (1989), Cruz-Rivera and Villareal (2006) and Parsons et al. (2010) were also found micro-filamentous turf algae are among macroalgae that harbored a high number of *Gambierdiscus* cells. This is because, turf algae with a variable palatability usually comprised of epilithic, small, filamentous and fast growing red (e.g., *Polysiphonia*, *Centoceras*, *Ceramium*), green (e.g., *Chladophora*, *Chaetomorpha*) and brown (e.g., *Giffordia*), as well as along with coralline algae, cyanobacteria and diatom (Kendrick, 1991; Cruz-Rivera & Villareal, 2006; Littler & Littler, 2013; Connell et al., 2014). On top of that, previous study from Yong et al. (2018) also found high abundance of *Gambierdiscus* associates with the microhabitat of turf algae in the coral reefs of Malaysia, which is by using the artificial substrate method. In fact, reviews from literature indicate that there is factors that influenced the *Gambierdiscus* preferences like surface of the algae (Yasumoto et al., 1979; Bomber et al., 1989a), structural architecture as well as the texture of the macroalgal thalli (Parsons & Preskitt, 2007). It is believed that the flexibility of the thallus of the filamentous algae has provided wide spaces around branches of the thalli that helps *Gambierdiscus* cells to swim and easily attached themselves when there is water disturbance (Nakahara et al., 1996).

Besides, palatability of the algae also may affect the preference of *Gambierdiscus* towards macroalgal host. This is because; some filamentous algae (like *Laurencia* which is palatable to unpalatable) have a physical features (e.g., having tough blades) that are protecting them against fish grazer (Cruz-Rivera & Villareal, 2006). This has become an advantage for *Gambierdiscus* that inhabit these macroalgae in order defense themselves macrograzers.

Apart from that, Rains and Parsons (2015) revealed that chemical cue present in macroalgae and influence the *Gambierdiscus* behavior towards macroalgae. Some algae released chemical cue in order to defend themselves against pathogens, herbivores, and epiphytes (Cetrulo & Hay, 2000). Likewise, chemical cue released has possibly inhibited the growth of the *Gambierdiscus* and cause cell death (Steidinger, 1983; Parsons et al., 2011). This might be because of the different chemical environments produced by macroalgae in the laboratory setup versus in the field. Chemicals produced by macrolagae in the field can be varied at different stages which help in maintaining the life cycle of the algae itself (Vergés et al., 2008). In contrast, some researchers suggested that host algae may provide beneficial exudates for *Gambierdiscus*, which could help in facilitating the trace metal uptake or remove toxic metals from the thallisphere (Withers, 1982; Steidinger, 1983; Bomber et al., 1989a; Parsons et al., 2011) and served as growth stimulatory compound. This may explained why *Gambierdiscus* species tested exhibited higher growth rates in algal host treatments when compared to controls (Figure 4.5). Although we did not conduct chemical examination on the extract produced by the hosts in this experiment, but from our observation it did demonstrate that *Gambierdiscus* relied on some nutrients provided by the hosts.

Similar with those reported by Nakahara et al. (1996), and Rains and Parsons. (2015), the attachment behavior of *Gambierdiscus* observed in this study was varied among the species and strains, as well as within individual strains of the same species. Earlier studies had reported on how *Gambierdiscus* attached itself to the host where cells were attached to macroalgae via the development of mucus membrane (e.g. Ballantine et al. 1988; Yasumoto et al. 1980) or embedded within the mucilaginous sheath on surface of the algae (e.g. Bomber et al. 1988). In our study, *Gambierdiscus* cells were observed to attach to host by forming mucus membrane (e.g. *G. balechii* and *Gambierdiscus* sp. type 7 in turf algae and *Laurencia* sp. treatments).

Besides, some of the *Gambierdiscus* cells seem to utilize the structure of the host where some of the cells hid under the blade or leaves of the algae (e.g. *G. caribaeus* in *Padina* treatments). This is because, *Gambierdiscus* prefers low light intensity for their growth (Yasumoto et al., 1980b; Morton et al., 1992). It is reported that *Gambierdiscus* cells prefer low irradiance (shade-adapted) for their optimum growth (Yasumoto et al., 1980a; Bomber et al., 1988; Morton et al., 1992; Xu et al., 2016), with maximal growths reported below <10% of surface irradiance (<200 µmol photons $m^{-}2$ $s^{-}1$) (Kibler et al., 2012). By having this kind of behavior, macroalgae can help cover and protect

Gambierdiscus from high light intensities and photoinhibition (Mustapa et al., 2019). This had been demonstrated by Nakahara et al. (1996) where *Gambierdiscus* cells were actively swam in daylight periods and attached to the host upon darkness. However, there were some data (e.g. Bomber et al. 1988; Morton et al. 1992; Parson et al. 2010) which showed that different *Gambierdiscus* species had different physiological responses to the light intensity regimes. This could be one of the reasons why some *Gambierdiscus* species has different preference in choosing the macroalgae as their host.

4.6 Conclusions

In conclusions, this study showed the assemblage of *Gambierdiscus* in Pulau Rawa can be found mainly associated with benthic substratum that covered by turf algae. From the host preference experiments, it was concluded that *Gambierdiscus* species tested does not have specific preference towards different macroalgal hosts, although the attachment behavior and host preference observed in different individual, strains and species of *Gambierdiscus* were varied.

CHAPTER 5: CONCLUSION AND RECOMMENDATIONS

Gambierdiscus strain T6PRGd03N, T6PRGd07N, T6PRGd12N, T6PRGam28 and T6PRGam29 from Pulau Rawa, Terengganu and strain GdPS03, GdPS04 and GdPS05 from Pulau Sibu, Johor were identified by using light and scanning electron microscopes, their genetic information in the large subunit ribosomal RNA genes were also characterized.

