PHYLOGENETIC RELATIONSHIP OF KAPPAPHYCUS AND EUCHEUMA IN INDONESIA

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ABSTRACT

Kappaphycus and *Eucheuma* are highly valued for the carrageenan hydrocolloid they produce. The production of these carrageenophytes is generally on the uptrend throughout the globe, with the supplies originating mainly from the Southeast Asian countries such as Indonesia, the Philippines ~~and~~, Malaysia and etc. Indonesia is currently the largest producer of *Kappaphycus* and *Eucheuma* in the world, generating massive incomes from the carrageenan marketed. Despite the massive carrageenan yields, the technologies involved in farming are conventional and taxing, with many still collecting *Kappaphycus* and *Eucheuma* from wild populations. However, with recent fundings and support from the local government, the seaweed industry in Indonesia stands a chance in really striving to become an advanced, high income and efficient entity. One of the aims for the improvement of the industry involves taxonomic studies on the morphologically plastic *Kappaphycus* and *Eucheuma* from Indonesia, to reduce misidentification and misplantation by farmers, thereby also reducing losses in overall carrageenan yields.

Earlier studies have shown the effectiveness of genetic markers in elucidating the phylogeny of *Kappaphycus* and *Eucheuma*. This study employs similar methodologies, with a main objective which is to phylogenetically reconstruct and elucidate the relatedness and ancestry of *Kappaphycus* and *Eucheuma* in Indonesia, as compared to those from elsewhere. The study also attempts to supplement morphological observations of these carrageenophytes in Indonesia with molecular data, which hopefully will help with the pre-existing taxonomic confusions, particular those associated with local varieties.

Thirty samples described based on local descriptions, consisting of *Kappaphycus alvarezii*, *K. striatum* and *Eucheuma denticulatum* were collected from various Indonesian Islands: Lombok, Sumbawa, Bali, Kalimantan, Sulawesi, Sumatra, Madura, Nusa Tenggara Timur and Irian Jaya. Distinguishing of local varieties proved challenging with the paucity of clear and distinctive identification criteria. Interspecific and intergeneric identifications were relatively easier, where *Eucheuma* specimens are different from *Kappaphycus* with the presence of pinnate or pectinate spines. *K. striatum* can be distinguished from *K. alvarezii* by the dense, short and angular branching patterns, forming a somewhat dorsally symmetrical bunch.

Molecular results based on the *cox*1 and *cox*2-3 spacer DNA markers have shown that all samples collected in this study were conspecific with the most common *K. alvarezii, K. striatum* and *E. denticulatum* cultivars in the world. Local varieties were not valid based on molecular data. Results based on both the *cox*1 and *cox*2-3 spacer genetic markers have shown that the *K. alvarezii* from the Hawaiian Islands were genetically distinct from the main commercial *K. alvarezii* plants and may require further investigations. The *cox*2-3 spacer genetic marker had also shown the existence of *K. alvarezii* which are unique to Africa. Two genetic strains of *K. striatum* were also observed in this study which are to date, undistinguishable based on morphology. Although not collected in the present study, the *Endong* variety of *E. denticulatum* as described by Ganzon-Fortes and co-workers on the year 2011 was shown to be available in Indonesia as well based on earlier records by Zuccarello and colleagues (2006). *E. denticulatum* originating from Africa were again genetically different from the *E. denticulatum* found in South East Asia and Hawaii. The decent phylogenetic resolution of both the *cox*2-3 spacer and *cox*1

gene had also suggested the potential use of these genetic markers as DNA barcodes for *Kappaphycus* and *Eucheuma* in the future.

Considering the relatively limited gene pool associated with commercial cultivars of *K. alvarezii*, *K. striatum* and *E. denticulatum*, further studies should emphasize on the collection of wild *Kappaphycus* and *Eucheuma* specimens revolving around the numerous Indonesian islands to better represent the distribution as well as species diversity of these economically important red algae. This is especially true considering the richness in biodiversity around the Coral Triangle.

ABSTRAK

Kappaphycus dan *Eucheuma* merupakan dua genera yang amat bernilai disebabkan hidrokoloid karagenan yang mereka hasilkan. Pengeluaran *carrageenophytes* ini umumnya menunjukkan pola yang meningkat di seluruh dunia, yang mana negara-negara Asia Tenggara seperti Indonesia, Filipina, Malaysia dan sebagainya merupakan pengeluar–pengeluar utama. Indonesia, kini sebagai pengeluar terbesar *Kappaphycus* dan *Eucheuma* di dunia, menjana pendapatan yang lumayan dari karagenan yang dipasarkan. Walaupun hasil karagenan adalah tinggi, teknologi penanaman yang digunakan adalah konvensional dan sukar, tatkala ramai yang masih mendapatkan *Kappaphycus* dan *Eucheuma* dari populasi liar. Namun begitu, dengan bantuan kewangan dan sokongan daripada kerajaan tempatan, industri rumpai laut di Indonesia kini berpeluang menjadi sebuah entiti yang maju, berpendapatan tinggi dan cekap. Salah satu matlamat dalam pembangunan industri melibatkan kajian taksonomi ke atas *Kappaphycus* dan *Eucheuma* dari Indonesia yang bermorfologi plastik, untuk mengurangkan pengenalpastian dan penanaman yang salah oleh para petani, tatkala mengurangkan hasil keseluruhan karagenan.

Kajian awal telah menunjukkan keberkesanan penanda-penanda genetik dalam penghuraian filogeni *Kappaphycus* dan *Eucheuma*. Kajian ini menggunakan kaedah yang sama, yang mana objektif utama ialah untuk membina semula hubungan filogenetik dan menjelaskan perkaitan dan keturunan *Kappaphycus* dan *Eucheuma* di Indonesia, berbanding sampel-sampel dari tempat lain. Kajian ini juga bertujuan untuk melengkapi pemerhatian morfologi *carrageenophytes* di Indonesia dengan data molekul, yang diharapkan akan membantu dalam kekeliruan taksonomi yang sedia ada, khususnya yang melibatkan varieti tempatan.

Tiga puluh sampel yang diberi nama berdasarkan deskripsi tempatan, merangkumi *Kappaphycus alvarezii, K. striatum* dan *Eucheuma denticulatum* telah dikutip dari beberapa Kepulauan Indonesia: Lombok, Sumbawa, Bali, Kalimantan, Sulawesi, Sumatera, Madura, Nusa Tenggara Timur dan Irian Jaya. Pembezaan varieti tempatan terbukti mencabar berikutan kekurangan kriteria pengenalpastian yang jelas dan bercirian tersendiri. Pengenalpastian antara-spesies dan antara-genus adalah lebih mudah, yang mana spesimen *Eucheuma* berbeza daripada *Kappaphycus* dengan kehadiran duri yang tersusun di kedua-dua belah paksi atau seperti gigi sikat. *K. striatum* boleh dibezakan dari *K. alvarezii* melalui corak dahannya yang lebat, pendek dan bersudut, lantas membentuk jambakan yang agak bersimetri dorsal.

Keputusan molekular berdasarkan kedua-dua penanda genetik *cox*1 dan *cox*2-3 spacer telah menunjukkan bahawa kesemua sampel dalam kajian ini merupakan spesies yang sama dengan kultivar-kultivar *K. alvarezii, K. striatum* and *E. denticulatum* yang biasa dijumpai di seluruh dunia. Varieti tempatan adalah tidak berasas berdasarkan keputusan molekular. Keputusan dari penanda genetik *cox*1 dan *cox*2-3 spacer menunjukkan bahawa *K. alvarezii* dari kepulauan Hawaii mempunyai genetik yang berbeza daripada *K. alvarezii* yang komersial dan berkemungkinan memerlukan siasatan lanjut. Penanda genetik *cox*2-3 spacer, juga menunjukkan kehadiran *K. alvarezii* yang unik dari Afrika. Dua baka genetik *K. striatum* juga diperhatikan dalam kajian ini yang mana sehingga kini masih tidak dapat dibezakan berdasarkan morfologi. Walaupun tidak diperolehi dalam kajian ini, *E. denticulatum* varieti "*Endong*" seperti yang dihuraikan oleh Ganzon-Fortes dan rakan sekerja pada tahun 2011 didapati hadir di Indonesia berdasarkan rekod awal oleh Zuccarello dan rakan-rakan (2006). *E. denticulatum* dari Afrika didapati berbeza daripada *E. denticulatum* dari Asia Tenggara dan Hawaii. Resolusi filogenetik

bagi kedua-dua gen *cox2*-3 spacer dan *cox*1 yang memuaskan juga mencadangkan potensi penggunaan penanda genetik tersebut sebagai kod bar DNA untuk *Kappaphycus* dan *Eucheuma* ~~di~~ pada masa depan.

Memandangkan kolam gen kultivar komersial *K. alvarezii*, *K. striatum* dan *E. denticulatum* agak terhad, kajian lanjut perlu menitikberatkan kutipan spesimen *Kappaphycus* dan *Eucheuma* yang liar di sekitar kepulauan Indonesia bagi mendapatkan taburan serta kepelbagaian spesies yang lebih menyeluruh untuk alga merah ini yang mempunyai kepentingan ekonomi. Ini amatlah ketara sekiranya kekayaan biodiversiti di sekitar Segitiga Terumbu Karang diambil kira .

