DIVERSITY OF MARINE FUNGI ISOLATED FROM SELECTED SCLERACTINIAN CORALS AND THEIR SECONDARY METABOLITES

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INSTITUTE OF ADVANCED STUDIES
UIVERSITI MALAYA
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ABSTRACT

Scleractinian corals (also known as hard corals) are organisms comprised of colonies polyps that harbor assemblage of microbes, known collectively as coral holobionts which comprises of algae, bacteria, virus, fungi and archaea. Despite the prevalence of coral fungi, not much is known regarding their role and interaction with its host, especially their secondary metabolites aspect. The marine environment hosts fungal community with a contrasting metabolic profile as compared to its terrestrial counterparts. In current study, a total of 31 marine fungi has been isolated from eight scleractinian corals. *Penicillium* and *Neodevriesia* both frequently occurred in the collected scleractinian corals. Using multigene phylogeny approaches, selected strains were identified as *Colletotrichum siamense* (from *Porites* sp2) and *Neodevriesia* sp. 4 (from *Galaxea astreata*) for studies of their secondary metabolites. Fungal strains were cultured in potato dextrose broth for 30 days and mycelium were filtered and macerated in methanol. Methanol extract were further partitioned with ethyl acetate and water. Ethyl acetate extract were undergone further purification using column chromatography and preparative thin-layered chromatography to isolate secondary metabolites. Partially purified isolates were evaluated using NMR and methylated lipid layers were analysed for fatty acid composition. Investigated strains of fungus showed contrasting FAME profiles making it potential identification markers to fungus. Crude extracts and fractions of *C. siamense* were inactive against bacterial strains. ^{1}H- NMR results showed that majority of the fatty acids isolated are variation of very-long-chained fatty acids.

ABSTRAK

Terumbu karang adalah organisma menpunyai polyp yang melenggarakan sekumpulan mikrob dikenali sebagai “coral holobiont”, mengandungi algae, bakteria, virus, kulat dan archaea. Walaupun kulat tersebar luar di terumbu karang, fungsi mereka di terumbu karang masih kurang dan tidak difahami, khususnya mengenai metabolit sekunder. Komuniti kulat dalam marin menpunyai profil metabolit yang beza berbandingkan dengan kulat daratan. Dalam projek ini, sebanyak 31 kulat marin telah diasingkan dari 8 terumbu karang dan edua-dua Penicillium dan Neodevriesia merupakan kumpulan dominan. Isolat-isolat yang terpilih telah dikenalpastikan dengan penggunaan pelbagai jenis gen, dan dikenali sebagai *Colletotrichum siamense* (dari *Porites* sp2) and *Neodevriesia* sp. 4 dari *Galaxea astreata*). Kedua-dua kulat tersebut telah dibiakkan dalam kaldu kubi dextrose selama 30 hari. Selepas itu, mycelium telah diasingkan dari kaldu dan menghamburkan dalam metanol. Estrak methanol telah dibahagikan kepada dua bahagian menggunakan etil asetat dan air. Estrak etil asetat selanjutnya diasingkan dengan ‘column chromatography’ dan ‘preparative thin-layered chromatography’. Sebatian tulen yang terpilih telah diterangkan dengan ‘NMR’ dan ‘GCMS’. Kedua-dua kulat menpunyai profil asid lemak yang berbeza. Untuk kajian tentang ciri anti-bakteria, estrak kasar dan bahagian *C. siamense* telah diujikan terhadap lima bakteria dan menunjukkan keputusan negatif. Keputusan NMR dan GCMS pun membuktikan bahawa kebanyakan sebatian diasingkan ialah terbitan asid lemak dengan rantaian panjang.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATION

^{o}C	Degree Celsius
β-Tub	Beta-tubulin
µl	Microlitre
µg	Microgram
28S rDNA	28S Ribosomal DNA
A	Adenine
Act	Actin
AIC	Akaike information criterion
AMF	Arbuscular mycorrhizal fungi
amu	Atomic mass units
BGCs	Biosynthetic gene clusters
BI	Bayesian Inferences
BLAST	Basic Local Alignment Search Tool
BMC	Beneficial Microorganisms for Corals
bp	basepair
C	Cytosine
Cal	Calmodulin
$CDCl_3$	Deuterated chloroform
CFD	Coralline fungal diseases
CHS-1	Chitin synthases
CIN	Chemotherapy-induced neutropenia
CIPRES	Cyberinfrastructure for Phylogenetic Research Science Gateway
cm	centimetre
CO1	Cytochrome c oxidase I
Chl	Chloroform

CMA	Corn Meal Agar
Da	Dalton
dNTPs	Deoxynucleoside triphosphate
DNA	Deoxyribonucleic acid
DNRA	Dissimilatory Nitrate Reduction to Ammonia
DSS	Dark Spots Syndrome
EDTA	Ethylenediaminetetraacetic acid
EMEA	European Medicines Agency
ESS	Effective sample size
EtOAC	Ethyl acetate
FAMEs	Fatty acid methyl esters
FDA	Food and Drug Administration
G	Guanine
g	Gram
GADPH	Glyceraldehyde 3-phosphate dehydrogenase
GC-MS	Gas Chromatography Mass Spectrometry
gDNA	Genomic DNA
gen. nov	genera nova
Hex	Hexane
ITS	Internal transcribed spacer
LC-MS	Liquid chromatography mas spectrometry
LSU	Large subunits
MAAs	Mycosporine-like amino acids
MAFFT	Multiple alignment program for amino acid or nucleotide sequences
MCMC	Markov Chain Monte Carlo
MEGA X	Molecular Evolutionary Genetic Analysis

208	MeOH	Methanol
209	mg	Miligram
210	$MgCl_2$	Magnesium chloride
211	MHA	Mueller Hinton agar
212	MHz	Megahertz
213	MIC	Minimal inhibitory concentration
214	ml	Mililitre
215	ML	Maximum likelihood
216	mM	Millimolar
217	mm	Milimetre
218	MNPs	Marine natural products
219	MRSA	Methicillin-resistant *Staphylococcus aureus*
220	N	Nitrogen
221	NCBI	National Center for Biotechnology Information
222	ng	nanogram
223	NGS	Next-generation sequencing
224	NH_4^+	Ammonium
225	NJ	Neighbour-joining
226	nm	Nanometre
227	NMR	Nuclear Magnetic Resonance spectroscopy
228	NO_3^-	Nitrate
229	NO_2^-	Nitrite
230	NR(I)	Nitrogen regulation protein
231	NSCLC	Non-small cell lung cancer
232	MUFA	Monounsaturated fatty acid
233	PCR	Polymerase chain reaction
234	PDA	Potato Dextrose Agar

PDB	Potato Dextrose Broth
pH	Potential of hydrogen
PGPR	Plant growth promoting rhizosphere
PTLC	Preparative thin layer chromatography
pp	Posterior probability
PUFA	Polyunsaturated fatty acid
SFA	Saturated fatty acids
s.lat	sensu lato
s.str.	sensu stricto
T	Thymine
TAE	Tris base, acetic acid and EDTA
TLC	Thin-Layered Chromatography
Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloride
rpoN	RNA polymerase nitrogen -limitation N
R_f	Retention factors
RNA	Ribonucleic acid
U.S	United States
VLCMFA	Very long-chain monounsaturated fatty acid
VLCFAs	Very-Long-Chained-Fatty-Acids

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 Marine fungi in scleractinian corals

Marine fungi can be defined as any fungus that is recovered repeatedly from marine habitats because: 1) it is able to grow and/or sporulate (on substrata) in marine environments; 2) it forms symbiotic relationships with other marine organisms; or 3) it is shown to adapt and evolve at the genetic level or be metabolically active in marine environments" (Pang et al., 2016). Fungi are known to associate with marine organisms, either as disease causing pathogens or as beneficial and mutualistic symbionts. The presence and association of fungi with sessile marine animals such as corals and sponges has been well established, yet information on the extent of their diversity and role is still limited (Yarden, 2014).

Scleractinian corals (order Scleractinia), also known as hard corals or stony corals are sessile marine invertebrates in the class Anthozoa of the phylum Cnidaria. Hard corals are comprised of polyps with six tentacles, hence also known as hexacorals (subclass Hexacorallia). Most scleractinian corals are hermatypic coral species that deposit hard calcareous material to form their skeletons and essentially forming the reef system. Most scleractinian corals form an intricate symbiotic relationship with the photosynthetic algae (*Symbiodinium* spp) called zooxanthellae. In this symbiotic relationship, metabolic respiratory requirement of the coral is being fulfilled by the photosynthesis outputs of zooxanthellae, while the algae themselves may obtain inorganic nutrients from waste metabolites of corals or other coral holobionts such as symbiotic cyanobacteria or even

from the seawater column (Muller-Parker et al., 2015). Coral holobionts refers to the complex assemblage comprising the cnidarian host and a diverse range of microbes such as bacteria, archaea, viruses, algae, protozoa and fungi (Rohwer et al., 2002; Bourne et al., 2009). This assemblage is dynamic and subject to microbial communities-shifts caused by changes in environmental conditions, host types, and tempo-spatial gradients (Morrow et al., 2012). Unlike other holobionts, the relationship of fungi and hard corals are allegedly detrimental as fungi are long deemed as parasitic prior to recent studies that proven otherwise. The term 'BMC' (Beneficial Microorganisms for Corals) is a concept proposed to define symbionts such as fungi that play a role in promoting coral health (Peixoto et al., 2017).

1.2 Marine natural products from marine fungi

Marine natural products (MNPs) are generally secondary metabolites, often containing biologically active compounds with potential to modulate biological activities such as anti-fungal, anti-viral, anti-bacterial, anti-inflammatory and anti-cancer (Mayer et al., 2017). Despite the sheer volume of terrestrial natural products isolated, marine natural products are superior to terrestrial natural products in terms of chemical novelty (Kong et al., 2010) and cytotoxicities (Munro et al., 1999). Hence, there has been a tremendous increase of bioprospecting in the oceans and marine environments, screening and extracting myriad of new MNPs from marine organisms spanning from marine invertebrates, marine plants to marine micro-organisms (Williams & Andersen, 2020).

Marine fungi exhibit a tremendous potential for the discovery of novel bioactive secondary metabolites. There are approximately 31,700 MNPs in the MarinLit database, mainly sourced from marine organisms of the phyla Porifera, Cnidaria and Ascomycota

which accounted for 29%, 18% 16 % of the inventory, respectively (Carroll et al., 2020). Since the last two decades, there has been an exponential increment of MNPs from Ascomycota (**Figure 1.1**) with approximately 4,708 new compounds reported from marine fungi (Carroll et al., 2019). Marine fungal-derived metabolites consist mainly of polyketides and followed by prenylated polyketides, meroterpenoids, terpenoids, peptides including diketopiperazines, alkaloids and other nitrogen-containing metabolites. Issues concerning cultivability, dormancy of biosynthetic gene clusters (BGCs) under laboratory culture conditions generally hampered the efforts to fully utilize marine fungi as drug resource (Tasdemir, 2017). Thus, marine fungi constitute a group of underrepresented resource for discovering novel therapeutics (Imhoff, 2016).

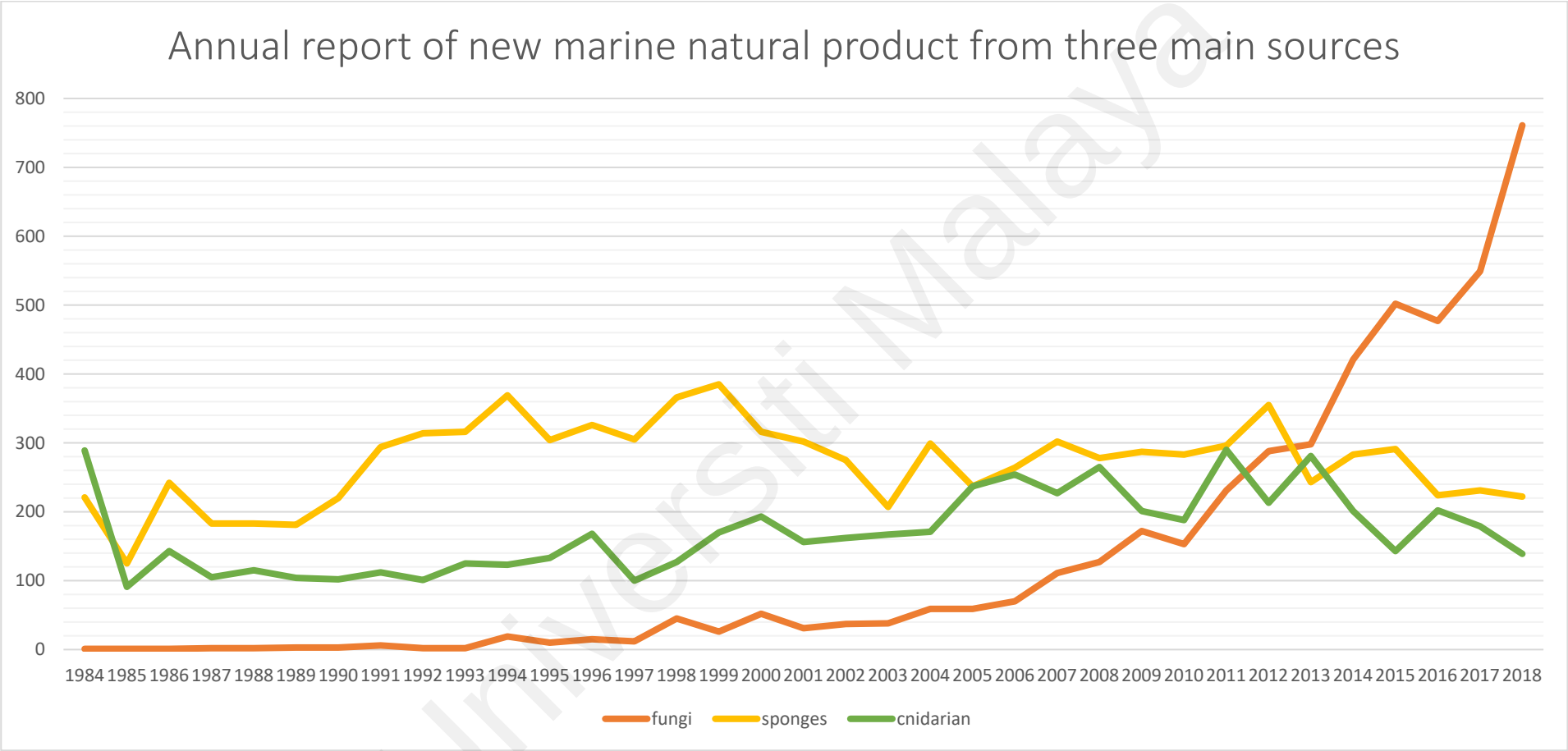


Figure 1.1: Annual report of new natural products from three major source marine-derived fungi from 1970s until 2018 (Faulkner et al, 1984 – 2002, Blunt et al., 2003 – 2018, Carroll et al., 2019, 2020)

1.3 Problem Statement

The Perhentian Islands are a group of islands located 21km off the coast of Terengganu, Malaysia and fall within the Coral Triangle that is the global centre of coral mega biodiversity regions with more than 605 species of reef-building corals (Green and Mous, 2003). Perhentian islands, gazetted as Marine Park, is one of the ecotourism hubs in east coast of Peninsular Malaysia which crucial for the livelihood and economy contribution of the local community. Due to the increase anthropogenic activities, coral cover in Perhentian Island has been gradually shrinking (Reef Check Malaysia, 2019). Thus, there have been rigorous efforts done in providing a comprehensive inventory of scleractinian corals for systematic conservation plan (Affendi & Rosman, 2012; Akmal et al., 2019). However, prior surveys rely solely on morphology features such as colonies shape and corallites, molecular cataloguing of corals remained meagre. Genomic tools are becoming integral for conservation researchers to obtain valuable and crucial insights in the highly complex and diverse ecosystems (Dybas, 2018).

Similarly, while there have been some extensive studies on marine fungi from mangroves in Malaysia (Siti et al., 2010; Zainuddin et al., 2010), studies of marine fungi from scleractinian corals are scant. Most of the studies are primarily revolved around other marine invertebrates of soft coral and sponges, which are the main source of marine natural products. There are growing interests in deciphering the relationship between scleractinian corals and marine fungi concerning their resilience and health.

This study aims to identify the marine derived fungi and their scleractinian coral hosts selected from the Perhentian Islands and to investigate the bioactive potential of the fungal secondary metabolites. The genomic sequences, both marine fungi and scleractinian corals, generated in this study will provide a baseline inventory data for further study. These data can act as an impetus for a more exhaustive research in

understanding the niche of marine fungi in scleractinian corals. In fact, contemporary research used metagenomic approach to investigate the diversity of marine fungi in scleractinian coral and try to decipher their role as one of the holobionts (Wegley et al., 2007; Amend et al., 2012).

1.4 Objectives

1. To identify the coral-associated fungal species and their scleractinian coral hosts based on morphology and DNA sequences (CO1 for hard corals, ITS for fungi).
2. To further identify the two selected fungal strains (*Colletotrichum siamense,* and *Neodevriesia* sp. 4) using multiple genetic markers (ITS, Cal, GADPH, β-Tub, CHS-1, Act, LSU sequences).
3. To investigate the bioactive potential of secondary metabolites from crude extracts of *Colletotrichum siamense.*
4. To identify the chemical compounds from the purified metabolites of *Colletotrichum siamense.*

CHAPTER 2

LITERATURE REVIEW

2.1 Diversity and distribution of marine fungi

Since the first description of marine fungi in the 1850s, their definition has been debated whether it should emphasize on their ecological and physiological characteristics instead of taxonomical features (Jones et al., 2015). The most widely accepted definition has distinguished marine fungi as 'obligate' if they grow and sporulate exclusively in a marine or estuarine habitat or 'facultative' if they were from freshwater or terrestrial habitat but able to grow (and possibly also to sporulate) in a marine environment (Kohlmeyer & Kohlmeyer, 1979). However, the well-established Kohlmeyers' definition has come under scrutiny due to its overly narrow definition that generally excludes fungi isolated from marine sediments (Jones et al., 2015). For example, the repeated success of isolating terrestrial species belonging to the genera *Aspergillus*, *Penicillium* and *Trichoderma* from the marine environments have advocated the idea of grouping them as marine (Jones et al., 2015). Consequently, a marine fungus is now accordingly defined as "any fungus that is recovered repeatedly from marine habitats because: 1) it is able to grow and/or sporulate (on substrata) in marine environments; 2) it forms symbiotic relationships with other marine organisms; or 3) it is shown to adapt and evolve at the genetic level or be metabolically active in marine environments" (Pang et al., 2016).

To date, there are approximately 1, 689 documented species (in 696 genera) of marine fungi (marinefungi.org accessed on 17 May 2020) (Jones et al., 2019). The number of described marine species is small compared to that of terrestrial fungi, which

is estimated at 120,000 to 140,000 (Hawksworth & Lücking, 2017). Most marine fungal species belong to the phyla Ascomycota and Basidiomycota, and some in Blastocladiomycota and Chytridiomycota (Jones et al., 2019) (**Figure 2.1**). Studies have shown that marine fungi have been isolated from open water columns (Richards et al., 2015), deep-sea sediments and hydrothermal vents (Le Calvez et al., 2009; Rédou et al., 2015), anoxic environments (Jebaraj et al., 2010), marine invertebrates (Yarden, 2014) and seaweeds (Gnavi et al., 2017).

With culturable isolates identified based on morphology and/or genomic sequences, environmental DNA-based surveys, despite their challenges, would allow for the screening of unculturable species (Amend et al., 2019). Jones (2011) estimated that there are probably more than 10,000 taxa which are not yet discovered and still considered as '*terra incognita*', such as the deep-sea fungi, cryptic species, planktonic fungi, unculturable fungi, marine-derived fungi isolated from different substrata. There are few existing fungal-specific clone libraries for marine fungi and only reveals a much simpler fungal community compared to terrestrial fungal community (Buée et al., 2009; Amend et al., 2019). Information on global distribution of marine fungi is also limited (Tisthammer et al., 2016). Prior studies limited to regional scale, such as varying depth across water column in Hawaii (Gao et al., 2010), deep-sea sediment from the East Indian Ocean (Zhang et al., 2014), and deep-sea sediment in Central Indian Oceans (Singh et al., 2011).

In order to establish a vigorous and thriving framework of documenting the diversity and distribution of fungi in the world's oceans, Amend et al. (2019) suggested three recommendations: 1) the establishment of a standardized procedure dedicated for marine fungi, comprising of set primers and protocols with outputs from continual cooperation among marine mycologists and interdisciplinary researchers; 2) the

emulation of diversity hotspots surveys on a global scale with possible partial contributions from ‘citizen scientists’; 3) the consolidation and streamlining of archives consisting of genomic sequences, vouched culture collections of marine origins, whole genomes, and marine fungus model systems analogous to the model yeast systems. An example of such initiative is the development of the marine fungi website, “www.marinefungi.org” by Jones et al. (2019), which establishes a curated database dedicated to marine fungi.