The morphology of *Gambierdiscus* strain T6PRGd03N, T6PRGd07N and T6PRGd12N were consistent with *Gambierdiscus* sp. type 6 as described in Xu et al. (2014). The taxonomic status of *Gambierdiscus* sp. type 6 has been revised and now assigned as *Gambierdiscus balechii* (this study; (Dai et al., 2017). Morphological molecular characterizations of *Gambierdiscus* strain T6PRGam28 and T6PRGam29 showed that the strains are *Gambierdiscus caribaeus* as described by Litaker et al. (2009). *Gambierdiscus* strain GdPS03, GdPS04 and GdPS05 were designated as a new ribotype, referred as *Gambierdiscus* sp. type 7 based on the distinct grouping in the phylogenetic inferences. Although these strains had overlapping morphological features when comparing to *G. balechii*, the phylogenetic relationships showed that the *Gambierdiscus* strains formed a distinct lineage. Further analysis is needed to verify the identity of *Gambierdiscus* sp. type 7. This study provides the first record of the occurrence of these *Gambierdiscus* in Malaysian waters.

Many studies on taxonomy, physiology and toxicity of *Gambierdiscus* had been carried out over the past decades. Plasticity in the morphology of many benthic dinoflagellate species has been identified and caused difficulty in precise identification. Mapping of the morphological characteristics onto the molecular phylogeny has demonstrated a certain degree of ambiguity in the morphology of *Gambierdiscus*. In this study, reconciling molecular phylogeny and morphological character states revealed several characters that are taxonomic informative. Analysis of character state evolution in this study has strengthened the current well-established morphology-based taxonomy of *Gambierdiscus* and provides basis for future character-state sampling. In fact, the web-based interactive key that developed in this study could be used as a tool to assist researchers in species identification of *Gambierdiscus*.

The *Gambierdiscus* assemblage in Pulau Rawa, Terengganu, was associated with benthic substratum covered mainly by turf algae. Consistently, laboratory experiments on the habitat preference also showed maximum yield of *Gambierdiscus* cell observed in turf algae and *Laurencia* treatments. This experiment demonstrated no specific preferences of *Gambierdiscus* species towards macroalgal host tested, with different attachment behaviors in different host treatments. This indicates that all species of *Gambierdiscus* might have equal chance inhabit different host algae, and not exclusively host-dependent. Apart from the characteristics of the host algae, it is believed that environmental factors (e.g. nutrient availability, allelophatic, light and turbulance) could also influence the preferences of habitats for *Gambierdiscus* species. Naturally, this will affect the abundance of *Gambierdiscus* species in the coral reef ecosystem. There is possibility that ciguatoxins are likely to be transferred through the marine food web as many palatable macroalage (turf algae, *Laurencia* and *Dictyota*) were found in fringing reef of Malaysia (e.g., Yong et al. 2018), thus exposed the potential risk of CFP. Additional studies on the role of macroalgae to *Gambierdiscus* are needed to better understand on how they could contribute and affect the growth and toxicological differences among *Gambierdiscus* species or/and strains.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

Publications:

1. **Mustapa, N. I.**, Teng, S. T., Tan, T. H., Lim, H. C., Lim, P. T. & Leaw, C. P. (2015). Character evolution of benthic thecate dinoflagellate, *Gambierdiscus* (Dinophyceae), with an introduction of the interactive key to species. **Malaysian Journal of Sciences**, 34, 33-42.
2. Dai, X., Mak, Y. L., Lu, C. K., Mei, H. H., Wu, J. J., Lee, W. H., Chan, L. L., Lim, P. T., **Mustapa, N. I.**, Lim, H.C., Wolf, M., Li, D., Luo, Z., Gu, H., Leaw, C.P. & Lu, D. (2017). Taxonomic assignment of the benthic dinoflagellate *Gambierdiscus* sp. type 6 as *Gambierdiscus balechii* (Dinophyceae), including its distribution and ciguatoxicity. **Harmful Algae**, 67, 107-118.
3. Yong, H. L., **Mustapa, N. I.**, Lee, L. K., Lim, Z. F., Tan, T. H., Usup, G., . . . Leaw, C. P. (2018). Habitat complexity affects benthic harmful dinoflagellate assemblages in the fringing reef of Rawa Island, Malaysia. **Harmful Algae**, *78*, 56-68.
4. **Mustapa, N. I.**, Yong, H. L., Lee, L. K., Lim, Z. F., Lim, H. C., Teng, S. T., . . . Lim, P. T. (2019). Growth and epiphytic behavior of three Gambierdiscus species (Dinophyceae) associated with various macroalgal substrates. **Harmful Algae**, 89, 101671.

Papers presented in conferences/ seminars/ symposiums:

1. **Mustapa, N. I**., Yong, H. L., Lim, P. T. & Leaw, C. P. 2016. Taxonomy and habitat preferences of *Gambierdiscus* (Dinophyceae) from Rawa Island, Terengganu, Malaysia. 21st Biological Sciences Graduate Congress.

2. **Mustapa, N. I.**, Yong, H. L., Mak, Y. L., Chan, L. L., Lu, C. K., Lim, P. T. & Leaw, C. P. 2017. Identification of ciguateric benthic dinoflagellate, *Gambierdiscus* species (Dinophyceae) from Rawa Island (Terengganu, Malaysia) and their host preferences.10th WESTPAC International Scientific Conference.