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SIMBOLS AND ABBREVIATIONS

Symbol	Information
%	Percent
ºC	Celsius degrees
cm	Centimeter
Kg	Kilogram
Ppm	Parts per million
ml	Milliliter
µl	Microliter

Abbreviations	Information
*Cox*2-3s	Cytochrome *c* oxidase subunit 2-3 intergenic spacer
*Cox*1	Cytochrome *c* oxidase subunit 1
USD	United States Dollar
FAO	Food and Agriculture Organization
DNA	Deoxyribonucleic acid
RFLP	Restriction fragment length polymorphism
RAPD	Random amplified Polymorphic DNA
PCR	Polymerase chain reaction
RubisCO	*Ribulose-1,5-bisphosphate* carboxylase oxygenase
AFLP	Amplified fragment length polymorphism
cDNA	Complementary DNA
ITS	Internal transcribed spacer
UPA	Universal plastid amplicon
*rbc*L	ribulose-bisphosphate carboxylase
RNAse	Ribonuclease
dNTP	Deoxyribonucleotide triphosphate
bp	Base pair
rpm	Rotation per minute

CHAPTER 1: INTRODUCTION

1.1 Economic importance of seaweeds

Seaweeds, commonly known as macroalgae in the scientific world are a member of the plant kingdom. Despite looking like a plant, it does not display a true leaf, stem, and root. The entire biomass as a whole is known as a thallus (Lembi & Waaland, 1988). The commercialization of seaweeds, both raw and processed is not new, especially in food industries. For many centuries seaweeds have been used a food source in China, Japan, and South Korea. With the advancement of technology, research and development, many new applications of seaweeds were discovered apart from being a sole food source. These include potential properties in the pharmacy, agriculture, and paper industries.

Ecologically seaweeds are very important being the main oxygen producers in aquatic areas. Seaweeds usually thrive within the rocky intertidal areas and tend to occupy rock surfaces in shallow, subtidal areas in temperate or cold areas. Even though most occur 8-20 m in most oceans, some are found in depths of more than 40m in particularly clear waters (Guiry, 2012).

Seaweeds are collected from the wild, or farmed for the extraction of its gelatinous substances such as alginate, agar, and carrageenan. These substances which are so called hydrocolloids or phycocolloids are commercially important, particularly in the production of food or food additives. Not just the food industries but also the pharmacy industries exploit the gelling, emulsifying, water retention, and other physical properties

displayed by these phcocolloids. Agar is extensively used as culture media in the fields of microbiology. Carrageenan is used in the preservation of meat and fish, dairy items and baked food. Alginate, which is often used similarly like Carrageenan, can be applied as coatings for paper, or used in would dressings. Recent researches have proceeded in using seaweeds as a fertilizer and a potential source of bioethanol for biofuel production (Meinita et al., 2011).

1.2 The importance of taxonomy study

It is already well known that seaweeds have many uses and benefits to humans. Researches and studies on seaweeds utilization are also on the rise, which could be seen by the increasing number of seaweed cultivation all over the globe.

The history of seaweed cultivation first started when Dr. Maxwell S. Doty from the University of Hawaii visited the Philippines in the 1960s (Bindu & Levine, 2010; Doty, 1988; Doty & Norris, 1985b). Ecological surveys of the coastal areas where wild populations of seaweeds reside were conducted. The global needs of seaweeds which outweighs the production from seaweeds collected from the wild, enticed the idea of seaweed cultivation. Cultivation studies were conducted with different types of farming techniques. Since then these practices for seaweed cultivation became the basis for commercial farming trials in different parts of the country. Until now, seaweed farming has been introduced into other countries around the globe such as Hawaii, Vietnam, Fiji, Columbia, Singapore, Indonesia, Malaysia and so on (Ask et al., 2001; Bindu & Levine, 2010; Conklin et al., 2009; Dang et al., 2008; Doty, 1985a, 1988; Doty & Norris,

1985b; Halling et al., 2012; Phang et al., 2010; Tan et al., 2012; Zuccarello et al., 2006).

Malaysia started the farming of *Kappaphycus* (Doty) Doty ex P.C Silva and *Eucheuma* J. Agardh in 1978. The taxonomy grouping of the cultivated Eucheumatoids were based on morphology (Hurtado & Critchley, 2006, Phang et al., 2010). However the taxonomic classification of *Kappaphycus* and *Eucheuma* proved to be challenging when solely based on morphological features, rendering different varieties and strains difficult to morphologically identify; this has thus resulted in a shift towards molecular approaches to elucidate the taxonomy of these seaweeds, and their associated phylogeny (Conklin et al., 2009; Dang et al., 2008; Ganzon-Fortes et al., 2011; Halling et al., 2012; Tan et al., 2012; Zhao & He, 2011; Zuccarello et al., 2006). From being harvested directly from the natural populations, the *Kappaphycus* and *Eucheuma* industry of the Indonesia archipelago has since the early 1960s (Trono, 1987), underwent rapid increment in terms of farming and carrageenan production, and is now the largest producer of Eucheumatoids in the world (Bixler & Porse, 2010). There were beliefs that the *Kappaphycus* and *Eucheuma* cultivated in Indoensia were originally brought in form the Philippines, but these beliefs are up until today, unverified (Gerung, 2006); and despite being the top producer in terms of carrageenan yield, taxonomic studies on the species and varieties these seaweeds were very limited, if not non-existent entirely. The prospects of molecular systematics are thus applicable here, and will be covered in this study.

1.3 Objectives of research

The objectives of this study are:

1. To elucidate the taxonomic confusion associated with the varieties and different species of *Kappaphycus* and *Eucheuma* in Indonesia using the mitochondrial-encoded *cox*2-3 spacer and *cox*1 genetic markers.

2. To determine the phylogenetic relationship between Indonesian varieties and species of *Kappaphycus* and *Eucheuma* and those from within and outside Indonesia.

CHAPTER 2: LITERATURE REVIEW

2.1 Rhodophyta

Rhodophyta, also known as the red algae is a group of algae characterized by the presence of phycoeryhtrin, phycocyanin, and allophycocyanins, accessory pigments which are capable of photosynthesis. The members of this division also are lacking flagella and centrioles (Woelkerling, 1990). Red algae possibly have the largest population compared to the other divisions with an estimation of up to 6000 species. Most species of this division predominate along the coastal and continental shelf areas, regardless of the temperates. (Luning, 1990). Apart from being primary producers, red algae also offer structural habitats for other organisms dwelling within the marine environment. Red algae are also of significant importance in the establishment and maintenance of coral reefs. There are three main divisions of algae which are red algae (Rhodophyta), green algae (Chlorophyta), and brown algae (Phaeophyta) and among these three groups, the red algae (Rhodophyta) are the most important commercially.

A special characteristic in algae is the existence of a polymer inside the cell wall of its thallus. Each division of algae exhibits a specific polymer displaying unique biochemical functions. Red algae are known to produce hydrocolloids that are economically important to the food and cosmetics industry due to their gelling, emulsifying or thickening properties (Doty & Norris, 1985b). This has led to the large-scale cultivation or natural harvesting of red seaweeds all over the world (Phillips, 1996).

Red algae display certain characteristics that differentiates them from other eukaryotic groups. According to (Garbary & Gabrielson, 1990), some among the main characteristics are:

- Lack of flagella and centrioles
- Floridean starch as a the storage for starch
- Accessory pigments phycoerythrin, phycocyanin, and allophycocyanin
- Absence of stacked thylakoids in plastids
- Lack of endoplasmic reticulum in chloroplasts

Carrageenan, a type of polysaccharide hydrocolloid is classified as a hydrophilic colloid exhibiting a wide range of functions. There are different types of carrageenan, which differs in composition and structure, thus displaying varying rheological properties that are commercially exploited. Different carrageenan cover a wide spectrum of gel properties and gel behavior ranging from viscous thickeners to thermally reversible gels that can be soft and elastic to firm and brittle. The two most commonly used carrageenan are *kappa* carrageenan (extracted from *Kappaphycus* (Doty) Doty ex P.C Silva) and *iota* carrageenan (extracted from *Eucheuma* J. Agardh); where the former is of greater economical value, owing to the fact that it is capable of interacting synergistically with other type of gums, which can result in modification to the existing gel texture (Thomas, 1997). The specific interaction between *kappa* carrageenan and *kappa* casein can also be exploited in stabilizing dairy goods (Stancioff & Renn, 1975).

2.2 Economic importance of *Kappaphycus* and *Eucheuma*

Kappaphycus and *Eucheuma* are widely cultivated throughout the globe particularly in the tropical areas. These two species are highly valued for the carrageenan they produce. The term Eucheumatoids is often used to describe these two species together (Aguilan et al., 2003). Aquaculture of these Eucheumatoids had begun since the 1960s in the Philippines. Intensive farming of *Kappaphycus* and *Eucheuma* initiated because of the high carrageenan content which these Eucheumatoids possess, and also the rapid growth as well as robustness. These Eucheumatoids are known to demonstrate high growth rates (15 to 30 days harvestable) essential for mass production (Ask et al., 2003; Ask et al., 2001; Bixler & Porse, 2010; Doty, 1988; Doty & Norris, 1985b; Hung et al., 2008; Hurtado et al., 2008; Neish I. C., 2003; Trono, 1992).