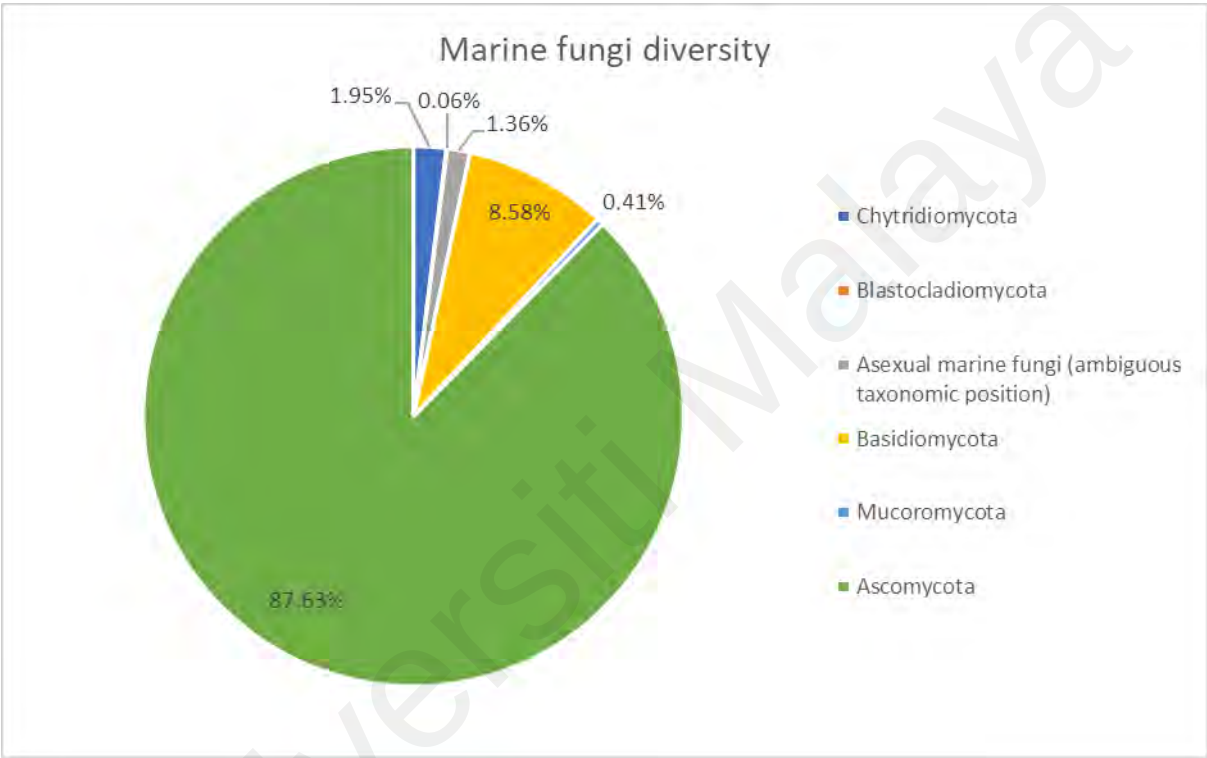


Figure 2.1 Distribution of fungal phyla in marine fungi (source from *www.marinefungi.org*) (Jones et al., 2019)

2.2 Ecological relationships between marine fungi and scleractinian coral hosts

Corals are marine invertebrates from the Class Anthozoa, which are distinguished as soft corals (subclass Octocorallia) and hard corals (subclass Hexacorallia). Hard corals (order Scleractinia) or scleractinians are characterized by their distinctively rigid calcium carbonate skeleton called corallite where individual polyp grows, having a cylindrical body crowned by an oral disc surrounded by a ring of tentacles. Hard corals are the building blocks of coral reef ecosystems known for their high diversity of biological species (Veron, 2011).

Like many marine invertebrates, hard corals also host a complex assemblage of diverse microbes that includes bacteria, archaea, viruses, algae, protozoa and fungi (Rohwer et al., 2002; Knowlton & Rohwer, 2003; Bourne et al., 2009). Thus, a host and its microbiota constitute a holobiont (Margulis, 1991) that is highly complex and dynamic because of shifts in microbial community structure, depending on environmental conditions, host types, and tempo-spatial gradients (Morrow et al., 2012). For example, corals expel the symbiotic algae (zooxanthellae of the genus *Symbiodinium*) living in their tissues when temperature gets too warm, causing corals to 'bleach' and turn completely white (Brown, 1997; Fujise et al., 2014). Unlike other holobionts, marine fungi were often deemed to have a harmful parasitic relationship with hard corals, unless proven otherwise (Yarden, 2014).

The ecological role of marine fungi is still inexplicit, with most studies attested for parasitism (Amend et al., 2019). Parasitic fungi such chytrids were found infecting marine phytoplanktons (Gutiérrez et al., 2016; Hassett & Gradinger, 2016; Lepère et al., 2016). Filamentous fungi such as *Aspergillus sydowii* were culpable for blight in sea fan (Geiser et al., 1998) and also perturbed photo-physiological performance of *Symbiodinium* (Hayashi et al., 2016). Coralline fungal diseases (CFD) which inflicted

lesion on crustose coralline algae, are well documented (Littler & Littler, 1998; Williams et al., 2014; Neal et al., 2020). However, new studies on mycoloop (Kagami et al., 2014), planktonic marine fungi (Cunliffe et al., 2017), bathypelagic marine snow (Bochdansky et al., 2017), deep sea sediment (Orsi et al., 2013) and anthropogenic hydrocarbons of oil spill (Bik et al., 2012) presented contrasting perspectives concerning their role in marine carbon cycle.

2.2.1 Nitrogen cycling

Coral holobionts play a vital role in nitrogen cycle including nitrogen fixation and nitrification (Rädecker et al., 2015; Pernice et al., 2020). Endolithic fungi are one of the candidates postulated to engage in partial denitrification process of Dissimilatory Nitrate Reduction to Ammonia (DNRA) (Wegley et al., 2007). DNRA is an anaerobic process in which NO_3^- is reduced to NO_2^- and then to NH_4^+, retain N source in the loop (Pajares & Ramos, 2019) where similar process helps to retain N in the soil matrix (Tiedje, 1988; Friedl et al., 2018). Wegley et al. (2007), using metagenome techniques, have discovered that several functional genes involved in nitrogen metabolic pathways are associated with fungi observed in *Porites astroides* genome such as nitrite reductase, ammonium transporter, ammonium transported family, uridylyltransferase, nitrogen regulation NR(I) and Sigma-54 factor rpoN (RNA polymerase nitrogen -limitation N). It is hypothesized that at least two processes of the nitrogen cycle namely, nitrate/nitrite ammonification and ammonia assimilation occurred within the coral are related to fungi (Wegley et al., 2007). Similar role of nitrification and denitrification has been proposed by Siboni et al. (2008) regarding the role of archaea in the mucus layer of these three hard corals of *Acanthastrea* sp., *Favia* sp. and *Fungia* sp. These roles could perhaps link the importance

of marine fungi to the rather obscure process of denitrification and nitrogen fixation in corals.

2.2.2 Coral Diseases

Coral fungi had been long deemed as parasitic and some pathogenic toward hard corals (Kendrick et al., 1982) causing coral diseases or syndromes as listed in **Table 2.1**. While investigating an outbreak of black-lined diseases in Caribbean corals, Ramos-Flores (1983) reported an invasion of unknown fungal hyphae afflicting necrosis in *Montastrea annularis* polyps, which also manifested as dark patches or lines on other hard corals. Bak and Laane (1987) documented the presence of a dark mycelial fungus with perithecia, appearing as black bands belong to Ascomycetes from the skeleton of *Porites* spp. Priess et al. (2000) reported the isolation of the endolithic Aspergillus-like conidiophore from the *Porites lobata* and *Porites lutea* in Mayotte Island of the Mozambique Channel of Indian Ocean, concordant with findings from Moorea Island of French Polynesia (Le Campion-Alsumard et al., 1995a; Le Campion-Alsumard et al., 1995b). Dark tannin-like substances were released by the fungi staining the algal filaments and skeleton herein lies the explanation of the black-band observed inside the coral skeleton (Priess et al., 2000). Such phenomena were suggested to be used as an indicator to date the occurrence of the invasion of the fungi akin to the tree ring as occurrence of dark band may signify stress on the coral (Le Campion-Alsumard et al., 1995a; Le Campion-Alsumard et al., 1995b). Bentis et al. (2000) used other hermatypic coral species such as *Pocillopora eydouxi, Acropora cytherea, Acropora humulis and Montipora cf. studeri* from Johnson Atoll to substantiate that the presence of fungi in coral are rife and affecting other hard corals as well. Using media inoculation method, presence of a septate dark brown mycelial fungus on the subsurface of dead patches on *Montipora tuberculosa*, *Goniapora* sp, *Poritea lutea* from the Andaman Islands in the

Bay of Bengal was identified as *Scolecobasidium* sp. (Raghukumar & Raghukumar, 1991). *Acropora formosa* from the Wistari and Heron Reef of the Great Barrier Reef, yielded pure cultures of the ascomycetous fungi (Yarden et al., 2007). Rapid wasting disease was characterized by fungal invasion after bitemark inflicted by parrotfish (Cervino et al., 1998).

However, most of the authors agreed that despite their parasitic actions, fungi are not the causal agents of diseases in hard corals, but rather opportunistic invaders on these sick hard corals. (Ramos-Flores, 1983; Bak & Laane, 1987; Le Campion-Alsumard et al., 1995a; Le Campion-Alsumard et al., 1995b; Cervino et al., 1998; Priess et al., 2000; Ravindran et al., 2001; Golubic et al., 2005; Yarden et al., 2007). All authors consistently advocate the hypothesis that fungi are secondary invader and aggravated by environmental stress.

Hitherto, the only coral disease presumably caused by fungi was reported by Sweet et al. (2013). They inferred that *Rhytisma acerinum* may have inflicted the Dark Spots Syndrome (DSS) in coral *Stephanocoenia interseptai* as this particular fungus was absent in healthy samples, while other bacterial candidates such as cyanobacteria of *Oscillatoria* sp. were detected in healthy samples. The similar symptoms of DSS with that of tar spot disease in terrestrial plants inflicted by the same fungus *Rhytisma acerinum* strongly underpinned this hypothesis (Sweet et al., 2013). Upon investigating the healthy hard corals as control, communities of fungi were present with no apparent sign of diseases on these hard corals, therein lies the notion that fungi might reside in hard corals as endoliths (Le Campion-Alsumard et al., 1995a; Le Campion-Alsumard et al., 1995b; Cervino et al., 1998; Bentis et al., 2000; Ravindran et al., 2001; Golubic et al., 2005; Yarden et al., 2007).

Coral skeletons and coral rubbles often provide porous substrates with suitable physicochemical conditions (eg. light, oxygen, pH and chemical gradient) for endolithic microbes such as algae, virus, bacteria, archea and fungi to grow and thrive (Rosenberg et al., 2007; Pernice et al., 2020). Evidence of endolithic fungi have been reported (Le Campion-Alsumard et al., 1995a; Le Campion-Alsumard et al., 1995b; Bentis et al., 2000) in coral skeletons, whereby the fungi established relationships with both the polyp host and endolithic algae too (Le Campion-Alsumard et al., 1995b). Conidiophores and hyphae of endolithic fungi have been observed and documented in hard corals skeletons using scanning electron microscopy, light microscopy and even resin replicas of boreholes in corals (Bentis et al., 2000; Priess et al., 2000; Golubic et al., 2005). The intrusion of fungal filaments into the polyp zone of the hermatypic coral *Porites lobata* is known to prompt a defense mechanism involving a dense deposition of skeleton, resulting in pearl-like structures, and exhibit rapid growth to match skeletal accretion (Priess et al., 2000).

Regardless of whether parasitic or not, it is apparent that endolithic fungi are ever-present in healthy corals (Bentis et al., 2000; Golubic et al., 2005). Environmental stress could disturb the innate equilibrium of the endolithic communities and compromise the health of corals due to inability to fend off other pathogens, hinder tissue regeneration or prevent recovery from recent bleaching events. (Bentis et al., 2000).

Table 2.1: List of fungal species and possible diseases or syndromes they cause in hard corals.

Putative fungi	Disease/ syndrome/ infection appearance	Infected hard coral	References
Unknown lower marine fungi	Black line disease	*Montastrea annularis*	Ramos-Flores (1983)
Halosphaeriaceae	Black band	*Porites* spp.	Bak & Laane (1987)
Scolecobasidium sp.	Dark necrotic patches	*Porites lutea,* *Porites lichen* *Montipora tuberculosa,* *Goniopora* sp. *Goniastrea* sp.	Raghukumar & Raghukumar (1991)
Unknown endolithic fungi	Perforation by fungal hyphae	*Porites lobata* *Porites lutea*	Le Campion-Alsumard et al. (1995b), Golubic et al. (2005)
Unknown fungi	Rapid wasting syndrome	*Montastrea annularis* *Colpophyllia natans*	Cervino et al. (1998)
Unknown endolithic fungi	Perforation by fungal hyphae	*Pocillopora eydouxi,* *Acropora cytherea,* *Acropora Humulis* M*ontipora* cf. *studeri* *Porites eydouxi*	Bentis et al. (2000)
Endolithic aspergillus-like conidiophore	Black band	*Porites lobata* *Porites lutea*	Priess et al. (2000)

Table 2.1: Continued.

Putative fungi	Disease/ syndrome/ infection appearance	Infected hard coral	References
Aspergillus sp. *Cladosporium* sp. *Labyrinthula* sp. *Chaetomium* sp. *Aureobasidium* sp.	Pink-line syndrome	*Porites lutea*	Ravindran et al. (2001)
Alternaria sp. *Aureobasidium pullulans* *Cladosporium* sp. *Fusarium* sp *Humicola fuscoatra* *Penicillium citrinum* *Phoma* sp.	Brown band syndrome and skeletal eroding band disease.	*Acropora formosa*	Yarden et al. (2007)
Unknown endolithic fungi	Dark spot syndrome (DSS)	*Siderastrea siderea* *Agaricia agaricites*	Renegar et al. (2008)
Unknown endolithic fungi	Dark sport disease-like	*Psammocora nierstraszi* *Pavona varians* *Montipora* sp. *Pavona maldivensis*	Work et al. (2008)
Rhytisma acerinum	Dark spot syndrome (DSS)	*Stephanocoenia intersepta*	Sweet et al. (2013)

2.3 Diversity of marine fungi in Hard Corals

Prior to the advent of metagenomic tool such as next-generation sequencing (NGS), marine fungal diversity studies relied predominantly on isolation via media. Reports on fungal diversity from the Australian Great Barrier Reef corals indicated the presence of fungal strains, comprising mostly of common terrestrial species, with approximately a total of 54 distinct fungal taxa were identified isolating from assorted media with seawater (Morrison-Gardiner, 2002). Raghukumar and Ravindran (2012) categorized the association of fungi with corals as non-native (**APPENDIX A**) and native (**APPENDIX B**), where the former is exclusively found in dead coral slabs and the latter are mostly terrestrial fungi cultured using media (Kohlmeyer & Kohlmeyer, 1979; Kohlmeyer & Volkmann-Kohlmeyer, 1987, 1988, 1989, 1990, 1991, 1992, 1998).

The family Koralionastetaceae from class Sordariomycetes was proposed by Kohlmeyer and Volkmann-Kohlmeyer (1987) based on five species (*K. angustus, K. ellipticus, K. ovalis, K. giganteus and K. violaceus*) recovered from coral slabs that form the single genus *Koralionastes*. These obligate marine mycetaen fungi are corallicolous; found exclusively in coral reefs, dead coral skeletons and coral slabs in shallow waters of the Atlantic Ocean, Belize, Central America and Pacific Ocean, Queensland, Australia (Kohlmeyer & Volkmann-Kohlmeyer, 1992; Jones, 2011). However, it is presumptive to classify these species from the family Koralionastetaceae as native to corals because some have yet to be isolated from living hard corals. For example, *Xenus lithophylli*, a parasitic fungus was observed mooching the calcified algae (*Lithophyllum* sp.) (Kohlmeyer and Volkmann-Kohlmeyer, 1992) but not directly on corals. *Halographis runica*, an endolithic lichenoid was discovered from corals and shells of living snails (Kohlmeyer & Volkmann-Kohlmeyer 1988, 1992), *Lulworthia calcicola* (Kohlmeyer & Volkmann-Kohlmeyer 1989).

Throughout the years, isolating fungi from corals using orthodox method of media culturing was run-of-the-mill routines. The non-selective element of the conventional plating method is one of the major disadvantages in fungi isolation practices. Plate culturing using media have higher propensity to isolate fast growing terrestrial counterpart such as *Aspergillus* and *Penicillium* as pointed by Jensen and Fenical (2000). Competition from fast growing terrestrial fungi, probable obligate and intrinsic symbiotic associations with hosts impede the effort of isolating the true coral-inhabiting fungi (Kohlmeyer & Volkmann-Kohlmeyer, 2003). Hence, molecular techniques such as NGS and pyrosequencing with multiple gene markers are preferential and cogency in the new era for searching new species of hard coral-inhabiting fungi and possibly unravel the association of these fungi with hard corals.

Recent metagenomic studies of holobionts of hard corals had revealed diverse communities of fungi residing in hard corals (Kohlmeyer & Volkmann-Kohlmeyer, 2003; Wegley et al., 2007; Thurber et al., 2009; Littman et al., 2011; Amend et al., 2012; Góes-Neto et al., 2020). According to Wegley et al. (2007), 454 pyrosequencing was used to sequence the metagenomes of the microbial communities residing in *Porites astreoides*, the mustard hill coral. Apart from the mitochondrial sequences that belong to the host coral *P. astreoides*, fungal sequences making up to 38 % of known sequences were identified as the major holobiont in the mustard hill coral. The fungal sequences analyzed matched portions of three major fungal phyla, Ascomycota, Basidiomycota and Chytridiomycota DNA sequences. Ascomycetes was found to be the primary fungal group among the detected three in *P. astreoides*. The class Sordariomycetes constitutes the major class of Ascomycetes, leading other classes such as Eurotiomycetes, Saccharomycetes and Schizosaccharomycetes (Wegley et al. 2007).

By exposing the healthy hard corals *Porites compressa* to four stressors, namely, increased temperatures, elevated nutrients, dissolved organic carbon loading and reduced pH, insalubrious conditions for the corals were created which caused shifts from a healthy-associated coral community to a community of microbes often found in diseased corals (Thurber et al., 2009). Majority of fungi-like sequences (96-100%) were assigned to Ascomycota and only ~7% assigned to Chytridiomycota (Thurber et al., 2009). Similar stress studies performed on *Acropora millepora* during bleaching event in Magnetic Island, Great Barrier Reef resulted in the detection of fungi-affiliated sequences dominated by Ascomycota (Littman et al., 2011).

Recent study by Amend et al. (2012) using the pyrosequencing of 28S rDNA technique of nucleic acid amplification on colonies from *Acropora hyacinthus* showed high diversity of Ascomycota (Sordariomycetes and Dothideomycetes) and Basidiomycota (Agaricomycetes and Ustilaginomycetes) sequences. Amend et al. (2012) suggests that these fungi are the likeliest candidates for an ecologically significant association within the *A. hyacinthus* holobionts. Contemporary studies by Bonthond et al. (2018) further corroborated the notion that fungal communities in hard corals are largely dominated by phyla Ascomycota. However, Góes-Neto et al. (2020) reported discrepancies between fungal communities of different hard corals; phyla Basidiomycota dominate in *Egrichipsammia*, *Porites* and *Leptoria*, Chytridiomycota dominate in *Pocillopora*, whereas Ascomycota dominate in *Montipora*, *Astrea*, *Galaxea*, *Goniastream Milepora* and *Montastrea.* Although Ascomycota are ubiquitous, different hard corals seemed to harbour different dominant groups of fungal communities. Studies also reported unassigned sequences that implied the possible presence of novel marine fungi lineages in hard corals (Amend et al., 2012; Góes-Neto et al., 2020)

2.4 Marine natural products

Marine natural products (MNPs) are generally secondary metabolites, often containing biologically active compounds with the potential to modulate biological activities such as anti-fungal, anti-viral, anti-bacterial, anti-inflammatory and anti-cancer (Mayer et al., 2017). Hence, there has been a tremendous increase of bioprospecting in the oceans and marine environments, screening and extracting myriad of new MNPs from marine organisms spanning from marine invertebrates, marine plants to marine micro-organisms (Williams & Andersen, 2020). Albeit the encouraging bioactivity properties of MNPs, only nine marine derived drugs are approved, to date, by most representative approving agencies of U.S. FDA, European Medicines Agency (EMEA), Japanese Ministry of Health and Australia's Therapeutic Goods Administration as shown in **Table 2.2** (Mayer et al., 2010; Gerwick & Moore, 2012; Newman & Cragg, 2016; Mayer et al., 2017; Mayer, 2018; Pereira & Aires-de-Sousa, 2018; Wiese & Imhoff, 2019; Le et al., 2020). Possible development of such MNPs into drugs could be hindered by their relatively high hydrophobicity, making them insoluble and unfavourable as drug design candidates (Kong et al., 2010). Thus, MNPs despite their novel scaffolds, required more attention and studies to further progress in drug development pipeline.

Table 2.2: List of approved marine-derived drugs to date. (Gerwick & Moore 2012; Newman & Cragg 2016, Pereira 2019, Wiese et al. 2019, Le et al., 2020).

Compound	Chemical class	Commercial trademark	Source (Initial)	Properties	Year
Cytarabine, Ara-C	Nucleoside	Cytosar-U®	Sponge, *Tethya crypta*	Anticancer (Leukimia)	1969
Vidarabine,Ara-A	Nucleoside	Vira-A ®	Sponge, *Tethya crypta*	Antiviral	1976
Ziconotide	Peptide	Prialt®	Marine snail, *Conus magus*	Analgesic	2004
Omge-3-acid ethyl ester	Omega-3 Fatty acid	Lovada®	Fish	Hypertriglyceridemia	2004
Eribulin mesylate (E7389)	Macrolide	Halaven®	Sponge, *Lissodendoryx* sp.	Anticancer	2010
Brentuximab vedotin	Antibody drug conjugate	Adcetris®	Sea hare, *Dolabella auricularia*	Anticancer	2011
Trabectedin, ET-743	Alkaloid	Yondelis®	Tunicate, *Ecteinascidia turbinata*	Anticancer	2015
Plitidepsin	Depsipeptide	Aplidin®	Ascidian, *Aplidium albicans*	Anticancer	2016
Polatuzumab vedotin	Antibody drug conjugate	POLIVY®	Mollusk/cyanobacterium	Anticancer	2019

The bioactive MNPs have been purified from marine fungi isolates acquired from various marine organisms, marine substrates and deep-sea sediments (Rateb & Ebel, 2011; Hasan et al., 2015; Sarasan et al., 2017; Tasdemir, 2017; Deshmukh et al., 2018; Zain ul Arifeen et al., 2020). Such profound increase was mainly attributed to 1) the discovery and unravelling of microbiomes of marine invertebrates in recent years through the advancement of metagenomic technology (Trindade et al., 2015; Miller et al., 2016; O'Brien et al., 2019; Storey et al., 2020); 2) the exploration of new marine environments that were previously inaccessible or understudied, such as the deep sea and the complex coral reef ecosystems (Yarden, 2014; Rédou et al., 2015; Nagano et al., 2017); 3) the development of new techniques such as biosynthetic gene cluster (BCG) mining, computational methods in exploring MNPs and strategies to elicit silent gene cluster (Brakhage & Schroeckh, 2011; van der Lee & Medema, 2016; Skellam, 2019; Tran et al., 2019); 4) growing demand for new drugs to fight the increased resistance of 'superbugs' such as the methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* (Coates et al., 2002; Cooper et al., 2004).

Despite the surging trends of isolating MNPs from marine fungi, only one compound derived from marine fungi has advanced to clinical trial stage. Plinabulin (NPI-2358) (**Figure 2.2**) is a synthetic chemical for the prevention of chemotherapy-induced neutropenia (CIN) and the treatment of non-small cell lung cancer (NSCLC). It was designed from two chemical analogues of natural products known as halimide and phenylahistin, which were isolated from marine *Aspergillus* sp. CNC-139 (Cheng et al., 1999) and *Aspergillus ustus* (Kanoh et al., 1997), respectively.

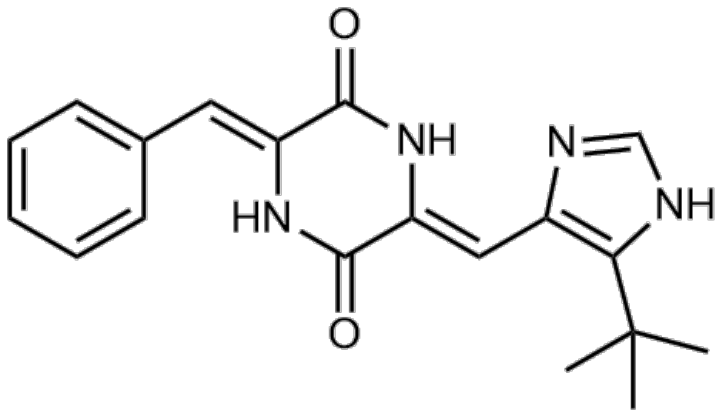


Figure 2.2 : Structure of synthetic analogue drug Plinabulin based on halimide and phenylahistin.

2.4.1 Origin of marine natural products from holobionts

Reports have shown that holobionts (an assemblage of microbes consists of diversity of bacteria, archaea, viruses, algae, protozoa and fungi) (Leal et al., 2014; Thompson et al., 2015) are the actual 'powerhouse' for manufacturing MNPs in marine invertebrates. Many MNPs and their derivatives from microbes are utilized by invertebrate hosts for their defence mechanism (such as allelopathy) against predators or competitors (Gerwick and Moore, 2012; Jiménez, 2018). For example, the compounds bryostatin and dolastatin 10; initially isolated from the bryzoan *Bugula neritina* (Pettit et al., 1982) and the sea hare *Dolabella auricularia* (Pettit et al., 1993; Sone et al., 1996), respectively; were in fact produced by their microbial symbionts which were the uncultivated γ-proteobacterium '*Candidatus* Endobugula *sertula'* in the bryzoans (Lopanik et al., 2006) and two cyanobacterial species *Lyngbya majuscula* and *Symploca* spp. in sea hares obtained via ingestion (Mitchell et al., 2000; Luesch et al., 2001).

Sessile animals such as hard corals have innately developed unique chemical defense mechanisms against predation, biofouling, diseases, environmental perturbations,

and other stressors throughout the millennia of evolutionary arm race. These chemicals are either synthesized by the organisms themselves or by their endobiontic microorganisms; since many of the secondary metabolites isolated from marine invertebrates exhibited striking structural similarities to those from the microbial origin. It was suggested that microorganisms are in fact the true sources; or at least involved in the biosynthesis of these respective metabolites; even though it is extremely difficult to definitively confirm the biosynthetic source of many marine natural products because of the complexity of associations in marine organisms (König et al., 2006). Unlike primary metabolism that is integral to the basic functioning of cells and common to all organisms, secondary metabolism is usually a strain-specific process strategically aimed for survival and is predominantly used by plants and microbes such as bacteria and fungi. Secondary metabolites serve as: (i) competitive weapons used against other bacteria, fungi, amoebae, plants, insects, and large animals; (ii) metal transporting agents; (iii) agents of symbiosis between microbes and plants, nematodes, insects, and higher animals; (iv) sexual hormones; and (v) differentiation effectors (Demain & Fang, 2000). Cnidarians which generally soft-bodied, morphologically simple organisms, sessile or have only limited mobility equipped with secondary metabolite for their defence mechanism. Cnidarians deploy a plethora of chemical arsenal for predation, protection and competition, especially in soft corals. Perhaps surprisingly, very little is known about the chemical arsenal employed by hard corals for survival, despite their expansive coverage and ecological prominence as keystone species in coral reefs.

To date, there are relatively limited bioactive compound isolated from hard corals compared to soft corals (from the order Alcyonacea) that constitute 94% of the 3,244 compounds discovered from cnidarians (Rocha et al., 2015). The lack of studies of hard coral metabolites may be due to the apparent lack of toxicity of most corals and the difficulties in obtaining sufficient biomass for biochemical work from these calcite-

fortified organisms. There is limited knowledge regarding the chemical defense mechanism of hard corals other than its epidermis and mucus that shed periodically to remove trapped microorganisms, phagocytic cells that can engulf and destroy microorganisms on contact, and soluble factors, including organic acids and antimicrobial products (Mullen et al., 2004). Prior to the recent concept of coral microbiome, corals were believed to lack adaptive immunity system and do not produce antibodies. Coral probiotic hypothesis proposed that the dynamic relationship of microbes, especially bacteria with its diverse population contingent on environmental condition could confer resistance to coral against disease and stresses (Reshef et al., 2006). The hologenome theory of evolution described hologenome as a consortium genome of host and associated symbiotic microorganisms that can change more rapidly than the host genome alone, thereby conferring greater adaptive potential to the combined holobiont organism, thus essentially responsible not only for the health and disease of individual higher organisms, but they also play a significant role in species survival and evolution (Rosenberg et al., 2007; Zilber-Rosenberg & Rosenberg, 2008). Understanding the mechanism concept of Beneficial Microorganism for Corals (BMC), a concept akin to the plant growth promoting rhizosphere (PGPR), could be important in coral reef conservation management, especially by proposing to manipulate the microbiome of corals for improving coral resilience, to ameliorate dysbiosis caused by environmental stress and to facilitate adaption against the shifting of environmental conditions (Peixoto et al., 2017). This proposed concept also highlight the possible dynamic roles of microbes such as fungi in corals other than the zooxanthellae and their plausible role in biosynthetic pathway of many marine natural products from the corals.

2.4.2 Mycosporines-like amino acids (MAAs)

MAAs are small (<400-Da), colourless, water-soluble compounds, imine derivatives of mycosporines. Due to the striking resemblance of core structures, MAAs are coined after the fungal mycosporines in which the former is prevalent in aquatic organisms and some terrestrial cyanobacteria (Karentz, 2001; Shick & Dunlap, 2002), while the latter are exclusively of terrestrial origin, from fungi (Leach, 1965; Trione et al., 1966; Favre-Bonvin et al., 1976; Bandaranayake, 1998; Gorbushina et al., 2003) with recent finds in rock-inhabiting cyanobacteria (Volkmann & Gorbushina, 2006).

To date, there are at least 27 known MAAs isolated from a wide range of marine organisms inclusive of phytoplankton, cyanobacteria, macroalgae and microalgae. (Bandaranayake, 1998; Karentz, 2001; Sinha et al., 2007; Rosic, 2019). Majority of them contain aminocyclohexenimine ring systems having UV absorption maxima between 310 and 360 nm with two exceptions of mycospporine-glycine and mycosporine-taurine containing aminocyclohexenone ring system similar to mycosporines (Bandaranayake, 1998) (**Figure 2.3**, **Figure 2.4**).

The symbiotic zooxanthellae of hard corals are ostensibly known to be the main contributors of MAA dubbed as 'microbial sunscreen' for the protection from ultraviolet radiation (Rosic & Dove, 2011). Even though 15 out of 20 MAAs been discovered across different genus of scleractinian corals (Jokiel et al., 1997; Dunlap & Shick, 1998; Shick et al., 1999; Shick, 2004; Yakovleva et al., 2004; Banaszak et al., 2006; Ferrier-Pagès et al., 2007; Carignan et al., 2009), the endosymbiont of corals, *Symbiodinium,* only able to produce maximum of five different MAAs in culture (Banaszak et al., 2000; Banaszak et al., 2006). Herein lies the reservation that *Symbiodinium* are the sole contributors of MAAs. There are possibilities that other coral holobionts such as marine fungi could contribute to the diversity of MAAs found in scleractinian corals, given the striking

homogeneity in the core structure of both MAAs and mycosporines. The discrepancies between zooxanthellae living in hospite produce more diverse MAAs compared to the same zooxanthellae cultured in vitro implies other roles of holobionts in contributing towards MAAs. Marine fungi are known to possess distinctive metabolism compare to their terrestrial counterpart (Reich &Labes, 2017). Their distinct and untapped BGCs may hold the clues to deduce MAAs production in hard corals.

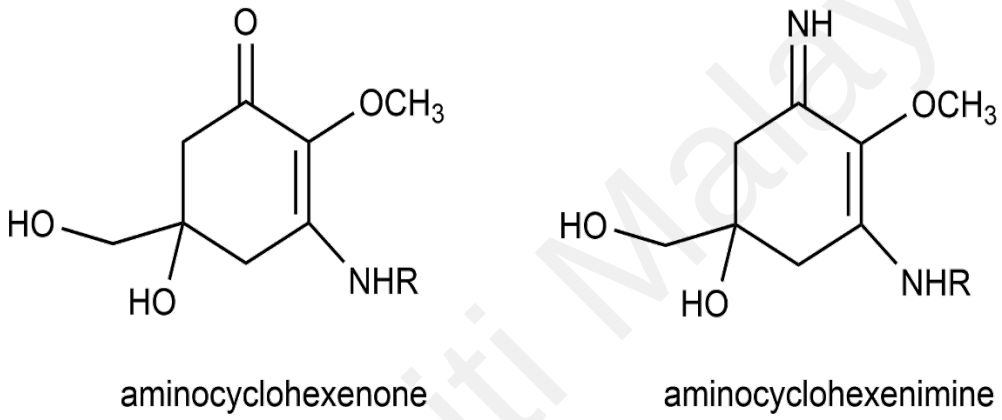


Figure 2.3: The core structure of fungal mycosporine (aminocyclohexenone)(left) and MAAs (aminocyclohexnimine) (right).

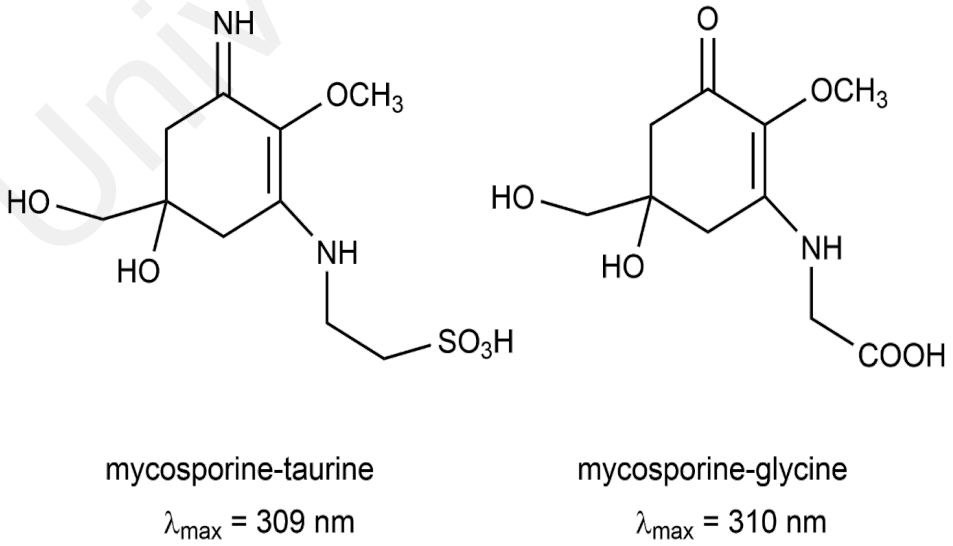


Figure 2.4: The only two exception of MAAs with core structure of aminocyclohexenone

CHAPTER 3

METHODOLOGY

3.1 Sample collections

Samples of scleractinian coral colonies from the Perhentian Islands were collected from surrounding coral reefs (**Figure 3.1**) by SCUBA diving, with their respective coordinates, depths and specimen identification number listed in **Table 3.1**. Scleractinian corals samples were chosen to represent the different growth forms commonly found in the islands, such as branching, solitary, meandering/plates and massive corals. Scleractinian corals were initially identified using the Coral Finder Guidebook during the underwater sampling. The scleractinian corals were photographed underwater using Olympus TG-5 digital camera for preliminary identification.

Scleractinian coral fragments (4~6 cm) were cut and placed into separately labelled plastic bags with seawater during underwater sampling. The coral samples were divided into two sets of labelled 50 ml Falcon tubes; one set containing absolute ethanol for molecular study and the other set in natural seawater for isolation of coral-associated fungi. The coral samples were kept cold in ice and transported back to the laboratory, where they were stored in 4 ^{o}C until needed for further identification work.

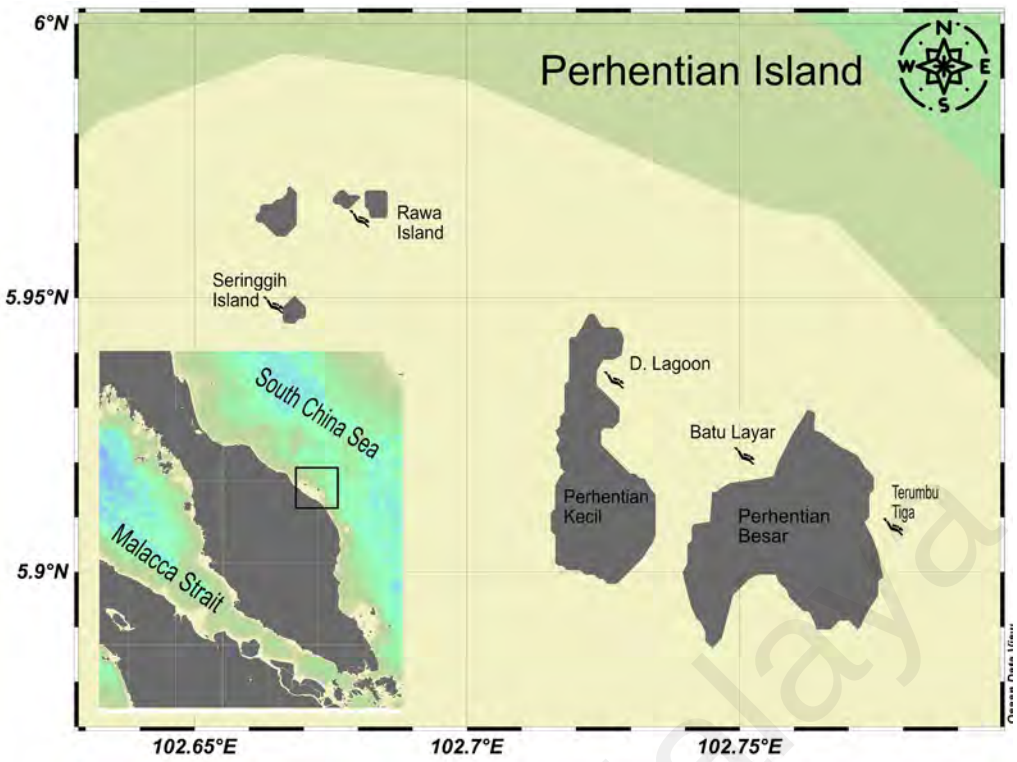


Figure 3.1: Map of five sampling sites (Rawa Island, Batu Layar, Seringgih island, D'Lagoon and Terumbu Tiga) in the Perhentian Islands, Terengganu where scleractinian coral samples were obtained by SCUBA diving. (Map was drawn in Ocean Data View).

3.1.1 Morphological Identification of Hard corals

The collected scleractinian corals were initially identified using the Coral Finder Guidebook during the underwater sampling. A total number of fourteen scleractinian corals were collected (**Table 3.1**). The coral fragments were sprayed with a powerful water jet to remove as much tissue as possible before being bleached in 5% sodium hypochlorite solution. Subsequent inspection and photography of the skeletons and corallites were done using an Encoded stereo microscope (Leica M125 C) with reference to Scleratinian of Eastern Australia Monograph Series (Veron & Pichon, 1976a, 1976b, 1977, 1980, 1984), Coral of the World Vol.1-3 (Veron, 2000) and Staghorn corals of the world (Wallace, 1999). The skeletons of the samples were deposited future references and examinations.

Table 3.1: List of scleractinian coral samples, their putative identification, growth forms, including the location and depth that they were collected from the five sampling sites in the Perhentian Islands, Terengganu.

Location	Sample ID	Putative coral genus/species	Growth form	Depth (m)	GPS coordinates
Rawa Island	PERC1701	*Acropora* sp1	Branching	4	102°40'54.59"E, 5°57'38.28"N
	PERC1702	*Pocillopora darmiconis*	Branching	5	
	PERC1704	*Acropora* sp2	Branching	4	
	PERC1706	*Acropora* sp3	Branching	4	
	PERC1707	*Fungia fungites*	Solitary	4	
Seringgih Island	PERC1709	*Pavona cactus*	Meandering	4	102°40'5.29"E ,5°56'27.66"N
	PERC1712	*Porites* sp1	Massive	5	
	PERC1713	*Fungia fungites*	Solitary	4	
D'lagoon	PERC1715	*Porites* sp2	Massive	7	102°43'23.21"E,5°55'56.34"N
	PERC1717	*Galaxea astreata*	Meandering	10	
Terumbu Tiga	PERC1718	*Tubastraea* sp1	Solitary	17	102°46'25.69"E ,5°54'2.76"N
	PERC1719	*Acropora* sp4	Branching	11	
	PERC1720	*Turbinaria peltata*	Meandering		
Batu Layar	PERC1722	*Porites* sp3	Massive	14	102°44'39.95"E,5°54'44.10"N

3.1.2 Genomic DNA (gDNA) extraction, PCR amplifications and DNA sequencing

For each sample, approximately 3 cm length of ethanol preserved coral fragment was ground and homogenized by using mortar and pestle. The homogenized tissue was transferred into a Falcon tube and centrifuged (500 x g at 44 °C for 5 mins) to separate the skeletal debris from the tissue. Extraction of gDNA was done using a DNeasy® Blood & Tissue kit (QIAGEN, Hilden, Germany) following the manufacturer's protocol. Quality of gDNA was checked via gel electrophoresis (2 % agarose gel in 1X TAE) and quantified using Nanodrop.

Mitochondrial cytochrome oxidase 1 (CO1) was amplified by polymerase chain reaction (PCR) using universal primers LCO1490: 5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3 and HCO2198: 5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′ (Folmer et al., 1994). PCR amplification was performed in a 2 X 20 µL total volume with a final concentration of 1X Reaction Buffer (GeNet Bio) (2 mM Tris-HCl, pH 9.0, 2 mM MgCl2), 0.25 mM dNTPs mixture, 2.5 U Taq DNA Polymerase (GeNet Bio), 0.1mM of each primer and 10ng of DNA template. PCR thermal cycling was performed using Applied Biosystems™ SimpliAmp™ Thermal Cycler following these profile conditions: 95 ^{o}C for 3 mins, followed by 35 cycles of 95 ^{o}C for 1 mins, 40 ^{o}C for 1 mins, 72 ^{o}C for 1.5 mins with a final extension step of 72 ^{o}C for 7 mins (Folmer et al., 1994; Hsu et al., 2014). The PCR products (5 µL) were visualised using 2 % agarose gel electrophoresis. The amplified products (150-200 ng in 15 - 30 µl volume) were sequenced in both direction by FirstBase Sequencing Services (Malaysia) using BigDye® Terminator v3.1 cycle (Applied Biosystem, USA).

3.1.3 DNA sequence analysis, multiple sequence alignment and phylogenetic analysis of scleractinian corals

DNA sequence reads and chromatograms were inspected using Sequence Scanner v1.0 (Applied Biosystem), edited using BioEdit ver. 7.2 (Hall, 1999) and aligned with Clustal-X 2.0 (Thompson et al., 1994; Larkin et al., 2007) to assemble the contiguous sequence for each coral sample. Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) was used to search for homologous CO1 sequences in GenBank for the initial species identification. Several plausible coral species identities were considered based on the BLAST results for each query sequence, depending on the score, similarity percentage, query cover. Some of these retrieved sequences were used for subsequent phylogenetic analysis, which inclusive of 118 COI reference sequences from Genebank/NCBI and Kitahara et al. (2010) (**APPENDIX C**).

Phylogenetic analysis was conducted to further identify the coral species. Our coral sequences were simultaneously aligned with other coral reference sequences (i.e type specimens and published vouchers), including sequences of anemones (Order Actinaria) and zoanthid (Order Zoantharia) as outgroups. Multiple alignment program for amino acid or nucleotide sequences (MAFFT) (Katoh et al., 2017) was used to align all the sequences with L-INS-i algorithms and default parameter. The aligned sequences were trimmed and analyzed for their nucleotide composition, sequence variation, and genetic distances using Molecular Evolutionary Genetic Analysis (MEGA X) (Kumar et al., 2018).