The valuable properties exhibited by *Kappaphycus* and *Eucheuma* had brought about huge demands worldwide, thus increasing the price of these Eucheumatoids. It has been reported that in the year 2004 these seaweeds could go up to average process of approximately USD 0.55/kg raw seaweed material, USD 3.95/kg semi-refined carrageenan and USD 8.68/kg of refined carrageenan (FAO, Fisheries and Aquaculture Department). Owing to the lucrative business generated, seaweed farms of *Kappaphycus* and *Eucheuma* have mushroomed all over the world e.g. South America, Africa, Hawaii, and Asian countries (Ask et al., 2001; Bindu & Levine, 2010; Bixler & Porse, 2010; Conklin et al., 2009; Doty, 1988; Doty & Norris, 1985b; Halling et al., 2012; Munoz et al., 2004; Neish I. C., 2003; Nguyen H. D. & Huynh Q. N., 1995; Phang et al., 2010; Tan et al., 2012; Zhao & He, 2011; Zuccarello et al., 2006).

The largest carrageenan producers are countries located around the coral triangle which has the most optimal environment conditions growth. Indonesia, being the number one producer of carrageenan, exported 102,415.93 tons of carrageenan worth 124.36 million US dollars, to countries such as Africa, America, Asia, Australia and Europe (Mchugh, 2001). Indonesia had also covered an approximate 13% of the world‘s carrageenan market as of 2007, 13% in 2008, 14% in 2009 and 15% in 2010 (Personal communication with Martin Huseini, the director general of the marine affairs and fisheries in Gorontalo, Sulawesi).

2.2.1 Carrageenan

Carrageenan is a polymer found in the cell walls of red algae. The carrageenan chemical structure consists of a backbone of galactose with a variation in location and proportion of ester sulfate groups and 3,6- anhydrogalactose. These variations would affect the texture, gel strength, and other properties of the carrageenan. Despite the lack of nutritional value, carrageenan has many potential functions. It could act as a thickening agent, stabilizing agent, and also emulsifying agent often used in food industries.

There are three main types of carrageenan that are used commercially, which are known as *iota*, *kappa*, and *lambda* carrageenan. The different types of carrageenan differ in the location and proportion of sulfation in the polymer. This difference would affect the properties of the carrageenan itself, such as the hydration gel strength, synersis, and synergism of the carrageenan (Thomas, 1997). *Kappa* carrageenan is mainly harvested from the red algae *Kappaphycus* while *iota* carrageenan is commonly harvested from

Euchuema.

As earlier mentioned, the types of carrageenan differ in its chemical structure, which in turn affects its rheological properties. *Kappa* carrageenan binds water to produce strong and rigid gels. This is because the *Kappa* carrageenan would mostly interact with potassium ions essential in forming firm gel structure. While the *iota* carrageenan also binds to water, it produces dry and elastic gels. This is because of the high intensity in the presence of calcium ions. *Kappa* carrageenan also interacts with calcium ions, resulting in a dry and elastic gel and that provides excellent freeze/ thaw stability (Rees, 1963).

Carrageenan is often used to induce thickening, gelling or create suspensions, ideal foremulsion stabilization, control of syneresis, as well as for bodying, binding and dispersion. Carrageenan is largely used in food industries particularly dairy applications. Carrageenan is also used in chemical and medicine industries recently, such as raw material for pill capsules and toothpaste.

Carrageenan is largely used in the production of chocolate milk, owing to its ability to suspend cocoa at low concentrations (~300ppm). Although not visible, a delicate milk gel structure is believed to keep the cocoa in suspension within the milk, and the uniformity of suspension is vastly influenced by the carrageenan type and quality (Lund & Bjerre-Petersen, 1952).

Much like *kappa*-carrageenan, *iota*- carrageenan displayed useful, and commercially exploitable characteristics. Often used in desserts, *iota*-carrageenan produces textures comparable to that produced by gelatin gels; and since carrageenan-derived gels have relatively higher melting point, they are especially popular in tropical regions, or in areas where refrigeration is not available. Gels made from *iota*-carrageenan also have the advantage of remaining soft upon aging, unlike gelatin gels that tends to solidify. Despite the many benefits, *iota*-carrageenan gels loses out in the different mouth-feel, as they do not melt like gelatins do (Phillips, 1996). *Kappa*-carrageenan is also extensively applied in gel desserts, often with locust bean galactomannan etc. to produce different textures (Anderson et al., 1968).

Carrageenan is also used in precise amounts within toothpastes to bind the formulation together and also to provide the quality of the texture and sheen. The better texture and cosmetics as well as immunity to cellulose enzymes displayed by carrageenan- derived toothpastes set them apart from gum produced from sodium carboxymethylcellulase (Dolan & Rees, 1965).

2.3 *Kappaphycus* and *Euchuema*

The two main genera used for carrageenan production is *Kappaphycus* and *Eucheuma*. *Kappaphycus* produces *kappa* carrageenan while *Eucheuma* produces *iota* carrageenan (Bixler & Porse, 2010).

The taxonomic classification of *Kappaphycus* and *Eucheuma* can be summarized as below (Guiry & Guiry, 2012):

Phylum	Rhodophyta
Subphylum	Eurhodophytina
Class	Florideophycea
Subclass	Rhodymeniophycidae
Order	Gigartinales
Family	Solieriaceae

2.3.1 Conventional taxonomy and systematics

Taxonomical studies on *Kappaphycus* and *Eucheuma* are fairly poor, with initial progress mainly hindered by the morphological plasticity of these two seaweeds. The taxa *Kappaphycus* did not exist and was referred and classified under the taxa *Eucheuma*. Maxwell Doty noticed the difference in *Kappaphycus* and *Eucheuma* in 1988, mainly from the different type of carrageenan produced. Until now there are five valid species of *Kappaphycus*, *Kappaphycus alvarezii* (Doty) ex P.C. Silva (type species); *K. cottonii* (Weber-van Bosse) Doty ex P.C. Silva; *K. inermis* (F. Schmitz) Doty ex H.D.Nguyen and Q. N. Huynh; *K. procrusteanus* (Kraft) Doty; *K striatus* (F.Schmitz) Doty ex P.C.Silva (biosynonym with *Eucheuma striatum* F. Schmitz).

For *Euchuema*, the taxonomical studies for the species under this taxa has already began since the 1917s. *Eucheuma denticulatum* (N.L.Burman) F.S.Collins and Hervey

is the type species and as of now there are a total of 51 species (and infraspecific) names recorded, of which 22 species are valid species (Guiry & Guiry, 2012).

2.3.2 Main cultivars

Kappaphycus species is a member to the Rodophyta division and are among the largest in biomass compared to other Rhodophytes. *Kappaphycus* has a high growth rate, and can be harvested in 15 to 30 days (Azanza-Coralles et al., 1992). This red alga has a very firm and tough thallus and can grow up to 2 meters tall. *Kappaphycus* plants are commonly known for its repeated branching patterns, with most branches primary while secondary branches grow between primary branches or entirely absent. The *Kappaphycus* species displays a considerably high variation in thalli colors ranging from shades of green, brown or even yellow (Abbott, 1999).

Kappaphycus plants are frequently found in the intertidal areas but they are also capable of surviving up to 20m deep. Wild *Kappaphycus* commonly grow attaching to a substrate or unattached altogether, floating loosely in shallow or deep waters. They are also capable of forming large moving mats of unattached thalli (Russell, 1989). Furthermore, *Kappaphycus* has a wide range of environment tolerance (Harrison & Lobban, 1994). These are the main reasons why *Kappaphycus* is a potential commodity for aquaculture and cultivation.

Due to its immense success, *Kappaphycus* is widely distributed throughout the Pacific by various entrepreneurs, governments and regional agencies. This has led to

increased concerns in the bioinvasion of *Kappaphycus* on local habitats. In the Solomon Islands, for example, attempts to farm *Kappaphycus* at two locations which were later abandoned resulted in the plant dispersing to and persisting at adjacent sites. Another example is the cultivation of *Kappaphycus* in the Gulf of Mannar (India) which became a threat to the coral reefs. The fast growing *Kappaphycus* plants prevented sunlight penetration essential for the coral reefs‘ survival (Krishnamurti & Joshi, 1970). Cultivation of *Kappaphycus* should be controlled and properly managed, especially in countries with commercially introduced strains, to avoid the cultivars from becoming bioinvasive pests to the local habitat.

Eucheuma plants are often differentiated from *Kappaphycus* plants based on the pectinate and pinnate branching patterns, production of *iota* carrageenan, as well as the arising of cystocarps associated only with lateral branches (Doty 1988; Neish, 2003). *Eucheuma* grows in a multitude of forms, which are highly influenced by environmental factors. The appearance might be different during the year depending on the changes in the environment. During the warm season, seaweeds might grow in terms of the girth, with less growth in terms of branching; whereas during the cooler months, the seaweed may undergo higher frequency of branching. This phenomenon can be ascribed to the lower temperatuire of the ocean during the cold season, which brings about moderate water currents and increased nutrients. (Foscarini & Prakarsh, 1991).

Wild populations of *Eucheuma* grow best in protected areas amongst coral reefs and require clear, but turbulent water. It thrives best at salinities of 30 to 35 ppt (i.e., close to

open seawater salinity)and commonly found attached to substratum, mostly rocks or hard corals, within the sub-tidal zones of the ocean (Rogers et al., 1981).