Phylogenetic reconstructions were performed with three different approaches, Neighbor-joining (**NJ**), Maximum likelihood (**ML**), and Bayesian Inferences (**BI**). ML and BI were performed on an online platform known as Cyberinfrastructure for Phylogenetic Research (CIPRES) Science Gateway V 3.3 (Miller et al., 2010). NJ was

performed using MEGA X with default settings of using the Kimura 2-parameter method with 1,000 bootstrap replicates. ML were performed using RaxML-HPC BlackBox 8.2.12/RaxML-NG (Kozlov et al., 2019), with 1,000 bootstrap replicates. BI was performed using MrBayes 3.2.7 (Ronquist et al., 2012). The best fitted evolutionary model were calculated by Akaike information criterion (AIC) using Modeltest-NG (Darriba et al., 2019), and GTR + Gamma + Proportion Invariant (GTR+G+I) model of DNA evolution was subsequently incorporated into ML and BI analysis. In BI analysis, Markov Chain Monte Carlo (MCMC) was used to estimate the posterior probability (PP) distribution executed using MrBayes v3.2.7 (Ronquist et al., 2012). The effective sample size (ESS) value was examined in Bayesian Evolutionary Analysis Sampling Trees BEAST v2.5 (Drummond et al., 2002; Bouckaert et al., 2019) and Tracer v1.7 (Rambaut et al., 2018) to determine the sufficient generations for MCMC. The CO1 dataset analysis was performed at 10,000,000 generations per run, sampling frequency of 1000 and PP was estimated with 25% burn-in.

3.2 Marine fungi from scleractinian corals

3.2.1 Isolation of marine fungi

For the isolation of marine-derived fungi, only eight of the fourteen scleractinians corals were chosen to represent different genera of corals. Prior to isolation, coral fragments stored in seawater were dipped in 90% ethanol for 3 minutes for surface sterilization and rinsed with sterilized artificial seawater. Coral fragments were ground and mixed with sterilized artificial seawater. The slurry was transferred in a Falcon tube and vortexed for 5 minutes and made into three sets of 10-fold serial dilution. 100 µl of each dilution was spread in three replicates of petri dishes containing Corn Meal Agar

(CMA) diluted in 70 % seawater spiked with Streptomycin. Plates were incubated at 26–28 ºC for 3–5 days. Plates were checked for any hyphal tips to signify successful culturing of fungi. The fungi were then isolated and sub-cultured on Potato Dextrose Agar (PDA) as pure culture. The pure fungal isolates were kept at 4 ºC for long term storage.

3.2.2 Morphological identification of marine-derived fungi

Pure fungal isolates were identified by using microscopy to observe the morphology of the spores and hyphae. Pure isolates were revived and cultured under continuous normal light on PDA for three weeks at 25 °C. Mycelial plugs (5 mm diameter) were cut from colony margins and placed into 9-cm-diameter petri dishes with two plates per isolate. Conidial production, shape and the size of conidia were examined daily up to three weeks on PDA incubated at 25 °C.

Appressoria were produced from hyphae using a slide culture technique described by (Riddell, 1950). PDA plugs (1 cm × 1 cm) were placed in clean petri dishes, and each agar plug was embedded with conidia, and a cover slip was placed over each plug. After 7 days, the micrographs were taken under a Nikon Eclipse Light microscope. Species identifications were based on (Kohlmeyer & Volkmann-Kohlmeyer, 1991; Hyde & Sarma, 2000; Webster & Weber, 2007). For each isolate, possible species were listed for further identification.

3.2.3 gDNA extraction and PCR amplifications of fungal strains

Isolates were incubated at 25 ^{o}C in Potato Dextrose Broth (PDB) for 7 days. After 7 days of growth, mycelia were harvested by filtering the broth and DNA was extracted using BioTeke Plant DNA purification kit (BioTeke, China) following manufacturer protocol. gDNA was amplified with universal barcoding marker for fungi, Nuclear ribosomal internal transcribed spacer (ITS) using primer pairs of ITS 5 (forward) (5'-GGA AGT AAA AGT CGT AAC AAG G-3') and ITS 4 (reverse) (5'-TCC TCC GCT TAT TGA TAT GC-3') (White et al., 1990). PCR amplification was performed in a 20 µl total volume with a final concentration of 1X Reaction Buffer (GeNet Bio) (2 mM Tris-HCl, pH 9.0, 2 mM MgCl2), 0.25 mM dNTPs mixture, 2.5 U Taq DNA Polymerase (GeNet Bio), 0.1mM of each primer, and 10ng of gDNA template. PCR thermal cycling was performed using Applied Biosystems™ SimpliAmp™ Thermal Cycler following these profile conditions: 95 ^{o}C for 5 mins, followed by 30 cycles of 95 ^{o}C for 1 mins, 55 ^{o}C for 1 mins, 72 mins for 1.5 mins with a final extension step of 72 ^{o}C for 10 mins (Kwiatkowski et al., 2012). The PCR products were electrophoresed in a 2 % agarose (Next Gene Scientific, Malaysia) gel in 1X TAE and were send for sequencing by FirstBase Sequencing Services (Malaysia) using BigDye® Terminator v3.1 cycle (Applied Biosystem, USA).

3.2.4 DNA sequence analysis, multiple sequence alignment and phylogenetic analysis of fungal ITS sequences for species identification

DNA sequence reads and chromatograms of 31 fungal isolates were inspected using Sequence Scanner v1.0 (Applied Biosystem), edited using BioEdit ver. 7.2 (Hall, 1999) and aligned with Clustal-X 2.0 (Thompson et al., 1994; Larkin et al., 2007) to

assemble the contiguous sequence for each fungal isolate. Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) was used to search for homologous ITS sequences in GenBank for species identification. NJ was performed using MEGA X with default settings of using the Kimura 2-parameter method with 1,000 bootstrap replicates.

3.2.5 Additional DNA sequencing and species verification of two selected marine derived fungi

Two marine-derived fungi were selected for metabolites study, namely *Colletotrichum* sp. (**PERF171521**) and *Neodevriesia* sp. 4 (**PERF171711**) that were isolated from the identified corals *Porites sp2* and *Galaxea astreata*, respectively. *Colletotrichum* was chosen to represent a fungal species that is known for its bioactive compounds, while *Neodevriesia* was chosen because it is a relatively new genus and less studied in terms of their metabolites.

(A) Molecular identification of *Colletotrichum* sp. (PERF171521) using multiple markers

Due to the poor resolution of ITS sequences to identify the species, five additional genetic markers (**Table 3.2**) were used: actin (ACT), chitin synthase (CHS-1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), calmodulin (Cal) and β-tubulin (TUB2). PCR amplifications were done in 2 X 25-µl volumes containing 4 mM MgCl2, 1 × PCR buffer, 0.5 units Taq DNA polymerase (GeNet Bio, Korea), 0.2 mM concentrations of each dNTP, 0.5 µM concentrations of each primer and 1 µl of template DNA (20 ng/µl). The PCR programs for ACT, CHS-1, GAPDH, ITS and Cal were set

following Weir et al. (2012). Amplified products were visualized using 2% agar gel electrophoresis and were outsourced for sequencing by FirstBase Sequencing Services (Malaysia). Sequences from the six genes were manually concatenated and re-aligned using SequenceMatrix (Vaidya et al., 2011). Best fitted evolutionary model for respective genetic regions were calculated by Akaike information criterion (AIC) using Modeltest-NG (Darriba et al., 2019)(). Reference sequences were obtained from Weir et al (2012) for the construction of the multigene phylogeny.

Table 3.2: Additonal five protein-coding markers used on resolve the identity of the strain PER171521.

Gene	Length	Primer		Sequence (5'–3')	References
Actin (act)	316bp	Act-512F	F	ATG TGC AAG GCC GGT TTC GC	Carbone & Kohn (1999)
		Act-783R	R	TAC GAG TCC TTC TGG CCC AT	
Beta-tubulin 2 (tub2)	716bp	TI	F	AAC ATG CGT GAG ATT GTA AGT	Glass & Donaldson (1995); O'donnell & Cigelnik (1997)
		Bt2b	R	ACC CTC AGT GTA GTG ACC CTT GGC	
Calmodulin (cal)	756bp	Cl1C	F	GAA TTC AAG GAG GCC TTC TC	Weir et al. (2012)
		Cl2C	R	CTT CTG CAT CAT GAG CTG GAC	
Chitin synthase (chs-1)	229bp	CHS-79F	F	TGGGGCAAGGATGCTTGGAAGAAG	Carbone & Kohn (1999)
		CHS-345R	R	TGGAAGAACCATCTGTGAGAGTTG	
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	308bp	GDF	F	GCC GTC AAC GAC CCC TTC ATT GA	Templeton et al. (1992)
		GDR	R	GGG TGG AGT CGT ACT TGA GCA TGT	

Table 3.3*:* Nucleotide substitution models used in phylogenetic analyses.

Markers	Gene	Nucleotide Substitution Model (AIC)
Act	Actin	HKY+I+G4
Tub2	β– Tubulin	TIM1+I+G4
ITS	Internal Transcribed Spacer	TRNEF+I+G4
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase	TIM3+I+G4
CHS-1	Chitin Synthase	TRNEF+I+G4
Cal	Calmodulin	TIM1+I+G4

BI were conducted using MrBayes 3.2.7 (Ronquist et al., 2012) with respective partitioned nucleotide substitution models of calculated in Modeltest-NG. Markov Chain Monte Carlo (MCMC) was used to estimate the posterior probability (pp) distribution executed using MrBayes v3.2.7 (Ronquist et al., 2012). The effective sample size (ESS) value was examined in Bayesian Evolutionary Analysis Sampling Trees BEAST v2.5 (Drummond et al., 2002; Bouckaert et al., 2019) and Tracer v1.7 (Rambaut et al., 2018) to determine the sufficient generations for MCMC. The CO1 dataset was performed at 20,000,000 generations per run, sampling frequency of 1,000 and PP was estimated with 25% burn-in, bootstrap support was calculated based on 1,000 iterations.

(B) Molecular identification of *Neodevriesia* sp. (PERF171711) using LSU and ITS

For *Neodevriesia* sp. 4 (**PERF171711**), LSU and ITS markers proved to be reliable markers to resolve the identity of the fungi *Neodevriesia* spp. (Frank et al., 2010; Li et al., 2013). 28S region using primer pairs NL1 (5’-GCA TAT CAA TAA GCG GAG GAA AAG-3’) with NL4 (5’-GGT CCG TGT TTC AAG ACG G-3’) (White et al., 1990).

PCR amplification was performed in a 20 µl total volume with a final concentration of 1X Reaction Buffer (GeNet Bio) (2 mM Tris-HCl, pH 9.0, 2 mM $MgCl_2$), 0.25 mM dNTPs mixture, 2.5 U Taq DNA Polymerase (GeNet Bio), 0.1mM of each primer, and 10ng of gDNA template. PCR thermal cycling was performed using Applied Biosystems™ SimpliAmp™ Thermal Cycler following these profile conditions: 94 ^{o}C for 2 mins, followed by 30 cycles of 94 ^{o}C for 15 seconds, 55 ^{o}C for 30 seconds, 68 ^{o}C for 2 mins with a final extension step of 68 ^{o}C for 5 mins (Kwiatkowski et al., 2012). The PCR products were electrophoresed in a 2 % agarose (Next Gene Scientific, Malaysia) gel in 1X TAE and were send for sequencing by FirstBase Sequencing Services (Malaysia) using BigDye® Terminator v3.1 cycle (Applied Biosystem, USA).

BI was conducted using MrBayes 3.2.7 (Ronquist et al., 2012) with respective partitioned nucleotide substitution models of calculated in Modeltest-NG, both are TIM2+I+G4 according to AIC. Markov Chain Monte Carlo (MCMC) was used to estimate the posterior probability (pp) distribution executed using MrBayes v3.2.7 (Ronquist et al., 2012). The effective sample size (ESS) value was examined in Bayesian Evolutionary Analysis Sampling Trees BEAST v2.5 (Drummond et al., 2002; Bouckaert et al., 2019) and Tracer v1.7 (Rambaut et al., 2018) to determine the sufficient generations for MCMC. The concatenated LSU and ITS dataset was performed at 10,000,000 generations per run, sampling frequency of 1,000 and PP was estimated with 25% burn-in, bootstrap support was calculated based on 1,000 iterations.

3.3 Bio-Chemical analysis of extract from marine derived fungi

3.3.1 Mass fermentation and extraction of mycelium from *Colletotrichum* sp. (PERF171521) and *Neodevriesia* sp. 4 (PERF171711)

Selected pure fungal isolates were cultured on Czepek's medium for 3 days at 28 °C to obtain the seed cultures. Seed cultures were then transferred into Erlenmeyer flasks (1L) (10 flasks) containing PDB for fermentation to obtain significant biomass for crude. Two strains of fungal, viz. *Colletotrichum* sp. (**PERF171521**) and *Neodevriesia* sp. 4 (**PERF171711**) were selected for mass culture. Both strains are fast growing strains and were identified based on morphological ID and DNA sequences. *Colletotrichum* spp. are known to produce antifungal and antibacterial bioactive compounds. *Neodevriesia* spp. however, on the other hand, is a recently discovered and lesser-known genus with no information available regarding the bioactive compound from the fungi. Strains were cultured in sterilized PDB in ten replicates. Cultures were fermented for 30 days before mycelium were filtered and immersed in methanol for extraction. The extract was filtered, and the filtrates were evaporated using a rotary evaporator to obtain the methanol crude extract and transferred to a vial. Methanol (MeOH) crude extract was then partitioned with mixture of ethyl acetate (EtOAc)/water, in 1:3 ratio, to separate the non-polar and polar layer. The ethyl acetate crude extracts were then further separated with silica gel (200–300 mesh) in column chromatography with solvent system of hexane (Hex) and EtOAc, with gradient elution of, 9:1, 8:2, 7:3, 6:4, 5:5, and final washing of chloroform (Chl)/MeOH/water with ratio of, 65/35/4.

3.3.2 Thin layer chromatography (TLC) profiling of extracts and NMR profiling

The ethyl acetate partition of fungal extracts, which contained the non-polar compound and its respective column chromatography fractions, were subjected to a thin layer chromatography to detect the presence of chemical constituents. Phosphomolybdic acid solution 5 wt. % in ethanol were used as a derivatization reagent for observation of phenolics, hydrocarbon waxes, alkaloids and steroids.

However, due to the low crude extract yield for *Neodevriesia* sp., only crude extract of *Colletotrichum* sp was further evaluated for presence of secondary metabolite using preparative thin layer chromatography (PTLC). Targeted isolates of interest crude extracts of *Colletotrichum* sp. were repetitively purified repeatedly using PTLC to obtain pure isolates, which were and finally subjected to nuclear magnetic resonance spectroscopy (NMR) to characterize the pure isolates. 1D-NMR (^{1}H,^{13}C) was done on a Bruker AVANCE III 600 MHz using deuterated chloroform, 99.95% (Merck) in 5mm diameter NMR tube.

3.3.3 Antibacterial assay

The evaluation of antibacterial potential of the *Colletotrichum* sp. fungal crude extract and fractions were assessed via disc diffusion antibacterial technique (Jorgensen & Turnidge, 2015) using three gram-negative bacteria; *Escherichia coli* ATCC 25922, *Salmonella enterica* serovar Typhi, *Serratia marsecens* and two gram-positive bacteria; *Staphylococcus aureus*, *Bacillus subtilis*. Bacterial colony was inoculated into sterile nutrient broth and incubated under aerobic condition for a period of 24 hours at 30 ºC. as nutrient broth is the suitable medium for reviving the bacterial strains used in this experiment. The optical density of the bacterial suspension was then adjusted to 0.5

MacFarland Index (0.105 ± 0.005 abs). Pre-prepared Mueller Hinton agar (MHA) plates was loaded with 100 µl of the adjusted bacterial suspension and spread evenly. Antibiotic discs were impregnated with 20 µl of fractions and extracts, which were diluted in methanol comprising with into five different concentrations (500 µg/ml, 250 µg/ml, 125 µg/ml, 62.5 µg/ml and, 31.25 µg/ml), respectively and were placed onto the MHA plates. Two different positive controls, Vancomycin (gram-negative strain positive control) and Imipenem (gram-positive strain positive control) were used. The plates were aerobically incubated at 30 ºC for 24 hours, followed by measurement of inhibition zones were measured to determine the minimal inhibitory concentration (MIC) of the extracts and fractions.

3.3.4 Fatty acid evaluation

The fatty acid fraction (column chromatography Fraction 1) of *Colletotrichum* sp. and *Neodevriesia* sp. fungal extracts were subjected to methylation and subsequently Gas Chromatography Mass Spectrometry (GC-MS) for profiling. Fraction 1 were converted to fatty acid methyl ester (FAME), in which 2.7 ml of hexane and 0.3 ml of sodium methylate solution were added to the fatty acids fraction in a methylation vial and stirred for 2 hours at room temperature. Then the solution was evaporated and chloroform-methanol (1:1) was added to dissolve and transfer all the residues from the methylation vial. The solution was evaporated to obtain orange-a yellowish-orange oil. The obtained FAMEs were evaporated, desiccated, and weighed, then and kept in freezer at -80˚C prior to analysis. The FAMEs were diluted in 2 ml of GC Grade (Merck) hexane and filtered using 0.45 µm filter before being used in GC-MS analysis., and then subjected to Gas Chromatography for analysis.

The conditions for GC measurements were as follows: Shimadzu QP2010 ULTRA (Shimadzu, Kyoto, Japan), column Restek™ Rtx™-5MS (29.6 m x 0.25 mm, 0.25µm thick) (Restek, Pennsylvania, USA); injection and detector temperature, 260 ºC; column temperature 50 ºC (5 min) - 4 ºC min-1 (25 min) - 280ºC (30 min). The injection volume was 1 µl at temperature 260 ºC, split ratio 1:30, helium as gas carrier and detector temperature was set at 260 ºC. FAME peaks were identified by comparison of their retention times with those of a standard mixture (Spelco 37 mixture FAME) (Spelco, UK). Shidmazu Class VP software (version 2.1) was used to integrate peak areas. The MS ion source temperature was set at 200 ºC, interface temperature at 280 ºC, scan range from 40 amu to 450 amu, event time of 0.07s, scan speed at 10,000 amu/s, with sampling time of 65mins.

CHAPTER 4

RESULTS

4.1 Morphological identification of scleractinian corals

A total of 14 coral samples were collected and identified (13 species, 8 genera). However, only 5 samples could be morphologically identified to species level (2 x *Fungia fungites, Pavona cactus, Galaxea astreata and Turbinaria peltata*). The other 9 samples were identified to genus level only (*Acropora* spp, *Porites* spp and *Tubastrea* sp) (**Table 4.1**). A more detailed description of the growth forms and corallites of the corals were shown in **APPENDIX D**.

Table 4.1: Identified scleractinian corals based on morphology.

ID	Hard corals species
PERC1701	*Acropora* sp.1
PERC1702	*Pocillopora* sp.1
PERC1704	*Acropora* sp.2
PERC1706	*Acropora* sp.3
PERC1707	*Fungia fungites*
PERC1709	*Pavona cactus*
PERC1712	*Porites* sp.1
PERC1713	*Fungia fungites*
PERC1715	*Porites* sp.2
PERC1717	*Galaxea astreata*
PERC1718	*Tubastraea* sp.1
PERC1719	*Acropora* sp.4
PERC1720	*Turbinaria peltata*
PERC1722	*Porites* sp.3

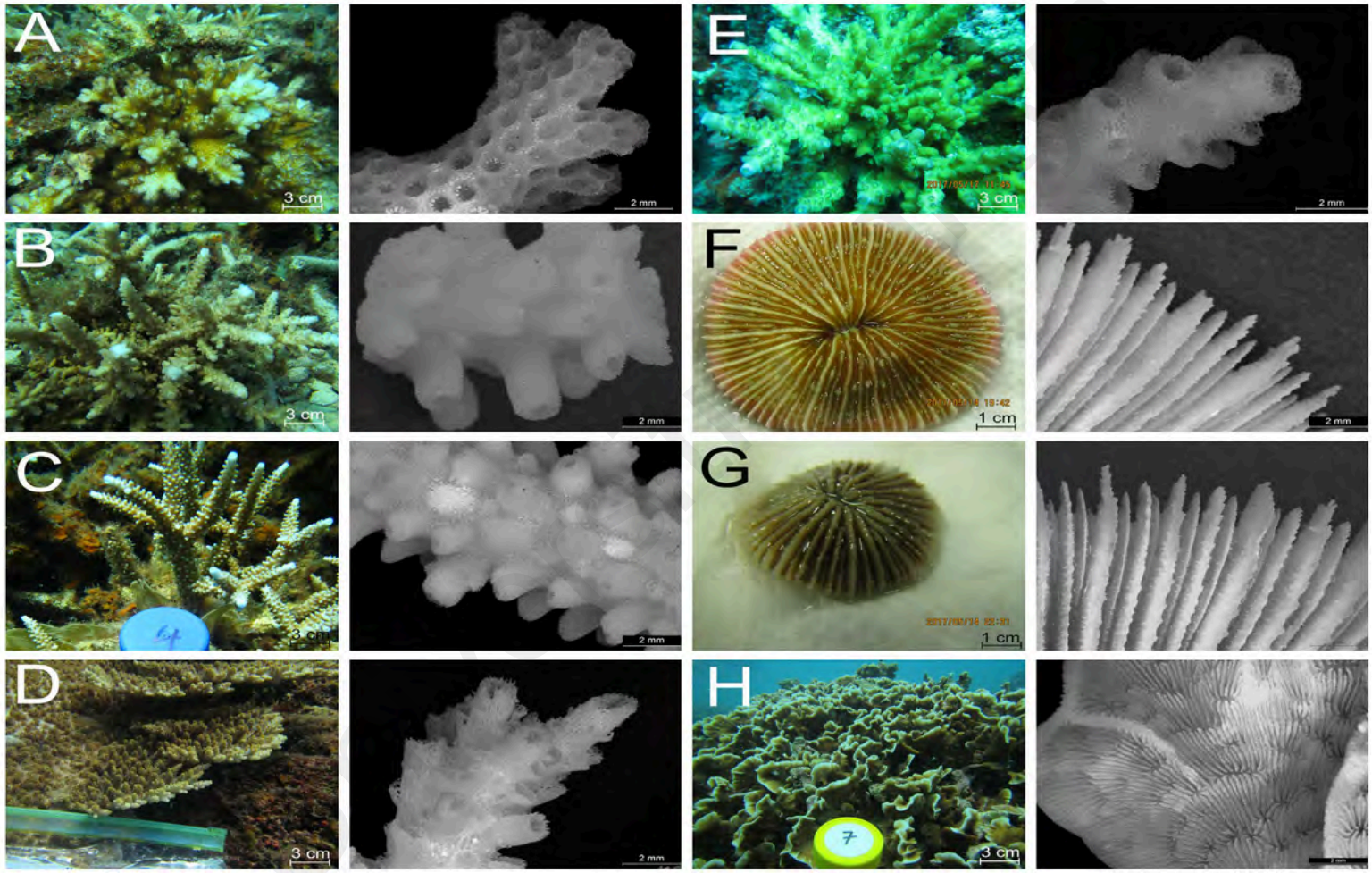


Figure 4.1: Morphology of Scleractinian corals with their growth form (left) corallites taken via Leica microscopy (right). **A)** *Pocillopora* sp1, **B)** *Acropora* sp1 **C)** *Acropora* sp2, **D)** *Acropora* sp3, **E)** *Acropora* sp4, **F)** *Fungia fungites*, **G)** *Fungia fungites*, **H)** *Pavona cactus*.

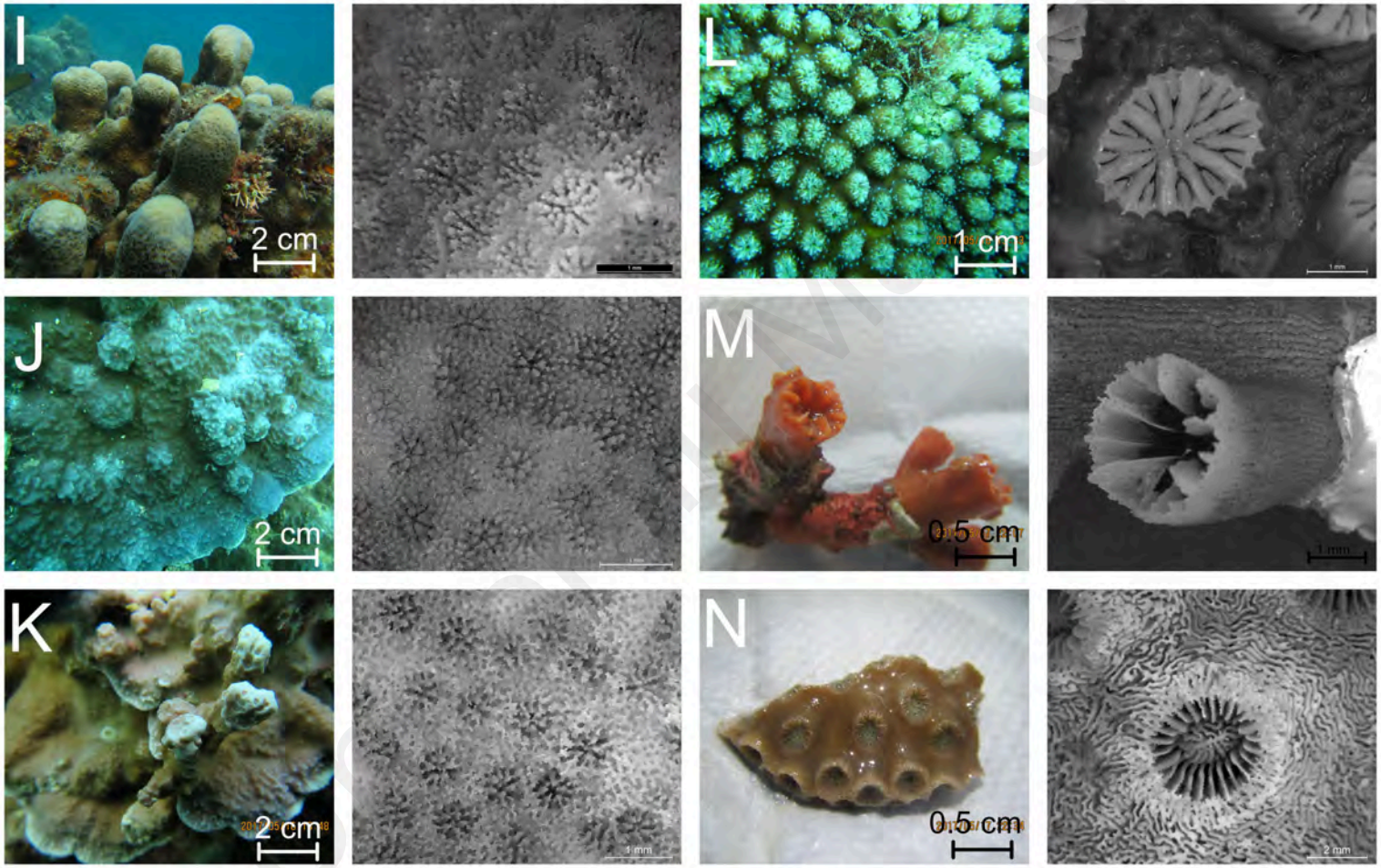


Figure 4.2: Morphology of Scleractinian corals with their growth form (left) corallites taken via Leica microscopy (right). **I)** *Porites* sp1, **J)** *Porites* sp2, **K)** *Porites* sp3, **L)** *Galaxea astreata*, **M)** *Tubastrea* sp1, **N)** *Turbinaria peltata*.

4.2 Scleractinian coral CO1 sequences and phylogeny

CO1 sequences were obtained as the standard hard coral barcode to complement the morphology identification. Fourteen coral samples sequences were generated from this study, ranging from 700~800bp in length. The final alignment length of 132 coral CO1 sequences was 660 bases (=220 amino acid residues) which contained 283 variable sites and 234 parsimony informative sites. Nucleotide composition of all sequences were similar, with the average of T (thymine) = 37.6, C (cytosine) = 17.7, A (adenine) = 21.2 and G (guanine) = 23.5 percent.

All the NJ, ML, and BI phylogenetic trees produced were congruent, and were rooted with *Zoanthus* sp., *Anemonia* sp. and *Stichodactyla* sp. **Figure 4.3** shows the consensus tree of NJ, ML, and BI analysis with their supporting bootstrap values indicated on the BI phylogram. The tree shows seven distinct and strongly supported scleractinian families (Fungiidae, Pocilloporidaem Dendrophylliidae, Poritidae Euphylliidae, Agariciidae, Acroporidae), in which the fourteen Perhentian island coral sequences were embedded. The identification of most samples had to rely on their morphology, especially when they could not be resolved phylogenetically.

Both **PERC1707** and **PERC1713** belong to family Fungiidae, and clustered with *Fungia fungites*, with medium to strong support values, especially for ML and BI analysis. **PERC1702** was found within the genus Pocillopora (family Pocilloporidae) with strong support. Although it clustered with some *Pocillopora damicornis* sequences, it could only be identified as *Pocillopora* sp.1 because monophyly was not established between the species and *P.edyouxi*. **PERC1718** and **PERC1720** are both members of family Dendrophylliidae. **PERC1720** clustered with type and reference sequences of *Turbinaria peltata*, with strong support, thus confirming its identity. Although,

PERC1718 could not be distinguished genetically between closely related *Tubastrea Dendrophyllia, Cladopsammia and Rhizopsammia*, the sample could be identified morphologically as *Tubastrea* sp1 based on the septa arrangements of the corallites. Samples **PERC1712, PERC1715, PERC1722** belong to the family Poritidae and strongly clustered within genus *Porites*. However, the CO1 marker was unable to differentiate betwwen *Porites* spp. Coupled with their corallites morphology and their **PERC1712, PERC1715, PERC1722** were assigned only as *Porites* sp.1, *Porites* sp.2 and *Porites* sp.3**,** respectively. **PERC1709** belong to the family Agariciiae, showed medium to strong phylogenetic affinity to either *Pavona cactus* or *Pavona clavus*, but was finally identified as *Pavona cactus* after considering its morphology. **PERC1717** clustered strongly in family Euphyllidae, clade containing genera *Euphyllia* and *Galaxea*, but was confirmed as *Galaxea astreata* based on morphology. Sequences **PERC1701, PERC1704, PERC1706, PERC1719** strongly clustered in the family Acroporidae and the genus *Acropora*. The topology shows the genus *Acropora* is a species complex and our coral sequences could not easily be identified. Furthermore, coupled with their similarity in corallites and branches morphology, we could only identify **PERC1701, PERC1704, PERC1706, PERC1719** as *Acropora* sp.1, *Acropora* sp.2, *Acropora* sp.3, *Acropora* sp.4.

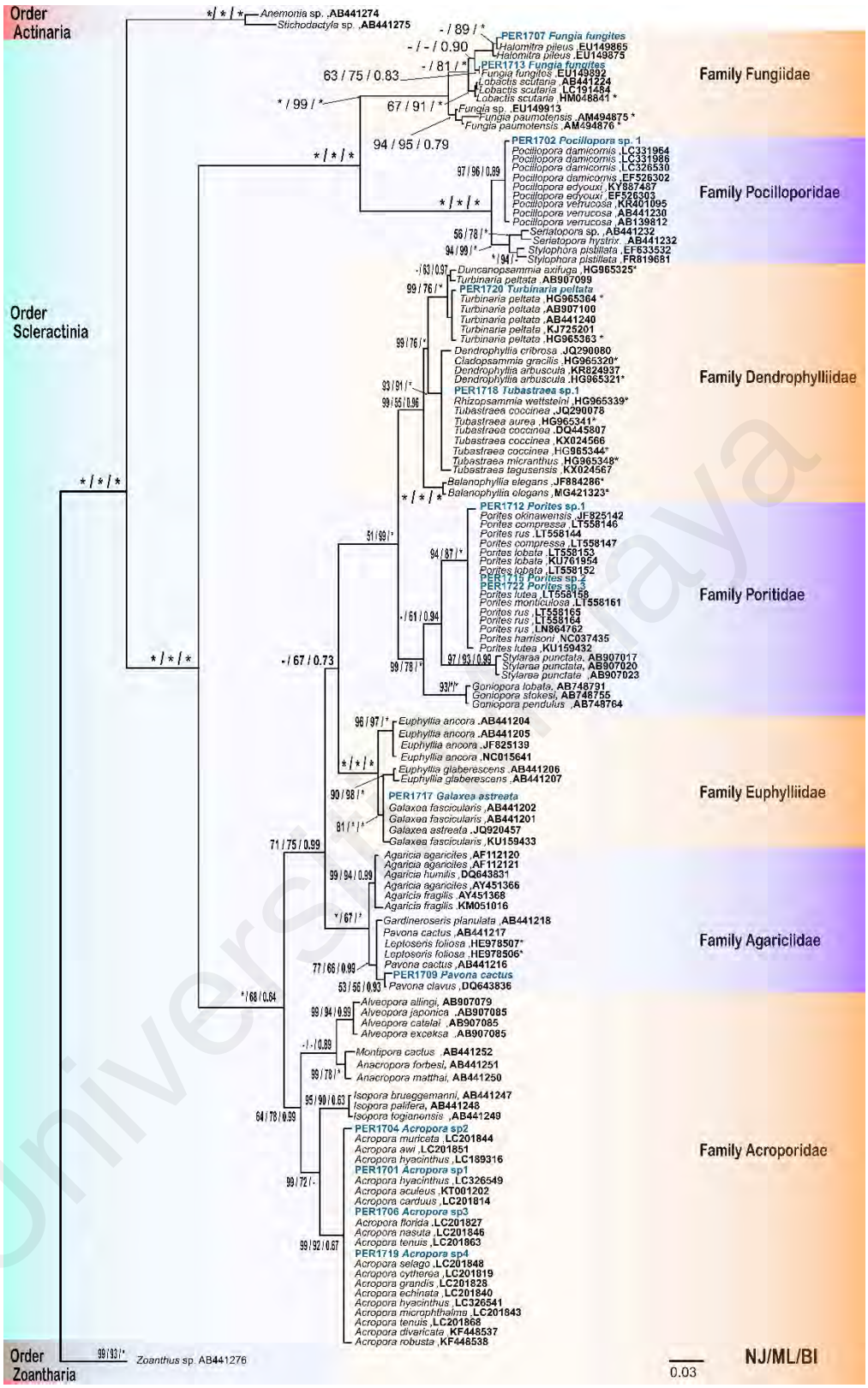


Figure 4.3: Consensus tree of NJ, ML and BI using the topology of BI tree based on the CO1 sequences (660bp) of scleractinian corals that was rooted with Zoanthus sp (Order Zoantharia), Anemonia sp. and Stichodactyla sp (both from Order Actinaria). Perhentian islands coral sequences clustered within the seven scleractinian families of Fungiidae, Pocilloporidaem Dendrophylliidae, Poritidae Euphylliidae, Agariciidae and Acroporidae. Bootstrap values (NJ/ML) and posterior probabilities (BI) are indicated above the branches. Only values above 50% or 0.5 were indicated and considered for intepretation. Asterisks indicating 100 and 1.0 support values.

4.3 Morphological and molecular identification of coral associated fungi

A total of 31 fungal isolates (represented in 18 genera) were successfully obtained and could only be identified up to the genus level using ITS sequences via NJ approach (see **APPENDIX E, F, G, H)**. The respective hyphae micrograph and plate culture photos were shown in **APPENDIX I.** Most of the isolated fungal strains were of terrestrial origin and cosmopolitan species. Species from the genus *Neodevriesia* was widespread, frequently found in five out of eight scleractinian coral samples, while *Penicillium* was the dominant genera, with a total of six isolates. ITS sequences (~600bp) of the 31 fungal isolates were compared against sequences in GenBank using BLASTn tool and NJ tree to further verify their identifications. However, both approaches could not confirm to the species level, despite having high similarity index of 97-100% and strong support because 1) Not all species of fungi have been barcoded using ITS marker, 2) Not all fungi in GenBank were identified, 3) ITS sequences show very little variations to distinguish some fungal species for species complex like *Aspergillus*, *Colletotrichum* and *Penicillium*. Due to relatively short length of ITS sequences (~600bp) and low variation, these sequences could not resolve the identification of the fungal isolates. The tentative identity of these isolates was summarized in **Table 4.2**.

Colletotrichum sp1 (**PERF171521**) from *Porites* sp. 2 and *Neodevriesia* sp. 4 (**PERF171711**) from *Galaxea astreata* were chosen for subsequent study. *Colletotrichum* spp. are known to produce various bioactive compound (Moraga et al., 2019) while *Neodevriesia* spp are a relatively novel fungal genus described and no bioactive metabolites have been reported from this particular genus.

Table 4.2: Isolated strains identified up to genus level from respective coral host using NJ tree and morphology for preliminary screening.

Host	Fungal species (Genus level)	Phyla
PERC1702	*Jaminaea* sp.1 (PERF170212)	Basidiomycota
Pocillopora sp1	*Aspergillus* sp.1 (PERF170221)	Ascomycota
	Acremonium sp.1 (PERF170231)	Ascomycota
	Sterigmatomyces sp.1 (PERF170213)	Basidiomycota
PERC1706	*Neodevriesia* sp.1 (PERF170613)	Ascomycota
Acropora sp1	*Letendraea* sp.1(PERF170621)	Ascomycota
	Pseudopithomyces sp.1 (PERF170611)	Ascomycota
	Pyrrhoderma sp.1 (PERF170612)	Basidiomycota
PERC1707	*Aureobasidium* sp.1 (PERF170712)	Ascomycota
Fungia fungites	*Letendraea* sp.2 (PERF170721)	Ascomycota
	Penicillium sp.1 (PERF170731)	Ascomycota
	Rigidoporus sp.1 (PERF170711)	Basidiomycota
PERC1709	*Pestaloptiopsis* sp.1 (PERF170931)	Ascomycota
Pavona cactus	*Neodevriesia* sp.2 (PERF170913)	Ascomycota
	Phomatospora sp.1 (PERF170912)	Ascomycota
	Rigidoporus sp.2 (PERF170911)	Basidiomycota
PERC1715	*Hyalocladosporiella* sp.1 (PERF171532)	Ascomycota
Porites sp2	*Colletotrichum* sp.1 (PERF171521)	Ascomycota
	Penicillium sp.2 (PERF171531)	Ascomycota
	Neodevriesia sp.3 (PERF171511)	Ascomycota
	Penicillium sp.3 (PERF171522)	Ascomycota
PERC1717	*Daldinia* sp.1 (PERF171721)	Ascomycota
Galaxea astreata	*Neodevriesia* sp.4 (PERF171711)	Ascomycota
	Penicillium sp.4 (PERF171731)	Ascomycota
PERC1718	*Cladosporium* sp.1 (PERF171821)	Ascomycota
Tubastraea sp1	*Hyalocladosporiella* sp.2 (PERF171822)	Ascomycota
	Neodevriesia sp.5 (PERF171811)	Ascomycota
PERC1720	*Penicillium* sp.5 (PERF172011)	Ascomycota
Turbinaria	*Penicillium* sp.6 (PERF172021)	Ascomycota
peltata	*Schizophyllum* sp.1 (PERF172022)	Basidiomycota
	Hyalocladosporiella sp.3 (PERF172031)	Ascomycota

4.3.1 Molecular identification of *Colletotrichum* sp. (PERF171521) using multiple markers

Due to the limitation of ITS sequences (300bp) and morphology to resolve the identity of *Colletotrichum* sp1 (**PER171521)**, a multi-gene phylogeny approach was taken by incorporating additional sequences of ACT (316bp), CHS-1 (229bp), GAPDH (308bp), Cal (756bp) and TUB2 (716bp) (Weir et al., 2012).

With concatenation of six marker genes of ITS, ACT, CHS-1, GAPDH, Cal and TUB2, a total alignment length of 2748bp was obtained with 78 reference sequences from Weir et al. (2012). The combined sequence alignment contained 963 variable sites and 745 parsimony informative sites. The average nucleotide composition for A: C: G: T was 21.6:28.8:23.1:26.5, respectively.

The BI tree (**Figure 4.4**) which was rooted with *Colletotrichum hippeastri* and *Colletotrichum boninense*, showed two major clades resembling to those by Weir et al (2012) as Clade Musae and Clade Kahawae, with strong support of PP values (>0.82). Isolate **PERF171521** clustered within the *C.siamense* subclade with strong support (1.00). The intraspecific divergence of the *C. siamense* clade was small, 0.85%. For interspecific divergences, *C. siamense* are closely related to *C. aeschynomenes* and *C. tropicale* as their divergences are comparatively small compare to other *Colletotrichum* spp., ranging from 1.5 - 1.6% of divergences. Since *C. siamense* appears monophyletic, **PERF171521** was identified as ***Colletotrichum siamense*.** Colletotrichum is one of the most important genera of plant pathogenic fungi with many of the 200 plus species known to cause disease in plant crops worldwide (Marin-Felix et al., 2017). Our findings of *C. siamense* in the corals are the first report of *Colletotrichum* spp. as endophytes in scleractinian corals.

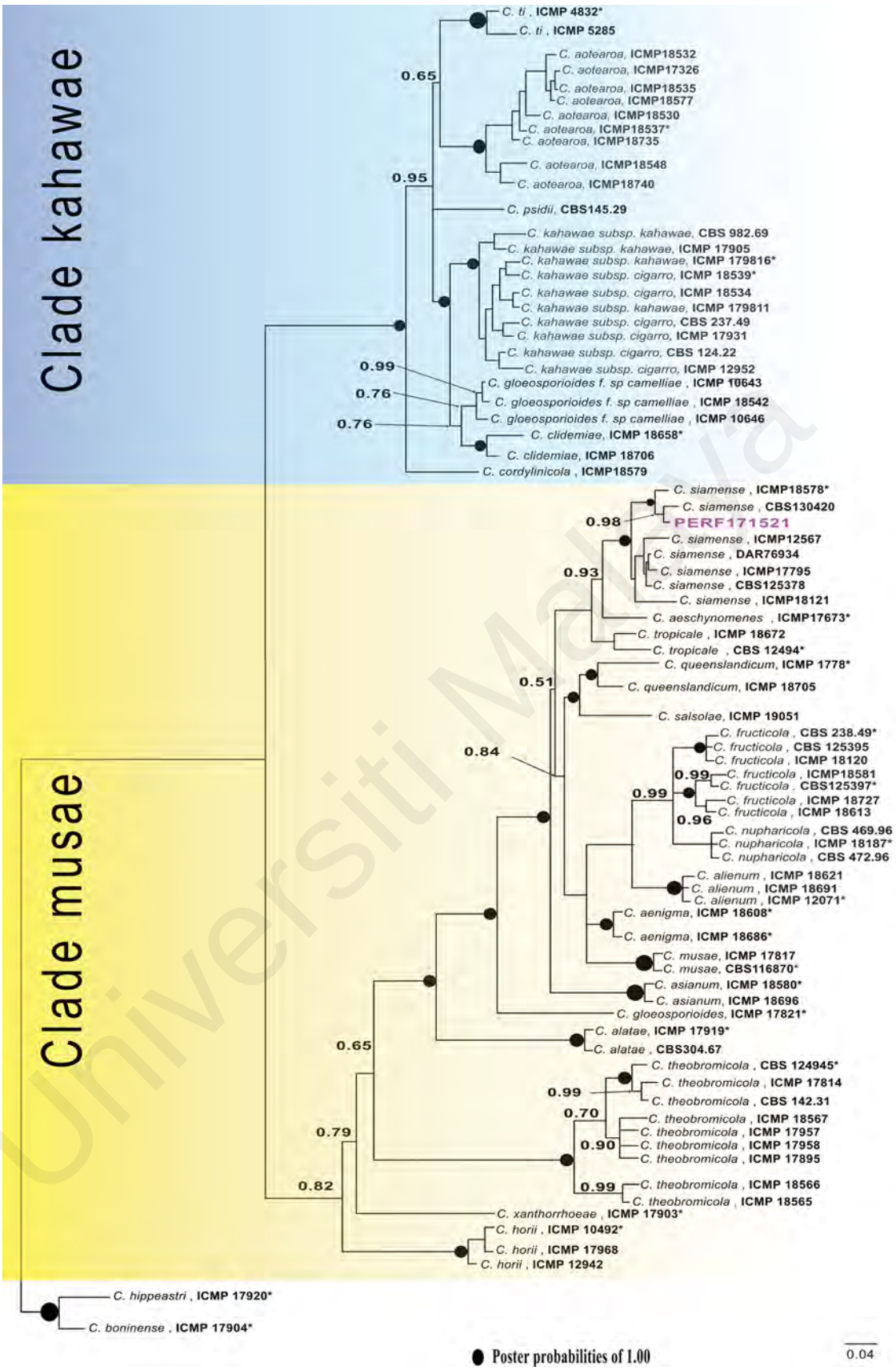


Figure 4.4: BI tree of *Colletotrichum* species and fungal isolate PERF171521 based on concatenated ITS, ACT, CHS-1, GAPDH, Cal and TUB2 sequences (2748bp). The tree as rooted with isolates with *C. hippeastri* and *C. boninense*. Posterior probabilities are indicated above the branches. Only values above 0.5 were indicated and considered for intepretation.