The cultivation method of *Eucheuma* is exactly the same as *Kappaphycus* since these two species have the same requirements for growth. *Kapppaphycus* and *Eucheuma* are well known by their commercial names as *cotonii* and *spinosum* respectively.

The three main cultivars Euchematoids in the world are *Kappaphycus* alvarezii, *K. striatum* and *Euchuema denticulatum.* In addition to the main species many different locally-derived varieties or forms are cultivated widely throughout the world. The main varieties of *K. alvarezii* that are cultivated in Philippines by their local names are A*dik Kalas, Adik Goma, Purdoy, Tambalang, Tambalang Milo, Tambalang Putan*, and *Vanguard Giant*. Similarly, *Kappaphycus striatum* may include variants displaying red and brown colour (*kab-kab* and *payaka*) as well as green and brown colour (*sacol*) (Hurtado & Critchley 2006). In Malaysia, the cultivars consisted of twelve varieties of *Kappaphycus*, locally named *Buaya* (Crocodile), *Tambalang* varieties (*Brown*, *Giant*, *Green*), *Tangan-tangan* (Loving Beauty), *Durian* varieties (Black, Red, Yellow), *Flower* varieties (Green and Yellow), *Aring aring*; and local *Eucheuma* variety called *Spinosum* (Tan et al., 2012) In Vietnam, the varieties of cultivars are: *Eucheuma denticulatum*: Green brown; *Kappaphycus striatum* (Payaka brown; Sacol brown and Sacol green) and *K. alvarezii* (dark green and brown with short branches) (Dang et al., 2008). In Mexico, three color strains of *K. alvarezii* (red, green and brown) were reported and cultivated (Munŏz et al., 2004).

In Indonesia, the main varieties of *K. alvarezii* cultivars display brown and green color (Indriani & Sumiarsih, 1995; Abidin, 2005). Local names of *Kappaphycus* and *Eucheuma* species in Indonesia are originated from the Philippines, which include *Tambalang, cottonii, sacol,* and *Maumere*. The local strain *K. alvarezii* var *Tambalang* are widely cultivated in several cultivation sites at Nusa Tenggara Barat. Earlier studies showed that the growth of *K. alvarezii* strain *Tambalang* and *Maumere* in this location are very good with high carrageenan quantity when harvested after 45 day (Widyastuti, 2008a). However different results were shown by the same strain of *K. alvarezii Tambalang* and *Maumere* at Muluk beach in which the carrageneenan quantity is low (Widyastuti, 2008b).

2.4 Molecular approaches in seaweed systematics

Molecular biology is a study on the biological activities at the molecular level using both physical and physiochemical methods (King & Stansfield, 1985; Kahl, 1995). A sub-discipline of systematics is the molecular systematics (Hilis et.al., 1990) which integrates molecular data into the analysis of phylogenetic relationships of organisms (McCourt et al., 1996). With the advancement of molecular technologies acquisition of molecular sequences, be it amino acid or DNA sequences have become relatively easy. For DNA, techniques employed include nucleic acid hybridization, random amplified polymorphism DNA (RAPD), restriction fragment length polymorphisms (RFLPs), DNA sequencing and others (Lim, 2003). The restriction fragment length polymorphism (RFLP) assay has proven its usefulness in measuring genetic diversities of organisms, and also to genetically link them (Botstein et al., 1980). However, the tediousness and time consuming protocols have rendered RFLP assays less favored as of late, especially when considering the advancement in other genetic methods.

Red algae are not real plants and are lacking in true leaves, stems or roots, thus it is generally difficult to differentiate the algae from one another. Because of this, molecular taxonomy has become more popular, offering valuable supplementary data to morphological descriptions in taxa classifications. Until now a variety of genes have been discovered for algae DNA barcoding and are widely used in the molecular systematics of algae (Graham & Wilcox, 2000).

2.4.1 Random amplified polymorphism DNA (RAPD)

The utilization of Polymerase Chain Reaction (PCR) technology in biotechnology and molecular research has become very common due to its simplicity, high yields as well as high success rates. However, the necessity of pre-derived DNA sequence information renders PCR assyas limited in terms of scope of application. The discovery of random primers that could amplify via PCR an unknown DNA sequence might be the reason why Random Amplified Polymerase DNA (RAPD) has become a trend in biotechnology researches. The exclusion of cloning and sequencing methodologies was also one of the reasons for the popularity of RAPD during its time.

However there are some limitations of the Random Amplified Polymerase DNA (RAPD) method, such as:

- Dominancy of RAPD markers. A DNA segment amplified cannot be determined as homozygous or heterozygous.
- PCR is laboratory dependent due to its large dependency on the quality and quantity of the template DNA, PCR components and PCR runtimes, which may be variable from one laboratory to the other, thus affecting the RAPD results.
- Primer mismatches will affect the final yields of PCR amplicons.

The RAPD method has been used also for seaweed molecular studies offering a way to identify those that are known to be highly plastic in terms of morphology. Research on

algae using the RAPD method has been frequently conducted, due to simplicity of the method. This is especially useful for wild algae species, in which the information of the DNA sequence is not yet known. Xiao et al., (2005), using RAPD technology to develop an effective primer on eleven samples of algae, showing their richness in genetic diversity. Another research is by Shalini et al., (2007) on the optimization of RAPD method for the evaluation of cyanobacteria biodiversity. Even though most of RAPD approaches for algae taxonomy employed cultured strains (Patwary et al., 1994; van Oppen et al., 1996), RAPD-PCR method has been applied to determine the genetic diversities within wild populations (Ho et al., 1995). Other successful analyses using RAPD on algae include the differentiationof morphological variants of *Graclairia salicornia* (Rhodophyta) (Lim et al., 2001) and the differentiation of male and female sporophytic thalli of *Gracilaria changii* (Sim et al., 2007).

2.4.2 Restriction fragment length polymorphism (RFLP)

Restriction Fragment Length Polymorphism (RFLP) is a technique which, on the basis of DNA segmentation, differentiates organisms in question.. When two organisms have similar segmentation sites, digestion by restriction enzymes, subjection to gel electrophoresis and Southern blot will produce similar products, where the patterns can be easily identified vice versa. These patterns can even be used to differentiate down to strains level (Budowle & Baechtel, 1990).

Although RFLP is said to be more accurate than PCR, the RFLP has a few disadvantages (Budowle & Baechtel, 1990), such as :

- Tedious and time consuming protocols
- Risk of exposure to radioactive probes Large quanities of materials required for experiments
- Requires high molecular weight compounds

Restriction Fragment Length Polymorphism (RFLP) was first used in genetic studies of markers based on hybridization of DNA, and in the year 1980 and 1990 has been applied to algal genetic research (Scholfield et al., 1991). Equivalent to RAPD, molecular algae researches using the RFLP method are also common. For example, a simple RFLP assay was designed to enable the ease of identification of common *Porphyra* species based on the RuBisCO spacer gene region (Teasdale et al., 2002).

2.4.3 Amplified fragment length polymorphism (AFLP)

Amplified fragment length polymorphism (AFLP) involves the specific amplification of restriction enzyme-digested DNA which produces a unique fingerprint for mapping genomes (Hongtrakul et al., 1997). First developed for plant studies, AFLP analysis is used for a variety of applications, such as:

- Genetic mapping for newly discovered species
- Determination of relatedness among cultivars
- Establishment of linkage groups in crosses
- Phylogenetic and genetic diversity studies

The AFLP markers are considered as good molecular markers, because they possess almost all the characteristics of good molecular markers, except for the codominance. According to Mueller & Wolfenbarger (1999), a good molecular marker should be/have:

- Mendelian inheritance : transmit from one generation to another
- Polymorphic: present in several alleles at the locus investigated (multiallelic)
- Co dominant: allow the discrimination between homo and heterozygotes
- Neutral: all alleles have the same fitness
- Not epistasis: one can determine the genotype of a phenotype irrespective of the genotype of the other loci
- Independent of environment: no phenotypic plasticity
- Frequent occurrence in the genome
- Even distribution throughout the genome
- Highly reproducible

Despite the advantages of AFLP method, there are also disadvantages based on Hakimizadeh et al. (2012), such as:

- Heterogeneity of the final products
- Cloning and sequencing required for identification
- cDNA required from the PCR amplicons

Amplified Fragment Length Polymorphism (AFLP) is a more modern technique compared to the traditional Restriction Fragment Length Polymorphism (RFLP) method. AFLP has been successfully applied to the molecular typing of bacteria (Lin et al., 1996), detection of diversity in fungal species (Majer et al., 1996), and also serve as a source of molecular markers for the red algae (Donaldson et al., 1998). There are also many other successful research on algae using AFLP method such as the analysis of genetic diversity in *Gracilariopsis lemaneiformis* (Pang et al., 2010) and the assessment of genetic stability of a range of terrestrial microalgae after cryopreservation (Muller et al, 2007).

2.4.4 DNA Sequencing

DNA sequencing is a technique to detect and identify the order and arrangement of nucleotides within a specific portion of DNA. The richness in terms of genetic information in these stretches of DNA has made DNA sequencing one of the most popular techniques in phylogenetics and ancestry studies. The massive, potential sizes of informative data are also, among others, important benefits of nucleotide sequencing. DNA sequencing has also led to the establishment of molecular systematics, where more in depth researches can be conducted in understanding gene evolution, convergence and selection, intraspecific and interspecific interactions, genetic diversity, biogeography, phylogenetics and so on (Hillis et al.,1996).

DNA is the main source of genetic information in bioorganisms, thus the DNA sequencing process has become a crucial part in biotechnology and molecular biology studies. DNA sequencing is achieved by utilizing labeled nucleotides incorporated into a

copy of the desired DNA. The DNA sequence can then be determined by the positions of the labeled nucleotides. The DNA of interest is first amplified via Polymerse Chain Reaction (PCR), followed by purification of the desired amplicons prior to DNA sequencing. The ABI SOLiD (Sequencing by Oligonucleotide Ligation and Detection) sequencing technique is currently the most common and economical, where the sequenced products can be subjected directly for further bioinformatics studies.

2.5 Molecular approaches in unraveling the taxonomy of *Kappaphycus* and *Eucheuma*

The taxonomy of *Kappaphycus* and *Euchuema* is confusing and challenging to resolve due to the lack of distinct characteristics, high morphological plasticity and also the misuse of commercial names by local farmers (Zuccarello et al., 2006; Tan et al., 2012). This has led to the use of molecular approaches in addressing the taxonomy-assciated issues pertaining to these carrageenophytes. The most common method used to elucidate the taxonomy and systematic of the these Eucheumatoids are via DNA sequencing of specific genetic regions such as at mitochondrial partial *cox*1 and the *cox*2–3 spacer genetic region; nuclear ribosomal internal transcribed spacer (ITS) and partial 28S large subunit; plastid *rbc*L, RuBisCO spacer and 23S universal plastid amplicon (UPA) (Conklin et al., 2009; Tan et al., 2012; Zhao & He, 2011; Zuccarello et al., 2006). Other commonly used genetic markers for the study of algae are shown in Table 2.1.

Zuccarrello and co-workers (2006) has conducted preliminary molecular systematic studies on *Kappaphycus* and *Eucheuma* using the *cox*2-3 spacer and RuBisCO spacer genetic markers, and have analyzed a large number of *Kappaphycus* and *Euchuema*,

be it cultivated and wild ones, from different countries: Philippines, Venezuela, Panama, Indonesia, Madagascar, Tanzania, Vietnam, Hawaii, Mauritius, United States of America and Kenya. This study has proven genetic markers to be feasible in phylogenetic reconstruction, and have provided valuable insights into the taxonomy of both *Kappaphycus* and *Eucheuma*. Tan et al. (2012), with the use of the *cox*2-3 spacer and RuBisCO spacer DNA markers, have shown that local Malaysian variety names lack support by molecular results, regardless of the variation in morphology or color.

Another technique that is used for taxonomy study of *Kappaphycus* and *Euchuema* is random amplified polymorphic DNA (RAPD) technique (Dang et al., 2008). Although not as popular as that of molecular markers, it managed to differentiate the different strains of *K. alvarezii*, *K. striatum* and *E. denticulatum* cultivated in Vietnam.

Table 2.1: A summary of nucleic acid regions used in the taxonomic studies of Algae.

Sequence region	Species/Genus/Family	Taxon/ division	References
*rbc*L	*Asterococcus*	Chlorophyta	Nakazawa et al., 2004
*rbc*L	*Prasiolales* Ulva	Chlorophyta	Sherwood et al., 2000
*rbc*L	*Pyramimonas*	Chlorophyta	Ciarán J.Loughnane et al., 2008
*rbc*L	Bryopsidaceae	Chlorophyta	Wilson and Ruenness., 1994
*rbc*L	Zygnemophyceae	Chlorophyta	Woolcott et al., 2000
*rbc*L	*Dictyotales*	Chlorophyta	Lewis & Mccourt, 2004
*rbc*L	Fucales	Phaeophyceae	Sun et al., 2012
*rbc*L	Ralfsiales	Phaeophyta	Rousseau et al., 1997
*rbc*L	Sphacelariales	Phaeophyceae	Lim et al., 2007
*rbc*L	Chorda	Phaeophyceae	Lin et al., 2001a
*rbc*L	Phaeophyceae	Phaeophyta	Gurgel & Fredericq, 2004
*rbc*L	*Chondracanthus*	Phaeophyta	Lin et al., 2001a
*rbc*L	*Botryocladia, Irvinea*	Rhodophyta	Schneider & Lane, 2005
	Gelidiales	Rhodophyta	Wilkes et al., 2006
*rbc*L	Gigartinaceae	Rhodophyta	Daugbjerg et al., 2000)
*rbc*L	*Gelidiales*	Rhodophyta	Hommersand et al., 1994
*rbc*L	Solieriaceae	Rhodophyta	Freshwater et al., 1999
*rbc*L	Gelidiales	Rhodophyta	Gavio & Fredericq, 2002
*rbc*L		Rhodophyta	Freshwater & Bailey, 1998

Table 2.1 (continued).

Sequence region	**Species/Genus/Family**	**Taxon/ division**	**References**
*rbc*L	*Acanthopeltis* and	Rhodophyta	Shimada et al., 1999
	Yatabella	Rhodophyta	Mclovr et al.,,2002
	Osmundae	Rhodophyta	Wang et al., 2000
*rbc*L	*Grateloupia catenata*	Rhodophyta	Vis & Entwistle et al., 2000
*rbc*L	Batachospermales	Rhodophyta	Draisma et al., 2001
*rbc*L	Deleseriaceae	Rhodophyta	Draisma et al., 2002
*rbc*L	*Opephyllum martensii*	Rhodophyta	Draisma et al., 2002
*rbc*L	Gracilariopsis	Rhodophyta	Gurgel et al., 2003 d
*rbc*L	Gracilariaceae	Rhodophyta	Zuccarello & Lokhorst (2005)
*rbc*L	Ceramiaceae	Rhodophyta	Daugbjerg & Guillou, 2001
*rbc*L	Rhodymeniales	Xanthopyceae	Gontcharov, 2003
*rbc*L	*Tribonema*	Heterokontophyta	Lange et al., 1994
SSU rRNA	Bolidophyceae	Streptophyta	Goff et al., 2004
18S rRNA	*Zygnemophyceae*	Prymnesiophyta	Adachi & Hasegawa., 1996
5.8S rRNA	*Phaeocystis*	Rhodophyta	Touzet et al., 2007
5.8S rRNA	*Gracilariopsis* and *Gracilaria*	Rhodophyta	Rousseae et al., 2000
LSU rRNA	*Alexandrium*	Dinophyceae	Lin et al., 2001b
	Alexandrium andersoni and *A.minutum*		

Table 2.1 (continued).

Sequence region	Species/Genus/Family	Taxon/ division	References
ITS	Delesseriaceae	Rhodophyta	Lin et al., 2002
ITS	*Gracilariopsis* and *Gracilaria*	Rhodophyta	Goff et al., 1994
ITS	*Alexandrium*	Chlorophyta	Adachi et al., 1996
ITS	*Caulerpa* species	Rhodophyta	Pillmann et al., 1997
ITS	*Batrachospermum gelatinosum*	Phaeophyta	Vis & Sheath, 1997
ITS	*Laminarionema elsbetiae*	Phaeophyta	Peters & Burkhardt, 1998
ITS	*Scytothamnale*	Rhodophyta	Lin et al., 2002
ITS	*Chorda* Nizymeniaceae	Chlorophyta	Peters, 1998
ITS	*Scytosiphon lomentaria*	Phaeophyta	Chiovitti et al., 1995
ITS 1	*Poryra suborbiculata, P. caroliensis* and *P. lilliputiana*	Phaeophyta	Cho et al., 2007
ITS 1	Gracilariaceae *Caulerpa racemosa Gigartina* and *Sarcothalia*	Rhodophyta	Tai et al., 2001
ITS 1		Rhodophyta	Broom et al., 2002
ITS 2		Rhodophyta	Bellorin et al., 2002
ITS 2		Rhodophyta	Famá et al., 2000

Table 2.1 (continued).