4.3.2 Molecular identification of *Neodevriesia* sp. (PER171711, PERF170613) using LSU and ITS markers

Neodevriesia sp.4 (**PERF171711**) obtained from *Galaxea astreata* was used for subsequent biochemical analysis. Additional strain of *Neodevriesia* sp.1 (**PER170613**) was added in the analysis to make a robust analysis. Only two strains of *Neodevriesia* were used as others failed to obtain both ITS and LSU sequences used for the analysis. Due to the limitation of the ITS sequences and morphology to resolve its identification, ITS (787bp) and LSU (577bp) sequences were concatenated to improve phylogenetic resolution as used by Wang et al. (2017). A final alignment length of 1,364 bp was obtained with 49 reference sequences from Wang et al. (2017). The sequence alignment contained 562 variable sites and 369 parsimony informative sites. The average nucleotide composition for A: C: G: T was 23.2 : 26.1 : 28.7 : 22.0 %, respectively.

The BI tree was rooted with *Parastagonospora nodorum* and showed six major clades that represented the family Neodevriesiaceae, Extremaceae and Teratosphaeriaceae, Mycosphearellaceae, and genus *Devriesia sensu stricto.* (**Figure 4.9)**. Both **PER170613** and **PER171711,** were clustered in clade Neodevriesiacea, which contain exclusively *Neodevriesia* spp. sequences. Our samples appeared as sister taxa of *Neodevriesia queenslandica* and *Neodevriesia shakazului* with strong support. Although **PER170613** and **PER171711** are closely related to *Neodevriesia queenslandica* and *Neodevriesia shakazului*, their phylogenetic position may imply a possible new species of *Neodevriesia* isolated from the hard corals.

The interspecific divergences of *N. shakazului* and *N. queenslandica* with our specimens (**PER170613** and **PERF171711)** is 4.3 and 4.5 % respectively, indicating albeit small but significant divergences among the sequences. *N. shakazului* is more closely related to *N. queenslandica* as their interspecific divergences of 2.6 % is the

lowest amongst other *Neodevriesia* spp. Therefore, the isolates **PER170613** and **PERF171711** could only be identified as *Neodevriesia* sp.1 and *Neodevriesia* sp.4, respectively.

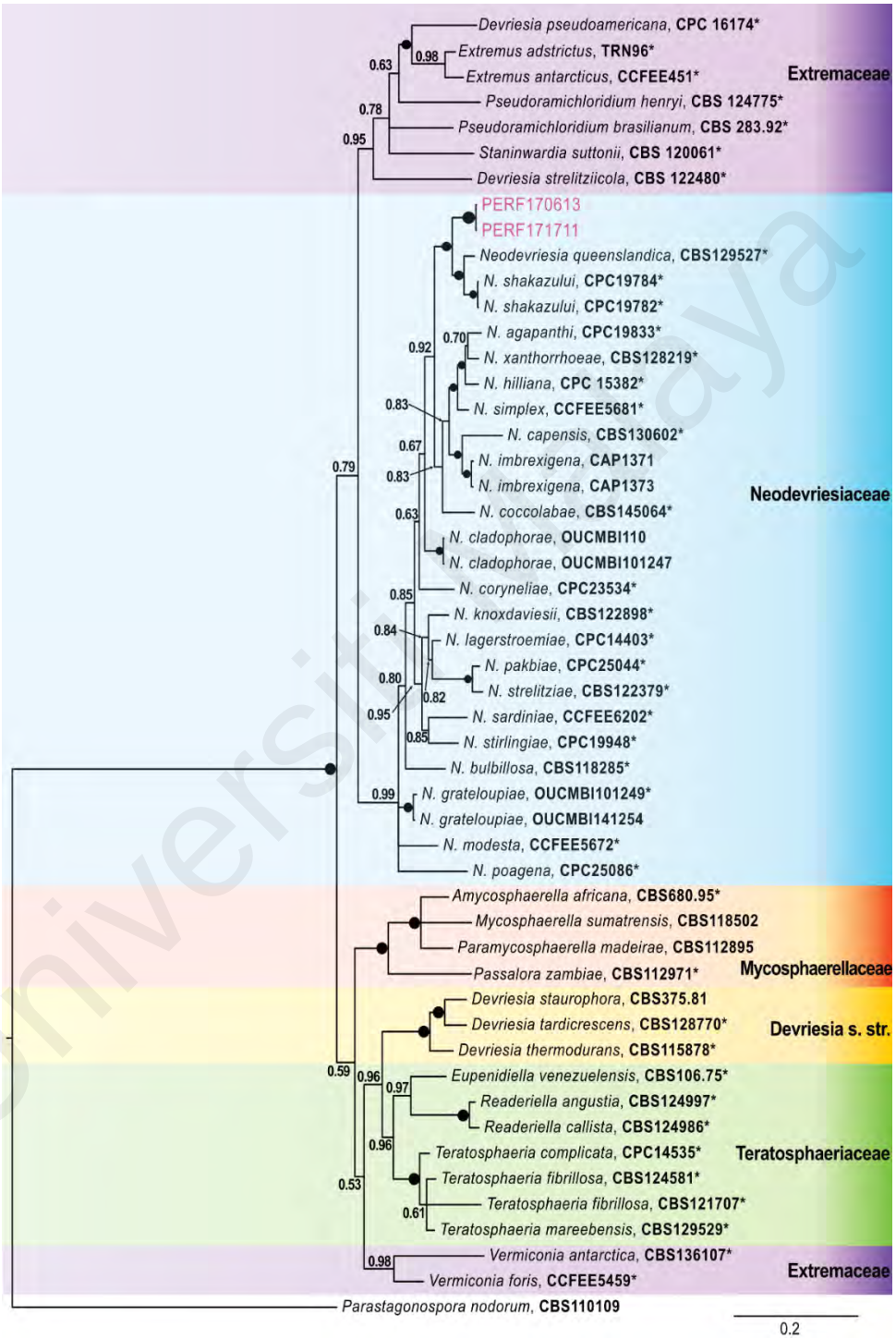


Figure 4.5: BI tree based on concatenated ITS and LSU sequences (1364bp) of fungal isolates **PERF170613, PERF171711** and other fungal reference sequences . The tree was rooted with Parastagonospora nodorum. Posterior probabilities are indicated above the branches. Only values above 0.5 were indicated and considered for intepretation. Dark circles indicat PP values of 1.00. TYPE and voucher specimen are indicated with '*'.

4.4 Chemical analysis of fungal strains

Fermentation of *Colletotrichum siamense* and *Neodevriesia* sp.4 yielded **2.3563g** and **0.8327g** of ethyl acetate-layer crude extracts, respectively. Approximately 0.8g of the crude extracts were subjected to fractionation via column chromatography with increasing gradient solvent system of hexane-ethyl acetate (Hex: EtOAc). A total of six fractions were obtained (**Table 4.3**). TLC profile of each fraction were elucidated using Hex: EtOAc, 3:1 (**Figure 4.6**).

Table 4.3: Show the weight of each fractions obtained from the two fungal strains via column chromatography fractionation.

Fraction	**Solvent ration (Hex:EtOAc)**	**Weight (mg)**	
		Colletotrichum siamense	***Neodevriesia* sp. 4**
1	9:1	561.5	570.9
2	8:2	257.7	34.6
3	7:3	29.6	8.5
4	6:4	30.9	9.0
5	1:1	131.3	5.0
6	Washing fraction (Chl:MeOH:H_2O) (65:25:4)	526.6	301.2

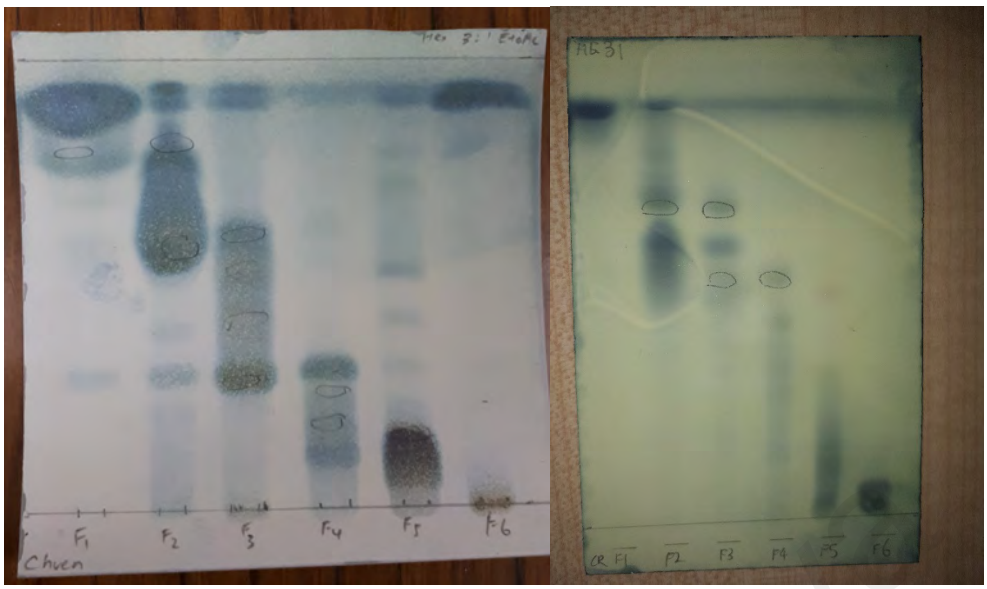


Figure 4.6: TLC profile of all six fractions of *Colletotrichum siamense* (left) and *Neodevriesia* sp.4 (right) crude extract using column chromatography with ratio of Hex: EtOAc, 3:1.

4.4.1 Profiling of each fractions from *Colletotrichum siamense*

To purify pure compounds from each fraction, solvent system suitable for separation of the compound were tested by trial and error of variety of solvent system involving Hex. EtOAc, Toluene, and Chloroform (Chl) as shown in **Figure 4.7**. Eight visible stained spots were observed with targeted compound of subfraction F1.2 spot at R_f value of 0.80. For fraction 2, a similar eight visible stained spots were observed when developed in Chl:EtOAc, 9.5:0.5. with our targeted subfraction of 2.6 at R_f value of 0.59. In fraction 3, using solvent system of Hex: EtOAc 7:3, two stained spots were observed. Fraction 4 with solvent system of Chl:EtOAc,1:1, five stained spots were observed and fraction 5 with Hex:EtOAc, 4:6., four stained spots were observed. Respective R_f values of each spot were listed in **Table 4.4**.

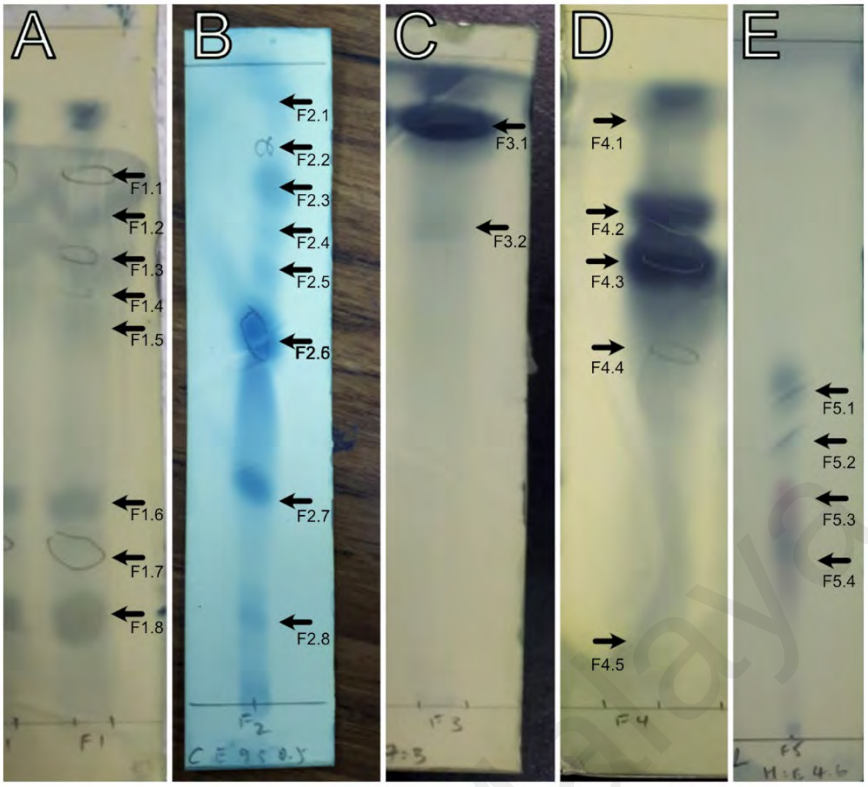


Figure 4.7: TLC profiles of fractions F1-F5 subjected to respective solvent system. (A) F1, 100 Chl (B) F2, Chl:EtoAc, 9.5:0.5 (C) F3, Hex:EtoAc, 7:3, (D) F4, Chl:EtoAc,1:1 (E) F5 Hex:EtoAc, 4:6.

Table 4.4: R_f values of respective stained spots (with different solvent system) on Fraction 1 to *F*raction 5.

Fractions	Spots	R_f values
F 1	1.1	0.87
	1.2	0.80
	1.3	0.68
	1.4	0.63
	1.5	0.62
	1.6	0.35
	1.7	0.26
	1.8	0.17
F 2	2.1	0.98
	2.2	0.90
	2.3	0.84
	2.4	0.77
	2.5	0.70
	2.6	0.59
	2.7	0.33
	2.8	0.13
F3	3.1	0.89
	3.2	0.73
F4	4.1	0.87
	4.2	0.74
	4.3	0.66
	4.4	0.54
	4.5	0.10
F5	5.1	0.77
	5.2	0.67
	5.3	0.54
	5.4	0.39

4.4.2 Isolation of metabolites from *C. siamense*

Neodevriesia sp. 4 was not subjected to secondary metabolite isolation due to low fraction yield compared to *C. siamense*. A total of two confirmed compounds were isolated from *C. siamense* through individual processing of each fraction via PTLC. Fraction 1 was subjected to PTLC and developed using 100% Chl, solvent system. **Figure**

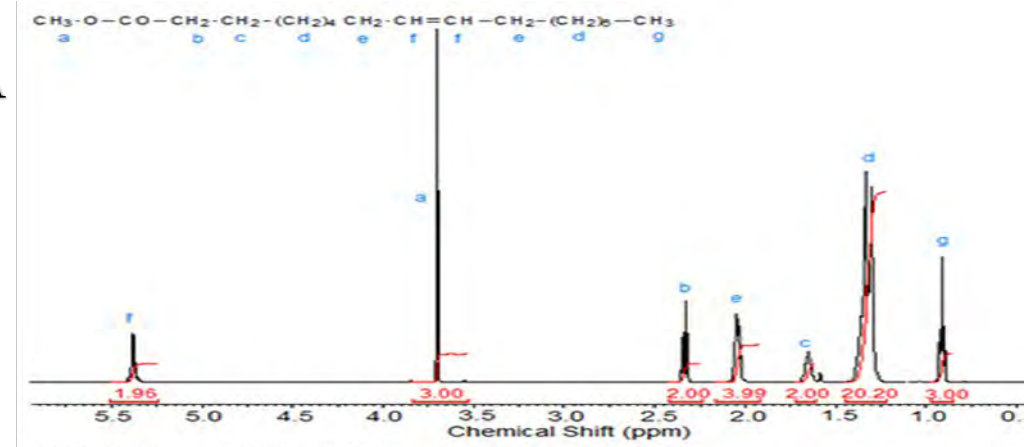
4.7 (A) shows the TLC of Fraction 1. Using the TLC as reference, PTLC isolation yielded eight isolates. The yield for each isolates of Fraction 1 is shown in **Table 4.5.**

Table 4.5: The weight of each subfractions obtained via PTLC using 100% Chloroform.

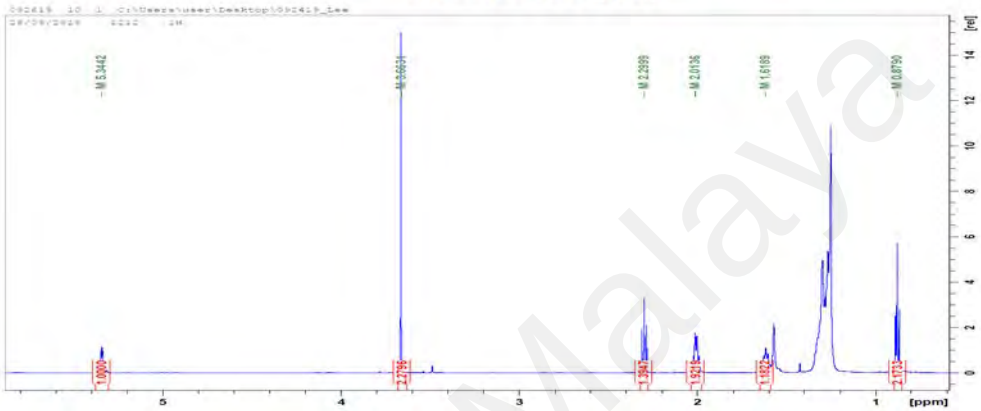
Subfraction	Weight (mg)
F 1.1	29.7
F 1.2	79.6
F 1.3	19.2
F 1.4	3.1
F 1.5	2.4
F 1.6	8.5
F 1.7	4.1
F 1.8	7.3

Upon purification using repetitive PTLC, we successfully isolated the first known compound as isolate 1-2-1-1, methyl oleate (**1**). The chemical shifts of compound 1, $C_{19}H_{36}O_2$; ^{1}H NMR (600 MHz, $CDCl_3$): δH 5.34 (m), 3.66 (s, 3H), 2.30 (t), 2.01 (q), 1.62 (m), 1.25 (m), 0.88 (t, 3H); 13C NMR (150 MHz, CDCl3): δC 174.1; 130.0; 51.4; 34.1; 31.9; 29.7; 29.6; 29.3; 29.0; 27.2; 24.9; 22.7; 14.1. Chemical data was confirmed based on chemical shifts from literature as well as splitting patterns of key signals in the proton chart (Wishart et al., 2017). **Figure 4.8 (A)** is an excerpt from the Human Metabolome Database showing the NMR spectra of oleic acid while **Figure 4.8 (B)** is the NMR spectra obtained from *C. siamense* extract F1. **Figure 4.8 (C)** shows the chemical structure of methyl oleate (**1**). Methyl oleate, the ester form of oleic acid, is one of the major unsaturated fatty acids found in marine benthic fungi (Das et al., 2007).

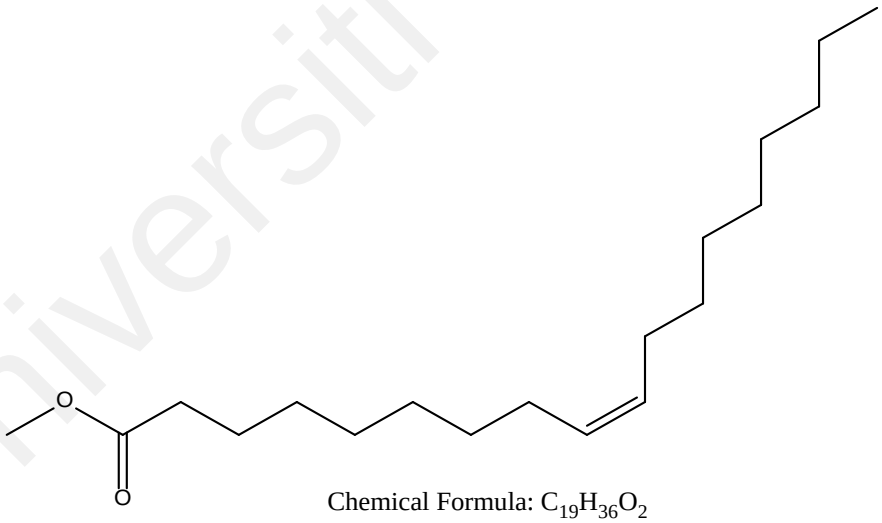
A



B



C



Chemical Formula: $C_{19}H_{36}O_2$

Methyl oleate (**1**)

Figure 4.8: Excerpt from the Human Metabolome Database showing the NMR spectra of methyl oleate (A) and ^{1}H spectrum obtained in this study from Colletotrichum siamense extract F1.2.1.1 (B) with their structure (C).

Fraction 2 was subjected to PTLC and developed using Chl: EtOAC, 9.5:0.5 solvent system. Using the TLC as reference, PTLC isolation yielded eight isolates (**Table 4.6**).