Sequence region	**Species/Genus/Family**	**Taxon/ division**	**References**
ITS 2	*Phaeocystis*	Prymnesiophyta	Hughey et al., 2001
Rubisco spacer	*Plocamium*	Rhodophyta	Lange et al., 2002
Rubisco spacer	*Ulva conglobata* and *U. Pertusa*	Phaeophyceae	Yano et al.,2004
Rubisco spacer	*Gracilaria verrucosa*	Rhodophyta	Kang & Lee, 2002
Rubisco spacer	*Gracilariopsis* and *Gracilaria*	Rhodophyta	Destombe & Doughlas, 1991
Rubisco spacer	*Gracilaria*	Rhodophyta	Hughey et al., 2001
Rubisco spacer	*Sphacelariales*	Phaeophyta Prymnesiophyta	Fujiwara et al., 2001 Draisma et al., 2002
Rubisco spacer	Phaeocystis	Phaeophyta	Lange et al., 2002
*psa*A, *psb*A *psb*A	Phaeophyceae	Rhodophytes,	Lee et al., 2002
*psa*A, *psb*A *cox*2-3	*Camplylaephora borealis*	Rhodophyta	Yoon et al., 2002
spacer *cox*1	*Griffithsia*	Rhodophyta	Seo et al., 2003
*cox*1	*Bostrychia calliptera, B. pinnata*	Rhodophyta	Yang & Boo, 2003
	Gracilaria changii	Rhodophyta	Yow et al., 2011
	Mastocarpus stellatus and *Gracilaria*	Rhodophyta	Robba et al., 2005

CHAPTER 3: MATERIALS AND METHODS

3.1 Sample collection

The samples of *Kappaphycus* (Doty) Doty ex P.C Silva and *Eucheuma* J. Agardh were collected from several localities in Indonesia. These localities are known as active producing seaweed cultivation sites in Indonesia. These locations are shown in Figure 3.1 are as follows, Lombok island: Pengantap (8° 49'37 37" South, 115° 53'58 37" West), Are Guling (8° 54'3 35" South, 116° 10'46 52" West), Srewe (8° 39'36 20" South, 116° 34'59 83" West), Ekas (8° 55'45 04" South, 116° 34'59 83" West), Sumbawa island: Kertasari (8° 43'55 34" South, 116° 47'6 50" West), Pulo Kaung (8° 28'57 62" South, 117° 22'6 15" West), Hu‘u (8° 50'38 73" South, 117° 22'6 17" West) , Wawaroda (8° 36'32 51" South, 118° 25'5 52" West), Bali island: Serangan (8° 49'52 06" South, 115° 6'0 95" West), Nusa Penida (8° 40'51 74" South, 115° 30'9 36" West), Kalimantan: Ketapang (0° 53'7 01" South, 109° 33'37 19" West), Sulawesi: Makassar (5° 8'58 21" South, 119° 26'40 27" West), Sumatra: Lampung (6° 50'48 59" South, 106° 4'15 73" West), Madura (8° 12'32 71" South, 119° 3'44 46" West), Nusa Tenggara Timur: Rote (10° 46'30 59" South, 112° 53'40 10" West), Kupang (10° 21'1 52" South, 112° 40'25 80" West), Irian jaya: Sorong (0° 59'32 06" South, 130°39'12 56" West).

Figure 3.1. *Kappaphycus* and *Eucheuma* sampling locations in Indonesia.

Collected samples were prepared as herbarium and deposited at the University of Malaya Seaweeds and Seagrass Herbarium for future references. For DNA extraction, about 5cm of the thallus of each sample was excised and preserved in silica gel.

3.2 Morphological Observations

The *Kappaphycus* and *Eucheuma* plants collected were washed with filtered seaweater and cleaned of epiphytes and foreign materials prior to the taking of photographs. Samples were ultimately press-dried into herbariums for future referencing. The morphology of collected plants was observed and described, with emphasis placed mainly on the plant color, size, branching patterns etc.

3.3 Molecular Analysis

3.3.1 DNA extraction

Total DNA was extracted with an i-genomic Plant DNA Extraction Mini Kit (iNtRON Biotechnology, Korea) following the manufacturer‘s instructions. First, the 5cm silica gel- preserved sample was grinded with Liquid Nitrogen in a 1.5ml tube using a micropestle. Then lysis buffer (Buffer PG), Enhancer solution, RNase A, and Proteinase K were added. The Enhancer solution functions to increase the transcription level of genes, whereas the other enzymes RNase and Proteinase K degrade RNAs and proteins respectively. The resulting mixture was then incubated at 65˚C for an hour, and vortexed at intervals of 5 to avoid gelation. The resulting solution was then sonicated to break up remaining intact cells. Buffer PPT was added and centrifuged at 13.000rpm for 5 minutes to precipitate the DNA. This was followed by washing of the mixture twice using spin columns. A final DNA elution of 50µl were kept under -20˚C for long term storage.

3.3.2 PCR amplification, purification and sequencing

The DNA successfully isolated from the algae specimen were then amplified with PCR using a Labnet MultiGene TM Gradient Thermalcycler (Labnet, USA). Two molecular markers were used for this study, namely the mitrochondrial-encoded markers *cox*2-3 spacer and *cox*1. The primer sets for *cox*2-3 spacer were (i) *cox*2-for: 5‘-GTACCWTCTTTDRGRRKDAAATGTGATGC-3‘; and (ii) *cox*3-rev: 5‘-GGATCTACWAGATGRAAWGGATGTC-3‘(Zuccarello et al., 1999, 2006). The primers

used for *cox*1 were (i) *cox*1 43F: 5‘-TCAACAAATCATAAAGATATTGGWACT-3‘; and *cox*1 1549R: 5‘-AGGCATTTCTTCAAANGTATGATA-3‘ from Geraldino et al. (2006).

Molecular markers were PCR amplified under the following conditions: 2µl PCR 10x buffer, 2µl each dNTP, 1µl each primer, 1.5µl of genomic DNA and 0.25µl of *Taq* DNA polymerase in a total volume of 50µl. Temperature profiles for PCR amplification is summarized in Table 3.1. Both genetic markers involved an initial denaturation step of 94˚C for 4 minutes, followed by 30-35 cycles of 94˚C denaturation, 50 ˚C extension and 72˚C elongation (1 minute each). A final extension step of 10 minutes was also added. Amplified products were electrophoresed through a 1%, SYBR ® Safe (Invitrogen, USA) stained gel and the desired amplicons were subsequently PCR or gel purified using a LaboPassTM Gel and PCR Purification Kit (Cosmo GenTech, Korea). Purified products were sent to Lucigen (Taiwan) for sequencing.

Table 3.1 PCR parameter for the *cox*1 and *cox*2-3 spacer molecular markers.

Process	***cox*1**			***cox*2-3spacer**		
	Temperature (ºC)	**Duration (Minutes)**	**Number of cycles**	**Temperature (ºC)**	**Duration (Minutes)**	**Number of cycles**
Initial denaturation	94	5		94	4	
Denaturation	94	0.5	35	94	1	30-35
Extension	50	0.5		50	1	
Elongation	72	2		72	1	
Final extension		10		72	10	

3.3.3 Phylogenetic analyses

Sequence results were manually assembled and edited in ChromasPro v1.5 (Technelysium Pty Ltd) and chromatograms were checked to confirm the validity of ambiguous nucleotides. For each marker, a multiple sequence alignment (MSA) which is inclusive of relevant GenBank sequences was generated with ClustalX (Thompson et al., 1994). The substitution models for each marker datasets were generated using Kakusan3 (Tanabe, 2007). The generated models were subsequently used for Maximum Likelihood (ML) and Bayesian Inference (BI) analyses. MP analyses were performed using default PAUP 4.0b10 (Swofford 2003) settings, with a bootstrap replicate of 1,000.

The Maximum likelihood (ML) model was used to construct the most likely tree from the data set using TreeFinder version Oct 2008 (Jobb et al., 2004). An initial phylogram was constructed using the *Reconstruct Phylogeny* preset within TreeFinder. Supports for tree nodes were then determined by bootstrap analysis with 1,000 ML bootstrap replicates (Felsenstein, 1985).

Bayesian Inference was performed using Mr. Bayes v3.1.2 (Huelsenbeck et al., 2001; Ronquist & Huelsenbeck 2003). The Markov chain Monte Carlo (MCMC) method was used with four chains of two independent runs of 2,000,000 generations. Trees were samples every 500^{th} generation, and a final 50% majority-rule consensus tree was outputted. All phylogenetic trees were viewed and analyzed using Treev32.

CHAPTER 4: RESULTS

4.1 Field sampling

Details of 30 *Kappaphycus* (Doty) Doty ex P.C Silva and *Eucheuma* J. Agardh samples collected throughout Indonesia are summarized as Table 4.1, inclusive of relevant GenBank sequences used for phylogenetic analyses.

Table 4.1 Samples used in the *cox*2-3 spacer and *cox*1 molecular analysis.

No	Species	Location	Collection number	Genebank Accession No.		Nature
				cox*2-3 *spacer	***cox*1**	
1	*K. alvarezii* Id110	Pengantap, Lombok	PSM12287-UMSS0422	JX898828	JX898798	Wild
2	*K. alvarezii "green"* Id22	Are Guling, Lombok	PSM1288-UMSS0428	JX898817	JX898787	Cultured
3	*K. alvarezii "brown"* Id26	Are Guling, Lombok	PSM12288-UMSS0428	JX898818	JX898788	Cultured
4	*K. alvarezii* Id32	Teluk Ekas, Lombok	PSM12290-UMSS0434	JX898819	JX898789	Cultured
5	*K. alvarezii* Id37	Teluk Ekas, Lombok	PSM 12290-UMSS0439	JX898820	JX898790	Cultured
6	*K. alvarezii* Id52	Kertasari, Sumbawa	PSM12292-UMSS0454	JX898821	JX898891	Cultured
7	*K. alvarezii* Id53	Kertasari, Sumbawa	PSM12292-UMSS0455	JX898822	JX898892	Cultured
8	*K. alvarezii* Id59	Kertasari, Sumbawa	PSM12293-UMSS0461	JX898823	JX898893	Cultured

Table 4.1 (continued).

No	Species	Location	Collection number	Genebank Accession No.		Nature
				***cox*2-3 spacer**	***cox*1**	
9	*K. alvarezii* Id72	Hu'u, Sumbawa	PSM12295-UMSS0474	JX898824	JX898894	Cultured
10	*K. alvarezii* Id81	Wawaroda, Sumbawa	PSM12296-UMSS0483	JX898825	JX898895	Cultured
11	*K. alvarezii* Id85	Wawaroda, Sumbawa Kalimantan	PSM12296-UMSS0487	JX898826	JX898896	Cultured
12	*K. alvarezii* Id89	Wawaroda, Sumbawa	PSM12296-UMSS0491	JX898827	JX898897	Cultured
13	*K. alvarezii "green"* Idl11	Rote, Nusa Tenggara Timur	PSM12301-UMSS0497	JX898828	JX898898	Cultured
14	*K. alvarezii "brown"* Idl12	Rote, Nusa Tenggara Timur	PSM12302-UMSS0498	JX898829	JX898899	Cultured
15	*K. alvarezii "dark brown"* Idl13	Rote, Nusa Tenggara Timur	PSM12303-UMSS0499	JX898830	JX898900	Cultured
16	*K. alvarezii* Idl21	Kupang, Nusa Tenggara Timur	PSM12299-UMSS0495	JX898831	JX898901	Cultured
17	*K. alvarezii* Idl32	Makassar, Sulawesi	PSM12306-UMSS0511	JX898832	JX898902	Cultured
18	*K. alvarezii* Idl41	Madura, Java	PSM12308-UMSS0513	JX898833	JX898903	18
19	*K. alvarezii* Idl62	Ketapang, Kalimantan	PSM12305-UMSS0513	JX898834	JX898904	19

Table 4.1 (continued).

No	Species	Location	Collection number	Genebank Accession No.		Nature
				***cox*2-3 spacer**	***cox*1**	
20	*K. alvarezii* Idl71	Lampung, Sumatra	PSM12306-UMSS0515	JX898835	JX898905	20
21	*K. striatum* Idl55	Kertasari, Sumbawa	PSM12292-UMSS0457	JX898836	JX898906	21
22	*K. striatum* Id101	Pulau Serangan, Bali	PSM12297-UMSS0493	JX898837	JX898907	22
23	*K. striatum* Id106	Nusa Penida, Bali	PSM12297-UMSS0494	JX898838	JX898908	23
24	*K. striatum* Id131	Makassar, Sulawesi	PSM12305-UMSS0510	JX898839	JX898909	Cultured
25	*K. striatum* Id181	Sorong, Papua	PSM12307-UMSS0516	JX898840	JX898910	Cultured
26	*K. striatum* Id106	Nusa Penida, Bali	PSM12297-UMSS0494	JX898841	JX898911	Cultured
27	*E. denticulatum* Id23	Are Guling, Lombok	PSM12288-UMSS0425	JX898842	JX898912	Wild
28	*E. denticulatum* Id43	Srewe, Lombok	PSM12291-UMSS0445	JX898843	JX898913	Wild
29	*E.denticulatum* Id133	Makassar, Sulawesi	PSM12307-UMSS0512	JX898844	JX898914	Wild
30	*E. denticulatum Id152*	Poto Tano, Sumbawa	PSM12304-UMSS0509	JX898845	JX898915	Wild

Table 4.1 (continued).

No	Species (Genbank Samples)	Location	Collection number	Genebank Accession No.		Nature
				***cox*2-3 spacer**	***cox*1**	
31	*Kappaphycus alvarezii* 13 *–Buaya*	Sabangkat, Sabah, Malaysia	PSM11996-UMSS0144	-	-	Tan
32	*Kappaphycus alvarezii* 18 *"Tambalang Giant"*	Sabangkat, Sabah, Malaysia	PSM12008-UMSS0164	-	-	Tan
33	*K. alvarezii* isolate E57	Venezuela	-	AY687427	-	Zuc
34	*K. alvarezii*	Madagaskar	-	AY687430	-	Zuc
35	*K.alvarezii* isolate 2614	Moku o Loe, Oahu, USA	-	FJ554862	FJ554853	Zuc
36	*K. alvarezii* isolate Reef4	Paje-Jambiani, Tanzania	-	JQ713901	-	Zuc
37	*K. alvarezii* Isolate UR13	Tanzania	-	JQ13902	-	Zuc
38	*K. alvarezii* 28 *"Tambalang Brown"*	Sabangkat, Sabah, Malaysia	-	JN663764	-	Tan
39	*K. alvarezii* 37 *"Tambalang green"*	Sabangkat, Sabah, Malaysia	-	JN663770	-	Tan
40	*K. alvarezii* 53 *"Tangan- tangan"*	Omadal, Sabah, Malaysia	-	JN663773	-	Tan

Table 4.1 (continued).

No	Species	Location	Collection number	Genebank Accession No.		Nature
				***cox*2-3 spacer**	***cox*1**	
41	*K. alvarezii* 103	Sabangkat, Sabah, Malaysia	-	JN663776	-	Tan
42	*K.* sp. isolate 919	Moku o Loe, USA	-	FJ554860	-	Zuc
43	*K. alvarezii* isolate E57	Kaneohe Bay, Oahu, USA	-	AY687432	-	Zuc
44	*K. alvarezii* isolate E71	Hawaii	-	AY687433	-	Zuc
45	*Kappaphycus* sp. 3955	Kaneohe Bay, Oahu, USA	-	FJ558461	FJ554857	Zuc
46	*K. striatum* E48	S.W, Sulawesi, Indonesia	-	AY687431	-	Zuc
47	*K.striatum* 60 *"green Flower"*	Bum-bum Island, Sabah, Malaysia	-	JN663778	-	Zuc
48	*K. striatum "yellow flower"*	Sabangkat, Sabah, Malaysia	-	JN663779	-	Tan
49	*K.striatum* 31	Sabangkat, Sabah, Malaysia	-	JN663780	-	Tan
50	*K.striatum* 83	Sabangkat, Sabah,	-	JN663781	-	Tan

Table 4.1 (continued).

No	Species (Genbank Samples)	Location	Collection number	Genebank Accession No.		Nature
				***cox*2-3 spacer**	***cox*1**	
51	*K. striatum* 105	Sabangkat, Sabah, Malaysia	-	JN663783	-	Tan
52	*K. striatum* isolate E117	Maratua island, Indonesia	-	AY687435	-	Zuc
53	*K. striatum* E89	Jolo, Philippines	-	AY687434	-	Zuc
54	*K. cottonii* isolate E108	Panglao, Bobol, Philippines	-	AY867426	-	Zuc
55	*K. cottonii* AOL186gz	Philippines	-	-	EU334417	Mon
56	*Kappaphycus* sp. Isolate 3954	Kaneohe Bay, Oahu, USA	-	-	FJ554856	Con
57	*Kappaphycus* sp. Isolate 3956	Kaawa, Oahu, USA	-	-	FJ554858	Con
58	*Kappaphycus* sp. Isolate 3957	Kaawa, Oahu, USA	-	-	FJ554854	Con
59	*Kappaphycus* sp HRM56gz *"Tambalang*	Philippines	-	-	EU334415	Mon
60	*Kappaphycus* sp. HRM11gz *"Bola-bola"*	Philippines	-	-	EU334416	Mon

Table 4.1 (continued).

No	Species (Genbank Samples)	Location	Collection number	Genebank Accession No.		Nature
				***cox*2-3 spacer**	***cox*1**	
62	*Kappaphycus* sp.14 *"aring aring"*	Sabangkat, Sabah, Malaysia	-	JN663784	-	Tan
63	*Kappaphycus* sp. 49 *"aring aring"*	Omadal, Sabah, Malaysia	-	JN663785	-	Tan
64	*Kappaphycus* sp.93 *"aring aring"*	Sabangkat, Sabah, Malaysia	-	JN663786	-	Tan
65	*E. denticulatum*	Indonesia	-	AY687437	-	Zuc
66	*E. denticulatum*	Indonesia	-	AY687429	-	Zuc
67	*E. denticulatum*	Mauritius	-	AY687439	-	Zuc
68	*E. denticulatum*	Tanzania	-	AYG87438	-	Zuc
69	*E. denticulatum*	Madagascar	-	AY687428	-	Zuc
70	*E. platycladum*	Tanzania	-	AY687423	-	Zuc
71	*E. platycladum*	South Korea	-	AY687422	-	Zuc
72	*Eucheuma sp.*	Tanzania	-	AY687424	-	Zuc
73	*Eucheuma sp.*	Hawaii	-	AY687425	-	Zuc
74	*B. phillipinensis* Isolate *E118*	Philippines	-	AY687417	-	Zuc
75	*Eucheuma sp.*	Kenya	-	AY687418	-	Zuc
76	*E. isiforme* isolate E2	-	-	AY687421	-	Zuc

Table 4.1 (continued).

No	Species (Genbank Samples)	Location	Collection number	Genebank Accession No.		Nature
				***cox*2-3 spacer**	***cox*1**	
77	*E. isiforme* isolate E37	Summerland Key, Florida	-	AY687419	-	Zuc
78	*E. isiforme*	Florida	-	AY687420	-	Zuc

Con Conklin et al., (2009), Llu Lluisma et al., (unpublished), Mon Montes et al., (2008), Zuc Zuccarello et al., (2006), Tan Ji et al., (2012).