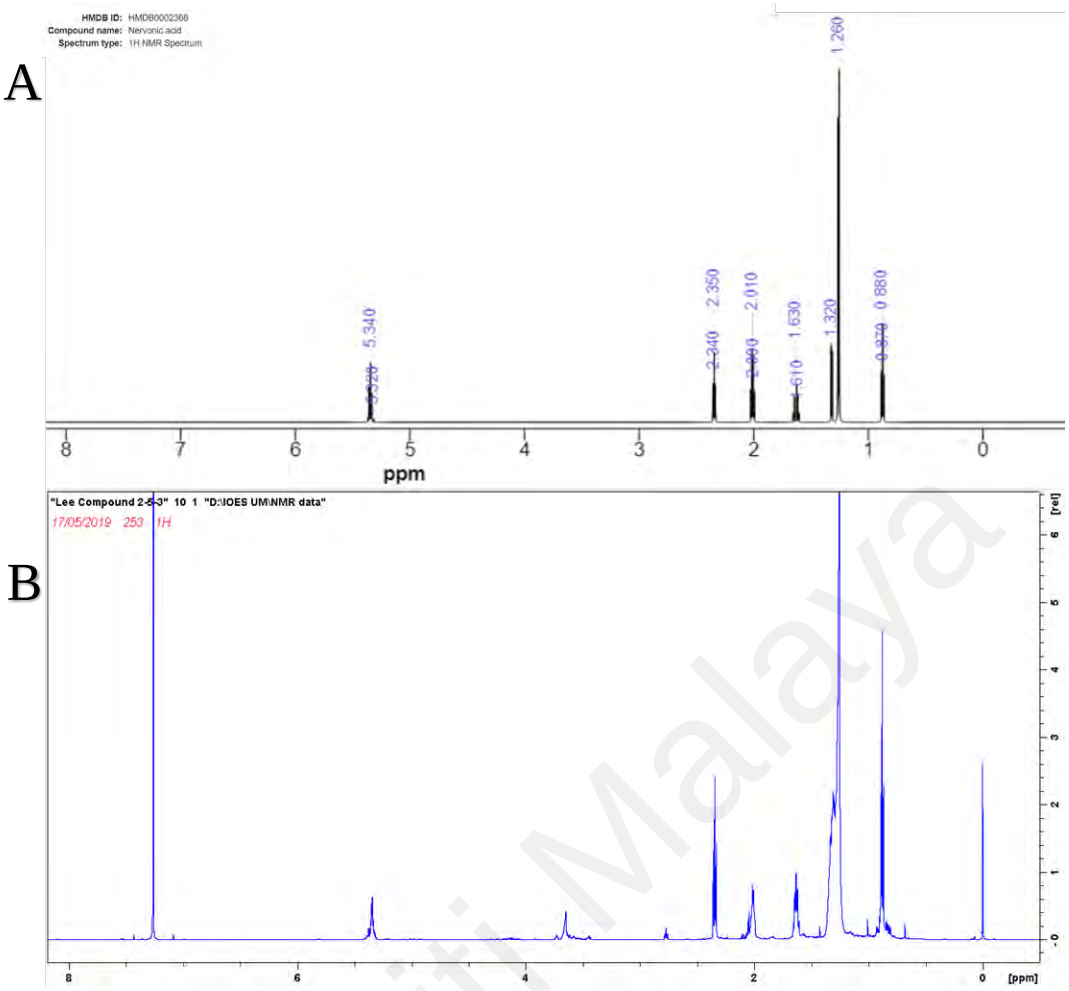
Table 4.6: The weight of sub-fractions purified from fraction 2 obtained via PTLC using Chl: EtOAC, 9.5:0.5.

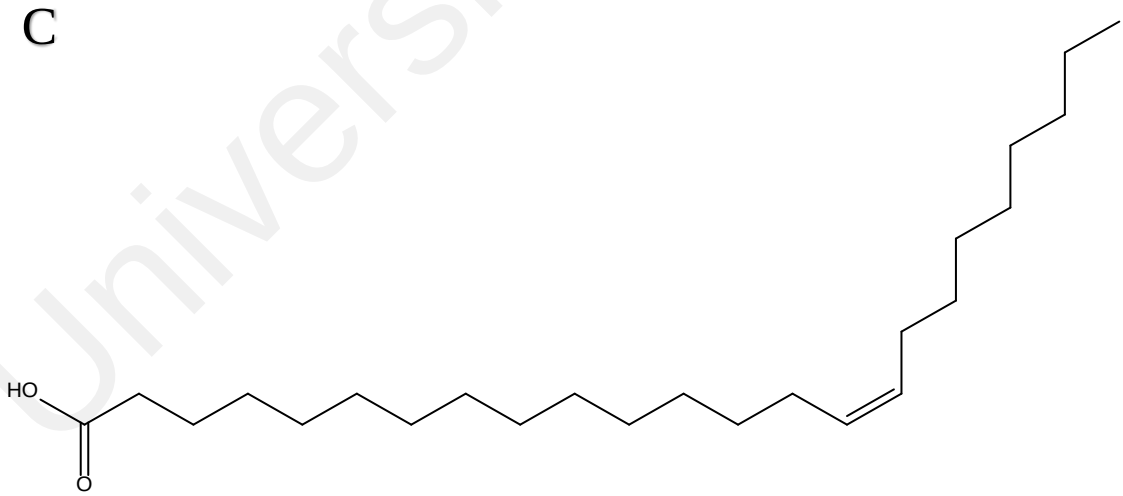
Subfraction	Weight (mg)
F 2.1	3.9
F 2.2	9.4
F 2.3	16.3
F 2.4	8.7
F 2.5	144.8
F 2.6	18.9
F 2.7	33.4
F 2.8	10.1

Upon purification using repetitive PTLC, the second compound as isolate 2-5-3, compound known as isolate F2.5.3 was identified as nervonic acid (**2**). The chemical shifts of compound 1, $C_{24}H_{46}O_2$; ^{1}H NMR (600 MHz, $CDCl_3$): δH 5.34 (m), 2.34 (t), 2.01 (m), 1.63 (m), 1.31 (m), 1.25 (m), 0.88 (t, 3H); 13C NMR (150 MHz, CDCl3): δC 179.2; 129.9; 33.9; 31.9; 29.8; 29.7; 29.5; 29.4; 29.1; 24.7; 22.7; 14.1. Chemical data was confirmed through searches in the National Library of Medicine database based on chemical shift and splitting patterns of key signals. **Figure 4.9 (A)** is an excerpt from the

Human Metabolome Database showing the NMR spectra of nervonic acid (Wishart et al., 2017) while **Figure 4.9 (B)** is the NMR spectra obtained from *C. siamense* extract F2.5.3.

After isolating methyl oleate (**1**) and nervonic acid (**2**) from the first two fractions, a similar pattern was observed in the ^{1}H-NMR spectrum of selected pure compounds from fraction 3 and 4 (**Figure 4.10**) indicating the possible presence of additional fatty acid metabolites. As such, we decided to analyze the composition of fatty acid in the isolated fungal extracts by methylating the fatty acid fractions into fatty acid methyl esters (FAME) and subjecting them to gas chromatography analysis.





Chemical Formula: $C_{24}H_{46}O_2$

Nervonic acid (**2**)

Figure 4.9: Excerpt from the Human Metabolome Database showing the NMR spectra of nervonic acid (A). ^{1}H spectrum obtained in this study from *Colletotrichum siamense* extract F2.5.3 (B) with their structure (C).

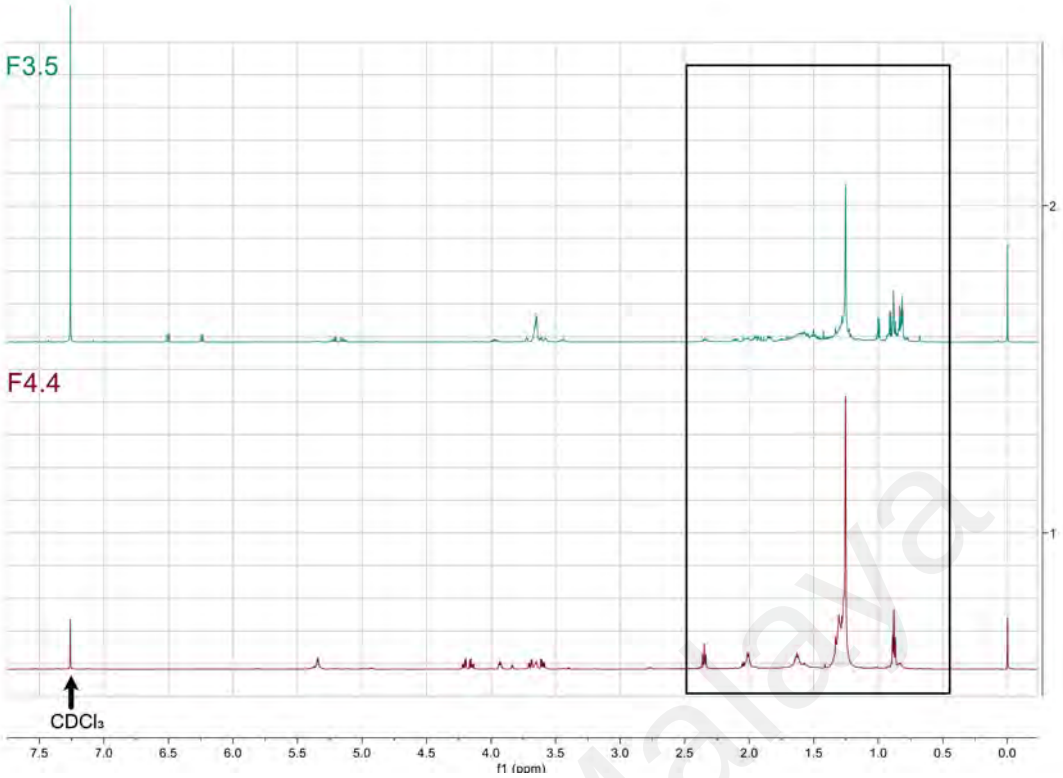


Figure 4.10: ^{1}H-NMR spectrum of selected compounds from fraction 3 and 5 indicating the presence of carboxyl groups akin to the spectrum of fatty acids.

4.5 Fatty acids (FAs) composition of *C. siamense* and *Neodevriesia* sp.4

The fatty acids profile of ***C. siamense*** and ***Neodevriesia* sp.4** exhibited a clear and distinct composition of FA species (**Table 4.7** and **Figure 4.11**). This characteristic shown indicates that the fatty acid composition can be used as an identity marker for fungal species. ***C. siamense*** contained high amount of palmitic acid and oleic acid, with 40.20 % and 52.76 % respectively. Tridecanoic acid and linoleic acid were dominant in ***Neodevriesia* sp.4**, with 12.07% and 18.51 % respectively.

Table 4.7: The relative concentration of detected fatty acids in two selected fungal strains of *Colletotrichum siamense* and *Neodevriesia* sp. 4 using GCMS.

Fatty acids	***Colletotrichum siamense* (%)**	***Neodevriesia* sp. 4 (%)**
Saturated Fatty Acid (SFA)		
Tridecanoic acid, C13:0	-	12.07
Palmitic acid, C16:0	40.20	5.15
Steric acid, C18:0	2.5	-
Heneicosanoic acid, C21:0	-	7.02
Monounsaturated Fatty Acid (MUFA)		
Oleic acid, C18:1 cis-9	52.76	9.82
Palmitoleic acid, C16:1ω7	2.86	-
Erucic acid, C22:1ω9	-	1.59
Polyunsaturated Fatty Acid (PUFA)		
Linoleic acid, C18:2 cis-9,12	0.65	18.51

- not detected

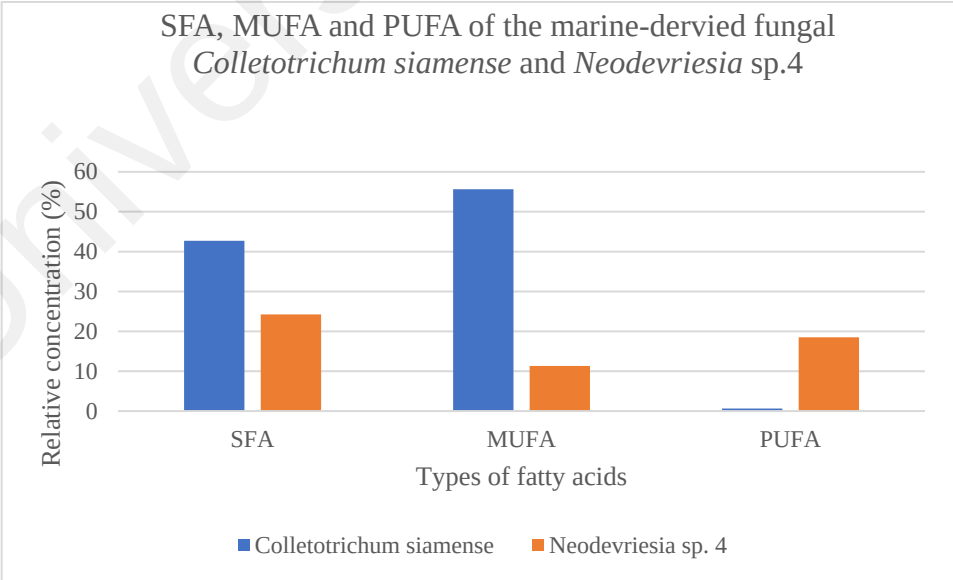


Figure 4.11: Comparisons of fatty acid composition of *C.siamense* and *Neodevriesia* sp. 4

4.6 Antibacterial assay

Antbacterial assay was done to assess the antibacterial properties of the crude extracts produced by *C. siamense* as fungi reportedly produce antibacterial compounds. The bacterial strains used three gram-negative bacteria; *Escherichia coli ATCC 25922*, *Salmonella serovar* Typhi, *Serratia marsecens* and two gram-positive bacteria; *Staphylococcus aureus*, *Bacillus subtilis* as all five of the strains are common human pathogen and frequently used for screening antibacterial properties of isolated compounds. A total of five strains of bacteria were used for antibacterial assay. The crude extract and fractions were of strain, *Colletotrichium siamense.* All the samples were tested negative across the five strains of bacteria (**Table 4.8**). minimum inhibitory concentration (MIC) was not found. Inhibition zone were not found across the five strains and the samples does not inhibit the growth of five bacteria. Positive control of antibiotic discs of Vancomycin and Imipenem exhibited zone of inhibition.

Table 4.8: Antibacterial assay of crude extracts and fraction of *C siamense* against five strains of bacteria.

Bacterial strain	***Escherichia coli* ATCC 25922.**					***Salmonella serovar* Typhi**					***Serratia marsecens***					***Staphylococcus aureus***					***Bacillus subtilis***				
Concentration used	500	250	125	62.5	31.25	500	250	125	62.5	31.25	500	250	125	62.5	31.25	500	250	125	62.5	31.25	500	250	125	62.5	31.25
CRUDE	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
F5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

- absence of activity

CHAPTER 5

DISCUSSIONS

5.1 Identification of scleractinian corals and marine-derived fungi

5.1.1 Scleractinian corals identification

Using both morphology and CO1 marker, only five out of fourteen corals have been identified up to species level. Based on the CO1 marker, it showed that CO1 marker are only effective in resolving corals up to genus or family taxon level but not species. Mitochondrial genes in anthozoans evolved slower than nuclear gene, thus much suitable for higher taxonomic rank phylogenies (Huang et al., 2008). Some specimens, with the helps of their distinctive corallite and growth form feature, are able to identified up to species. For instances, both *Pavana cactus* and *Galaxea astreata* has distincive corallites features, that able to distinguished them from their interspecific counterparts. Colonies of *Pavona cactus* are composed of thin, contorted, bifacial, upright fronds while *Pavona clavus* formed columnar or club-shaped columns. Similarly, what distinguised *Galaxea astreata* from *Galaxea fascicularis* lies in their characteristic corallites septa. *Galaxae astreata* have eight to twelve septa reaching corallite centre while *Galaxea fascicularis* have numerous septa reaching corallite centre. However, *Acropora, Porites, Pocillopora* and *Tubastrea* are species complex and have ambiguous corallite features that make it difficult to identify them using only CO1 marker and corallite features.

To date, molecular studies of hard corals are lacking in Malaysia as most studies resort to morphological feature of corals.Only two studies, by Robert et al. (2019) and Waheed et al. (2015) incorporated genetic markers in their coral studies to genetically

characterize the hard corals dominated in Sabah (East Malaysia). While in Peninsular Malaysia, studies are pivoted on reporting the diversity and morphology were used to identified them (Akmal et al., 2019). Thus, it is imperative to conduct a more extensive studies to genetically characterize the hard corals in Malaysia to fill in the knowledge gaps.

5.1.2 Marine-derived fungi identification

For marine fungi, ITS marker was used as a preliminary screening of the fungal species isolated from the hard corals. Using NJ approach, the isolated fungal strains can be tentatively identified up to genus level. Due to the limitation of morphology, the ITS ITS marker (via NJ approach) was used as dealbreaker in tentative screening of fungal strains. Morphological plasticity and discriminatory morphological characteristics limit the identification of marine fungi (Reich & Labes, 2017). Although ITS is the standard barcoding markers, the resolution power is poor in some fungal groups due to high sequence and length variability, for instance, marine fungi *Cladosporium, Penicillium* or *Fusarium* possess a shorter ITS sequence (~400 bp) including the (Reich & Labes, 2017). Marine fungi contain novel and distinct fungal lineage that remain elusive given that current database of marine fungi are still in its infancy stage.

5.2 Association of Ascomycota fungi with scleractinian corals

Scleractinian corals host an assemblage of microbes known as holobionts, comprised of bacteria, archaea, fungi, viruses, and protists and the dinoflagellate algae, Symbiodinium (Rohwer et al., 2002). While other holobionts such as bacteria, archae and

protists are the focal interest of microbiome research in coral, fungi remain an enigma. Prior to the introduction of metagenomic sequencing, studies of fungi in coral relied on orthodox method of plate culturing in media to retrieve culturable fungi (Morrison-Gardiner, 2002). With application of high-throughput amplicon and metagenomic analyses, studies were able to reveal the diverse fungal communities reside in the coral (Wegley et al., 2007; Thurber et al., 2009; Littman et al., 2011; Amend et al., 2012; Góes-Neto et al., 2020), with possible novel marine fungi lineage reside in coral.

In current study, the culturable fungi, obtained using plate culturing media, were consistent with previous studies that fungi from phyla Ascomycota are the dominant and ubiquitous in different taxa of corals. Majority of isolated fungi belong to phyla Ascomycota, comprising of thirteen different genera such as *Acremonium, Aspergillus, Aureobasidium, Cladosporium, Colletotrichum, Daldinia, Neodevriesia, Hyalocladosporiella, Letendraea, Penicillium, Pestalotiopsis, Phomatospora and Pseudopithomyces*. The ascomycetes such as *Acremonium, Aspergillus, Aureobasidium, Cladosporium, Penicillium* have been isolated from scleractinian corals in previous studies (**APPENDIX A**), however *Colletotrichum, Daldinia, Neodevriesia, Hyalocladosporiella, Letendraea, Pestalotiopsis, Phomatospora* and *Pseudopithomyces*, to our knowledge, has not been reported.

Ascomycetes such as *Acremonium, Aureobasidium, Cladosporium, Penicillium* are cosmopolitan fungi that known to be repeatedly isolated from marine environment and possibly occupy a niche in the marine environment (Amend et al., 2019). *Acremonium*, a pathogenic fungi genus isolated from *Pocillopora* in our investigation is consistent to reports by Fouillaud et al. (2017) where the *Acremonium* had been isolated from a living *Pocillopora* in La Réunion Island. It has also been isolated from different *Porites* spp. (Ravindran et al., 2001; Li et al., 2014). *Acremonium* were absent in both

healthy and diseased *Porites* but were found to colonize the dead patches of *Porites*. *Cladosporium*, isolated from *Tubastrea coccinea*, were repeatedly isolated from different corals across different regions in previous studies (Kendrick et al., 1982; Ravindran et al., 2001; Yarden et al., 2007; Góes-Neto et al., 2020). *Cladosporium* was found in both healthy and diseased acroporids, but with increase abundance in the latter condition (Yarden et al., 2007), hence suggesting fungi are ubiquitous and their abundance dictated by their health status. Similarly, *Aureobasidium* isolated from a solitary coral, *Fungia fungites*, were repeatedly found in other scleractinian corals (Ravindran et al., 2001; Yarden et al., 2007; Góes-Neto et al., 2020). Consistent to most of the studies on fungal communities in scleractinian corals (Kendrick et al., 1982; Li et al., 2014; Fouillaud et al., 2017; Abd El-Rahman et al., 2020), *Penicillium* was the dominant genera and prevalent amongst the specimens due to the fast growing, adaptability and ability to sporulate in expansive range of substrates (Overy et al., 2014). *Penicillium* has the most species (82 species hitherto) as recorded in the titular website of marinefungi.org (Jones et al., 2019).

The remaining genera of *Colletotrichum, Daldinia, Neodevriesia, Hyalocladosporiella, Letendraea, Pestalotiopsis* and *Pseudopithomyces* are known terrestrial strains but were the first record of isolating from scleractinian corals. Despite not previously isolated from corals, *Colletotrichum, Daldinia, Letendraea, Neodevriesia, Pestalotiopsis, Phomatospora* and *Pseudopithomyces* have been isolated from other marine sources. *Daldinia*, despite being primarily a woody plant endophyte (Stadler et al., 2014), did frequently occur in the marine environment as well. Tarman et al. (2012) isolated *Daldinia eschscholzii* from the red alga *Gracilaria* sp.SGR-1, collected from coast of South Sulawesi. *Daldinia eschscholzii* also been isolated from mangrove tree of *Bruguiera sexangula* var. *rhynchopetala* (Liao et al., 2019) and from branches of *Scaevola sericea*, also known as beach cabbage (Hu et al., 2014). isolated *D. eschscholzii*

from marine sponge, *Xestospongia* sp. while investigating on chemical diversity of secondary metabolites from marine microorganisms. In their ongoing search of novel compounds from marine-derived fungi, both Huang et al. (2019) and Xu et al. (2020) isolated *Letendraea* sp. 5XNZ4-2 from the gut of a marine crab.

Pestalotiopsis species are notoriously known as plant pathogens (Maharachchikumbura et al., 2014) but with recent spotlight on marine-derived fungi, more novel strains of marine *Pestalotiopsis* have been isolated. Marine *Pestalotiopsis* spp. have been isolated from different marine substrates and associated with different marine hosts. Kumar et al. (2018) sequenced the genome of novel marine *Pestalotiopsis* sp. isolated from the German Wadden Sea and revealed a unique and novel BGCs from the marine fungi. *Pestalotiopsis* sp. NCi6, a halotolerant and lignocellulolytic mangrove fungus was isolated from the trunks and prop roots bark of *Rhizophora stylosa* mangrove trees. In prospecting novel marine natural products from associated microbes from marine organism, *Pestalotiopsis* spp. have been isolated from sponges, *Phakellia fusca* (Lei et al., 2017), marine alga *Coelarthrum* sp. (Wang et al., 2020) and soft coral *Sarcophyton* sp. (Wei et al., 2013). In a review by Xu et al. (2014) where they focused on recent *Pestalotiopsis*-derived natural products, several new strains of marine Pestalotiopsis have been reported.

Phomatospora are aquatic ascomycetes that frequently isolated from submerged wood in both freshwater and seawater habitat such as intertidal zone or saltmarsh. There are six known fresh water *Phomatospora* species, vis. *P. aquatica* (Minoura & Muroi, 1978), *P. berkeleyi*, *P. muskellungensis* (Fallah & Shearer, 1998), *P. striatigera* (Scheuer, 1988), *P. triseptate* (Raja & Shearer, 2008) and *P. luteotingens* (Fournier & Lechat, 2010). The first marine *Phomatospora acrostichi* was described by Hyde (1988), and subsequently five more new marine *Phomatospora* spp. discovered from marine habitats,

namely, *P. kandelae* (Hyde, 1992), *P. nypae* (Hyde, 1993), *P. bellaminutai* (Kohlmeyer et al., 1995), *P. phragmiticola* (Poon & Hyde, 1998), *P. nypicola* (Hyde et al., 1999). Despite their prevalence in marine environment, information regarding their ecological niche or association with organism are scant. More research is needed to shed the light on this genus.

Pseudopithomyces Ariyawansa & K.D. Hyde gen. nov. is introduced as a new genus to distinguish between morphologically homogeneous but phylogenetically distinct of *Pithomyces chartarum* (renamed as *Pseudopithomyces chartarum*) and type species of *Pithomyces flavus* (Ariyawansa et al., 2015). *Pseudopithomyces chartarum* are known plant pathogen affecting wide range of medicinal plants and some crops (Tóth et al., 2007; Perelló et al., 2017). *Hyalocladosporiella* Crous & Alfenas, gen. nov. is a newly described genus that currently considered as *Chaetothyriales incertae sedis*, with the first holotype isolated from the leaves of teak tree, *Tectona grandis*. Hitherto, not much is known about both genera from marine as information is lacking.

5.2.1 Marine-derived *Neodevriesia* fungal species

Despite successful isolation of *Neodevrisia* strains from the present study, the species could not be identified. Nevertheless, it was confirmed belonging to the *Neodevrisia* genus and closely related to *Neodevrisia shakazului*, and *Neodevrisia queenlandica*, with the possibility of being a new species. *Neodevriesia* is a new genus described due to the polyphyletic genus of *Devriesia* containing four distinct clades, namely *Devriesia sensu stricto* (s. str.), and *Devriesia sensu lato* (s. lat.) 1, 2 and 3 (Crous et al., 2009; Frank et al., 2010). Eventually, *Devriesia* s. lat. 3 is designated as *Neodevriesia* (Neodevriesiaceae) with two plant associated species, both distinct from the

soil-inhabiting *Devriesia* s. str. (Teratosphaeriaceae) (Quaedvlieg et al., 2014). Quaedvlieg et al. (2014) established *Neodevriesia* (type *N. hilliana*) with morphological characters of medium brown and unbranched conidiophores, thick-walled, medium brown, rarely septate conidia, short and mostly unbranched conidial chains, and the absence of chlamydospores. *N. shakazului*, (previously known as *Devriesia shakazului*), a terrestrial species was first isolated from *Aloe* sp. and described by Crous et al. (2012). Albeit primarily a terrestrial genus, Wang et al. (2017) reported two new *Neodevriesia cladophorae* and *N. grateloupiae* that associated with marine algae, this is the first report of marine species associated with marine algae.

5.1.1 Marine-derived *Colletotrichum siamense*

Colletotrichum siamense was successfully identified in the present study, and one of the first to be isolated from a scleractinian coral. *Colletotrichum* is often associated with plant diseases; commonly referred to as anthracnose that primarily infects crop plant families like chili, strawberry, apple, citrus, and stone fruits, and major cereals such as maize and sorghum (Cannon et al., 2012). Not much is known regarding *Colletotrichum* in marine environment as it was regarded as one of the most important group of plant pathogenic fungi in the world (Dean et al., 2012).

Colletotrichum is considered primarily plant pathogen with rare occasions of implicating in disease such as causing keratitis and subcutaneous infections (Shiraishi 2011; Shivaprakash et al., 2011), and one report of *Colletotrichum* exhibited mycotic infection on the lungs and kidneys in sea turtle (Manire et al., 2002). Most of the species are of terrestrial origin, however, *C. typhae* is known to be associated with an aquatic weed, Southern cattail (*Typha domingensis*), an invasive macrophyte widespread over the

world (Maia et al., 2020). The occurrence of marine *Colletotrichum* is rare and it is highly possible that the fungus occurred as 'flotsam' in the marine environment. Manire et al. (2002) acknowledged that *C. acutatum* found in the sea turtle was probably an opportunistic pathogen that infect the turtle via airborne as other turtles in proximity did not develop the same infection.

Colletotrichum siamense is a recently described species, isolated from both healthy and lesion parts of coffee berries, implying opportunistic pathogenicity (Prihastuti et al., 2009). *Colletotrichum siamense* is a ubiquitous saprobe or endophyte that infected wide range of plants (James et al., 2014); found in citruses (Pérez-Mora et al., 2020), tropical woody plants (Zhang et al., 2020), tridax daisies (Jongsareejit et al., 2020) and black peppers (Munasinghe et al., 2017). Due to its new discovery, documentation regarding its ecology and epidemiology in terrestrial and marine environments are lacking.

Although they are virulent agents, some species from *Colletotrichum* are integral in plant symbiosis responsible for conferring disease resistance, growth enhancement, drought tolerance and stress tolerance (Redman et al., 2001; L. P. Singh et al., 2011). *Colletotrichum magna, C. gloeosporioides, C. orbiculare* were observed to provide drought resistance and enhanced growth to tomato plants (Redman et al., 2001), whereas endophytic *Colletotrichum tofieldiaei* promoted growth in maize and tomato plants without repercussion from the endophytes *per se* (Díaz-González et al., 2020). This exemplary fungal-plant symbiosis may imply similar role of fungi in sessile animals, such as corals or sponges, where holobionts are integral and vital for their survival in the nutrient poor environment of seawater.

5.3 Association of Basidiomycota fungi with scleractinian corals

Other than ascomycetes, basidiomycetes are the second largest fungal group found in the scleractinian corals (Amend et al., 2012; Góes-Neto et al., 2020). In our studies, *Jaminaea*, *Pyrrhoderma*, *Sterigmatomyces*, *Schizophyllum* are identified for the first time in corals while *Rigidoporus* has been isolated from *Porites pukoensis* in Xuwen coral reef, China (Li et al., 2014).

Jaminaea, a basidiomycetous yeast, have been isolated from intertidal zone driftwood (Mahdi et al., 2008) and the leaves of *Avicennia marina* (Nasr et al., 2017). The species isolated by Mahdi et al. (2008) as *Sympodiomycopsis lanaiensis*, was revised by Kijpornyongpan and Aime (2017) and currently named as *Jaminaea lanaiensis*. *Sterigmatomyces*, on the other hand, are common in marine environment with the first introduction and description of this halotolerant deuteromycetes collected from seawater (Fell, 1966). *Sterigmatomyces halopilus* is exclusively found in marine environment and it is widely known as obligate marine yeast (Zhang et al., 2014). Kaewkrajay et al. (2020) isolated eight genera of culturable marine yeast, inclusive of *Sterigmatomyces*, that frequently cultured from marine invertebrates. Both *Pyrrhoderma* and *Schizophyllum* are wood-inhabiting fungi that are not known to inhabit marine environment. However, the split gill fungus *Schizophyllum*, has been isolated from a cadaver of diseased harbor seal (*Phoca vitulina*) in which hyphae were seen in granulomatous lesions in the eyes, lung, heart, and lymph nodules (Hanafusa et al., 2016). *Schizophyllum* are widely deemed as the most common filamentous basidiomycetes reported from human infections, casing non-lethal allergic respiratory conditions to more adverse condition of brain lesions in both immunocompetent and immunocompromised host (Chowdhary et al., 2014). Despite the anomaly, the authors do not rule out the possibilities of new pathogenic strains

of *Schizophyllum* that can causes granulomatous endophthalmitis, pneumonia, and myocarditis in marine mammal (Hanafusa et al., 2016).

5.4 Fatty acid composition of marine fungi

Fungi are one of the oleaginous microbes that have the capability to accumulate the lipid content more than 20–30% in term of cell dry weight (Ratledge, 1991). Despite inhabiting different environment than their terrestrial counterpart, marine fungi too are oleaginous microbes. Some studies have demonstrated the prospect of marine fungi as feedstock for biodiesel production (Khot et al., 2012) and as a feed source for fish larvae (Khot et al., 2012; Barra et al., 2017) due to their high lipid contents. Feed encapsulated with *Rhodotorula* sp. (isolated from seawater) have significantly higher RNA/DNA ratio (proxy for nutritional state) than control group and could be replacing traditional feed of Artemia nauplii. (Barra et al., 2017). Mangrove -derived fungi *Aspergillus terreus* has the highest yield of microbial lipids amongst other strains and touted as potential candidates of feedstock for biodiesel (Khot et al., 2012).

Marine fungi have contrasting fatty acid profile and it can be a potential biomarker (Cooney et al., 1993; Ruess et al., 2002; Devi et al., 2006). *Colletotrichum simense* has relatively higher percentage of SFA and MUFA than *Neodevriesia* sp., while *Neodevriesia* sp. has relatively higher percentage of PUFA than *Colletotrichum siamense*. The profile of the fatty acid is contrasting amongst different strains of marine fungi. Cooney et al. (1993) reported the fatty acid profile of ten marine fungi stains of which, eight out of ten strains tested, have higher percentage of PUFA with linoleic acid (18:2n6) as the major fatty acid.

In Devi et al. (2006), all four strain of marine fungi have higher percentage of MUFA, with oleic acid (18:1n9) as the dominant fatty acid. Eight out of twelve strains of benthic marine fungi tested have oleic acid (18:1n9) as the dominant fatty acid, while three out of twelve strains have linoleic acid (18:2n6) as dominant fatty acid, and the remaining one strains have palmitic acid (16:0) as the dominant fatty acid (Das et al., 2007). Khudyakova et al. (2009) tested seven strains of marine fungi and majority of them (4 out of 7) have palmitic acid (16:0) as the dominant group. Despite the variation, linoleic (18:2n6), palmitic (16:0) and oleic (18:1n9) acids were the principal fatty acids found in this study, in which, consistent with studies that involve fatty acid profile of marine fungi (Cooney et al., 1993; Devi et al., 2006; Das et al., 2007; Khudyakova et al., 2009).

The major fatty acid, oleic acid, found in our samples, the marine derived *Colletotrichum siamense* are consistent with the profile terrestrial strains of Colletotrichum sp. In Dey et al. (2011) study on the potential of Colletotrichum sp. isolated from a medicinal herb, oleic acid constituted 58.1 % of the lipids content, followed by linoleic acid (23.2%) and palmitic acid (5.1%). The high content of oleic acids is a prospective feature for biodiesel production (Papanikolaou et al., 2004; Dey et al., 2011). As for *Neodevriesia* sp., the fatty acid profile is different from *Colletotrichum siamense*, as the linoleic acid is the major fatty acid, followed by tridecanoic acid. The contrasting profile of fatty acids could be used as a biomarker for the marine fungi.

5.5 Secondary metabolites from *Colletotrichum siamense*

Despite the negative results from our antibacterial assay with crude extracts of *Colletotrichum siamense*, methyl oleate and nervonic acid were detected from *C.siamense* in the present study. *Colletotrichum* spp. are known to produce many

structurally unique and biologically active natural products due to their pathogenicity (Kim & Shim, 2019; Moraga et al., 2019). Kim and Shim (2019) have listed 109 compounds that include nitrogen-containing metabolites, sterols, terpenes, pyrones, phenolics, fatty acids, and other miscellaneous compounds. For instance, colletotric acid, isolated from a liquid culture of *C. gloeosporioides* show antimicrobial properties against *Bacillus subtilis*, *Staphylococcus aureus*, and *Sarcina lutea* with the minimal inhibitory concentrations (MICs) of 25, 50, and 50 mg/ml, respectively (Zou et al., 2000). The fungal strains of *C. gloeosporioides* was isolated from *Artemisia monglica*, a plant known to have strong resistant against bacterial and fungal pathogens. Thus, endophytic fungi like *Colletotrichum* may perhaps play an important role in aiding the immune system of corals.

Methyl oleate are ester form oleic acids, organic compounds touted as biodiesel (Atabani et al., 2012). Studies have shown that fungi possess with the lipase to catalyst the esterification of fatty acid into ester through solid state fermentation (Oliveira et al., 2012; Martínez-Ruiz et al., 2018; Oliveira et al., 2020). Methyl oleate (free fatty acid ester) is an important storage lipids in fungal cells (Pan et al., 2018).

Nervonic acid, is a major very long-chain monounsaturated fatty acid (VLCMFA), one of the main components of the white matter of brains and myelin sheath of nerve fibers (Martínez & Mougan, 1998). Many fungi, particularly the oleagious species, able to synthesize nervonic acids as reviewed by Fan et al. (2018). Filamentous fungal strain *Mortierella capitata* RD000969 can accumulate up to 6.94 % nervonic acid of its total fatty acids and medium containing yeast extract, $CaCl_2$ and $MgSO_4 . 7H_2O$ was able to maximize the production of nervonic acid (Umemoto et al., 2014). In fungal cells, Very-Long-Chained-Fatty-Acids (VLCFAs) are the building blocks for sphingolipids and glycerophospholipids, important component of cell membranes (Erdbrügger & Fröhlich,

2021). VLCFAs can also be used as fuel via β-oxidation in peroxisomes (van Roermund et al., 2012).

5.5 Possible roles of recycling carbon of fungi to coral host

Lipids are stored as main long-term energy source by corals (Patton et al., 1983; Harland et al., 1993). Lipid content constituted a major portion in term of dry weight, averaging 24.2% and it varied according to seasonality, species and depths (Imbs, 2013). It is widely known and established hypothesis that, majority of carbon source was photosynthetically-fixed by the endocellular dinoflagellates, *Symbiodinium* and transferred to the coral host in the form of glycerol, glucose, amino acids, and lipids (Muscatine et al., 1981; Patton et al., 1983; Venn et al., 2008). Essential fatty acids such as MUFA, PUFA and SFA are synthesized by *Symbiodinium* and transferred to the host (Papina et al., 2003; Treignier et al., 2008; Teece et al., 2011) or obtained by the host via heterotrophic action (Al-Moghrabi et al., 1995; Houlbrèque et al., 2003; Radice et al., 2019). The synthesis of PUFA is long believed to be exclusively of dinoflagellates origin, due to the assumption of corals, as animals, lack specific enzyme (i.e., Δ9 Δ12 Δ15, methyl-end desaturases) required for the synthesis of PUFA (Dewick, 1997). However, recent studies have shown methyl-end desaturases gene are extensive in metazoan including scleractinian corals, and indeed capable of de novo biosynthesis of PUFA (Monroig et al., 2013; Kabeya et al., 2018; Monroig & Kabeya, 2018). A detailed probe into cnidarian-dinoflagellate symbiosis using lipidomic approach also challenge the long-standing translocation model of dinoflagellate to cnidarian in the context of PUFA (Dunn et al., 2012). The translocation of PUFA could be done reciprocally and it is not only a unidirectional model (Imbs et al., 2010; Imbs et al., 2014). Thus, other holobionts such as fungi can play a minute role in recycling the waste and distribute back to the coral host.

Other holobionts like fungi may involve in the recycling of wastes produced by the hosts and dinoflagellates in order for the corals to thrive in oligotrophic environments (Golubic et al., 2005). The coral skeleton provides a micro-environment for hosting holobionts (Ricci et al., 2019). The endolithic fungi may play a role in recycling nutrient (Wegley et al., 2007). Key fungal functional gene involved in metabolism of sugars and proteins were detected via metagenomic analysis of microbiome associated with Porites astreoides (Wegley et al., 2007). Inference of fungi role as saprotroph in the terrestrial ecosystem may help to explain the prevalence of marine fungi. Large portion of fungal biomass was detected in bathypelagic marine snow particles collected from the North Atlantic Ocean may suggest marine fungi's role in regulating or recycling of carbon in the ocean (Bochdansky et al., 2017), or even as part of coral holobionts (Thompson et al., 2015; Bourne et al., 2016; Amend et al., 2019). Parallel roles of carbon cycling has been proven in sponges where the sponge holobionts partake in the assimilation process (Rix et al., 2017). Perhaps there's a similar role akin to arbuscular mycorrhizal fungi (AMF) portrayed by marine fungi in cnidarian like corals in which they formed symbiosis with the coral hosts.

5.6 Future Studies

The findings from the present work may provide some foundation for future studies to further investigate on coral associated fungi, their ecological roles and discovery of novel compounds. Some of the immediate recommendations to further improve and expand the current work, which was somewhat preliminary, would be to: Include more extensive barcoding of corals species from different areas in Malaysia, using different genetic markers such as ITS, or perhaps using new technology of NGS to sequence whole mitogenome.

I. Intensive screening, isolation and identification of marine fungi cultures or even employing metagenomics for detecting unculturable marine fungi directly from the marine environments and organisms

II. A more detailed species description of *Neodevriesia* strains from marine environments

III. A more comprehensive metabolites study to purify all the metabolites and with the use of LC-MS and NMR for structural elucidation.

IV. Various bioactivities screenings with pure compounds instead of crude extracts

It is an irrefutable fact that fungi are part of the holobiont and they do play an important niche on par with other holobionts and even the exemplary endosymbiotic dinoflagellates (Symbiodiniaceae). Recent studies have proven that dinoflagellates aren't the panacea or the soloist that co-evolved with the coral enabling the corals to thrive in the oligotrophic seawater (Reshef et al., 2006; Rosenberg et al., 2007; Bourne et al., 2016; Peixoto et al., 2017; Rosenberg & Zilber-Rosenberg, 2018; van Oppen & Blackall, 2019). The laudable *Symbiodinium*-coral symbiosis are susceptible and perilous in face of climate change and could turn parasitism and hamper coral resilience (Baker et al., 2018).

The microbiome including fungi are essential for the corals despite its complexity and dynamic relationship with the host. Most of the studies done regarding the microbiome have been pivoted on bacterial community. The concept of coral probiotic hypothesis proposed by Reshef et al. (2006) is modeled by bacteria. The term microbiome, more often than not, has been appertained to bacterial community only while other microbes such as fungi have been overlooked. Although there are some breakthroughs deciphering the fungal community (Wegley et al., 2007; Littman et al., 2011; Amend et al., 2012; Sweet et al., 2013; Góes-Neto et al., 2020), more works are needed to explain

the nexus between fungi and the coral host such as their ecological niche in coral. Contemporary studies on bacteria community in corals using advanced technology such as metagenomic tools have been fruitful. Using metagenomic tools, researchers are able to verify the co-evolution between bacterial community and scleractinian corals (Pollock et al., 2018), create a fitting model predicting the implication of temperature on coral microbiome dysbiosis (Lima et al., 2020) and even amplify coral resistance against bleaching via microbiome manipulation (Rosado et al., 2019). Review by Thompson et al. (2015) also demonstrate how bacterial community (I) co-evolved with coral host, (II) complementing coral metabolism such as nitrogen fixation, (III) aid in development of coral from gametogenesis to settlement (IV) mediating homeostasis and (V) demonstrating interaction inclusive of competition. Perhaps, we can emulate the 'modus operandi' used in bacterial community to unfold the enigma behind mycobiome.

CHAPTER 6

CONCLUSION

In the present study, various fungal strains were isolated successfully from different coral growth forms. By using CO1 for coral and ITS for fungi together with their morphological features, only some of the scleractinian corals host and their respective marine-derived fungi were identified to the species level; seven out of fourteen collected scleractinian corals. Since *Acropora*, *Porites*, *Pocillopora* are species complexes, their identification was only done up to the genus level due to limitation of CO1 marker to resolve the complex, confounded also by their respective ambiguous morphological features of the corallites.

A total of 31 fungal isolates were derived from eight scleractinian corals. The isolated fungi were diverse and mainly ascomycetes and basidiomycetes, comprising of 18 fungal genera. Various studies done on the mycobiome of scleractinian corals have also shown that marine fungi inhabit in corals are diverse (Amend et al., 2012; Góes-Neto et al., 2020). Both *Penicillium* and *Neodevriesia* occurred frequently in our collected scleractinian coral samples. Occurrences of Penicillium are common in scleractinian corals while Neodevriesia are rare. There are also few new genera of fungi that have not been discovered previously in scleractinian corals, for instances, *Colletotrichum*, *Daldinia*, *Jaminaea*, *Hyalocladosporiella*, *Letendraea*, *Neodevriesia*, *Pestalotiopsis*, *Pseudopithomyces*, *Pyrrhoderma*, *Schizophyllum* and *Sterigmatomyce* are new occurrences in scleractinian corals. Multigene approach has done to identify two selected strain for studies of the secondary metabolites, namely PERF171521 as *Colletotrichum siamense* and PERF171711 as *Neodevriesia* sp. 4. Six markers (ITS, Cal, GADPH, β-

Tub, CHS-1, Act) were used to identify *Colletotrichum* spp., due to the species complexity, while only LSU coupled with ITS marker were used for *Neodevriesia*. With multi-locus phylogeny, PERF171521 was confirmed as *Colletotrichum siamense*, while PERF171711 and PERF170613 both, could be a new species from genus *Neodevriesia* as concatenated sequences of ITS and LSU show our samples are phylogenetically distinct from available *Neodevriesia* TYPE sequences.

Fatty acid analysis of the two strains *Colletotrichum siamense* and *Neodevriesia* sp. 4 show that majority extracts were of fatty acids. Two pure compounds (methyl oleate and nervonic acid) were isolated from *Colletotrichum siamense* and verified with 1H NMR experiment. Comparison of fatty acids of fraction 1 from both strains using GCMS shown contrasting profiles of fatty acids and it could be used as potential identification marker for the marine fungi. Antibacterial assay of crude extracts and fractions from *Colletotrichum siamense* showed negative results and no MIC was found. However, *Neodevriesia* is newly described fungal genus, with no reports on their metabolites so far, or even being found in marine organisms. Thus, future metabolite study can further exploit on this genus to discover more potential novel metabolites.

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