4.2 Morphological studies

The representative samples collected for this study are shown in Figure 4.1 to 4.5. Most of the samples collected turned out to be *Kappaphycus alvarezii*, which generally possess thalli that are fleshy or cartilaginous, ranging from greenish yellow to brownish red. The *K. alvarezii Tambalang* variety, much like those farmed in other countries, is the most preferred cultivar by seaweed farmers. Often observed with dense and cartilaginous thalli, this particular variety grows rapidly, with dense, inderterminate branchings. Degree of branching is often up to tertiary, where branching patterns range from irregular to unidirectional, depending on sunlight direction. Branch apices are numerous, and mostly flexuous and pointed. *Tambalang* varieties are usually identified based on color variations, i.e. *Tambalang* Green (Figure 4.1) and *Tambalang* Brown (Figure 4.2). The *Moumere* variety of *K. alvarezii* (Figure 4.3) is generally known for the blunt protrusions occurring throughout the thalli surface, which are particularly apparent on the main axis and primary branches. This feature resembled those observed in the *Buaya* varieties reported from Malaysia by Tan et al., (2012). The *Moumere* variety generally displays cylindrical thalli that are uneven and robust. Owing to the relatively high biomass, the *Moumere* plants were also preferred as alternatives to the local *Tambalang* varieties. Branchings were indeterminate and somewhat sympodial, with branching degrees up to tertiary and rarely quaternary. Terminal branches irregular to unidirectional, and are mostly pointed or tapering.

Kappaphycus striatum plants often occur in somewhat isodiametric, dorsally symmetrical, cauliflower-like bunches, hence the local name *Flower* (Figure 4.4). The

morphology of *K. striatum* cultivars are easily recognized, and local varieties are often named merely in accordance to color differences- shades of green to greenish yellow to brown. Thalli of plants are cylindrical, fleshy and robust. Thalli surface mostly smooth, with certain samples occasionally displaying scattering blunt protrusions on primary branches. The main axis is often short and apparent, which radiate outwards into primary branches. Degree of branching was relatively higher compared to *K. alvarezii*, recording up to a magnitude of 5 or 6 degrees. Branchings were frequent (less than 2cm apart) and somewhat predicable; and mostly angular, either bifurcating or trifurcating. Terminal branches are often short and tiny, with minor bifurcating or trifurcating patterns.

Eucheuma denticulatum (locally called *E. spinosum)* are generally smaller sized compared to *Kappaphycus*. The main characteristics which differentiate *Eucheuma denticulatum* (Figure 4.5) from *Kappaphycus* cultivars are the pinnate or pectinate, laterally-occurring spines growing predominantly on the main axis or primary branches. The commercial name *Spinosum* was coined because of the occurrences of these spines. Thalli of *E. denticulatum* plants are often cylindrical, tiny, rough, crisp and brittle. Thalli color often in shades of brown. Main axis apparent. Branching sympodial and regular. Most spines presumably determinate, whereas those that are indeterminate will arise as predominant primary or secondary branches. Spines may vary from pointed to rounded.

Figure 4.1 Indonesian *Kappaphycus* variety; *Kappaphycus* "*Tambalang* green‖. Are Guling, Lombok (scale: 3cm).

Figure 4.2 Indonesian *Kappaphycus* variety; *Kappaphycus –Tambalang* brown‖. Kertasari, Sumbawa (scale: 3cm).

Figure 4.3 Indonesian *Kappaphycus* variety; *Kappaphycus –Maumere*‖. Rote, Nusa Tenggara Timur (scale: 3cm).

Figure 4.4 Indonesian *Kappaphycus* variety; *Kappaphycus "flower* green‖. Nusa Penida, Bali (scale: 3cm).

Figure 4.5 Indonesian *Eucheuma* variety; *Eucheuma spinosum.* Makassar, Sulawesi (scale: 3cm).

Table 4.2 Morphological observations of *Kappaphycus* and *Eucheuma* sampled from Indonesia coastal areas.

Sample	Variant	Location	Nature	Characteristics nature		
				Color	**Maximum height**	**Branching pattern**
K. alvarezii	*Tambalang* green Id10	Pengantap, Lombok	Wild	Green	36cm	Thalli fleshy, cartilaginous and cylindrical (diameter < 1cm). Surface smooth to uneven. Holdfast not observed. Main axis generally sympodial. Branching open, up to tertiary degree, irregular to unidirectional. Branches indeterminate, gradually thinning with each subsequent degree of branching. Terminal branches often slender and tapering, occurring either singularly, or as bifurcates.
	Tambalang green Id22	Are Guling, Lombok	Cultivated	Green	42cm	
	Tambalang brown Id26	Are Guling, Lombok	Cultivated	Green	33cm	
	Tambalang brown Id32	Teluk Ekas, Lombok	Cultivated	Brown	39cm	
	Tambalang brown Id37	Teluk Ekas, Lombok	Cultivated	Green	45cm	
	Tambalang green Id52	Kertasari, Sumbawa	Cultivated	Brown	33cm	
	Tambalang brown Id53	Kertasari, Sumbawa	Cultivated	Brown	31cm	
	Tambalang Isolate Id59	Kertasari, Sumbawa	Cultivated	Green	29cm	
	Tambalang brown Id72	Hu‘u, Sumbawa	Cultivated	Brown	36cm	
	Tambalang brown Id81	Wawaroda, Sumbawa	Cultivated	Brown	32cm	

Table 4.2 (continued)

Sample	Variant	Location	Nature	Characteristics nature		
				Color	Maximum height	Branching pattern
K. alvarezii	*Tambalang* brown Id85	Wawaroda, Sumbawa	Wild	Brown	36cm	Thalli fleshy, cartilaginous, robust and mostly cylindrical (diameter < 1.5 cm). Thalli surface may range from smooth to rough, with occasional blunt protrusion throughout. Color generally dark brown or brown, with occasional shades of green. Holdfast not observed. Main axis apparent and may be sympodial. Primary branches dominant, with irregular, and somewhat short.
	Tambalang brown Id89	Wawaroda, Sumbawa	Cultivated	Brown	37cm	
	Maumere Id111	Rote, Nusa Tenggara Timur	Cultivated	Green	45cm	
	Maumere Id112	Rote, Nusa Tenggara Timur	Cultivated	Brown	44cm	
	Maumere Id113	Rote, Nusa Tenggara Timur	Cultivated	Brown	43cm	
	Maumere Id121	Kupang,Nusa Tenggara Timur	Cultivated	Brown	40cm	
	Tambalang brown Id81	Wawaroda, Sumbawa	Cultivated	Brown	32cm	
	Tambalang brown Id85	Wawaroda, Sumbawa	Cultivated	Brown	33cm	
	Tambalang brown Id89	Wawaroda, Sumbawa	Cultivated	Brown	37cm	

Table 4.2 (continued)

Sample	Variant	Location	Nature	Characteristics nature		
				Color	**Maximum height**	**Branching pattern**
	Maumere Id111	Rote, Nusa Tenggara Timur	Cultivated	Green	45cm	lateral, indeterminate branches. Branching degree mostly tertiary, and rarely quaternary. Branch apices flexuous and pointed
	Maumere Id112	Rote, Nusa Tenggara Timur	Cultivated	Brown	44cm	
	Tambalang brown Id89	Wawaroda, Sumbawa	Cultivated	Brown	37cm	
	Maumere Id113	Rote, Nusa Tenggara Timur	Cultivated	Brown	43cm	
	Maumere Id121	Kupang,Nusa Tenggara Timur	Cultivated	Brown	40cm	
	Tambalang green Id132	Makassar, Sulawesi	Cultivated	Green	37cm	
	Tambalang green Id141	Madura, Java	Cultivated	Green	32cm	
	Tambalang green Id162	Ketapang, Kalimantan	Cultivated	Green	31cm	
	Tambalang green Id171	Lampung, Sumatra	Cultivated	Green	30cm	

Table 4.2 (continued)

Sample	Variant	Location	Nature	Characteristics nature		
				Color	**Maximum height**	**Branching pattern**
Kappaphycus striatum	*Flower* green Id155	Kertasari, Sumbawa	Cultivated	Green	25cm	Plant dorsally symmetrical and compact. Thalli fleshy and cylindrical (<1.5cm). Thalli texture often smooth and even. Holdfast not observed. Main axis apparent, but short, furcating into primary branches. Branching dense, compact (<1.5cm apart), and mostly angularly bifurcating. Degrees of branching may be up to 5 or 6 degrees.
	Flower green Id101	Pulau Serangan, Bali	Cultivated	Green	26cm	
	Flower green Id106	Nusa Penida, Bali	Cultivated	Green	23cm	
	Flower green Id131	Makassar, Sulawesi	Cultivated	Green	28cm	
	Flower green Id181	Sorong, Papua	Cultivated	Green	24cm	
	Flower green Id106	Nusa Penida, Bali	Cultivated	Green	22cm	

Table 4.2 (continued)

Sample	Variant	Location	Nature	Characteristics nature		Branching pattern
				Color	Maximum height	
Eucheuma denticulatum	*Spinosum* Id23	Are Guling, Lombok	Cultivated	Green	25cm	Thalli cylindrical (<1cm) brittle and crisp. Holdfast not apparent. Main axis sympodial. Tiny spines occur throughout the thalli surface in a pinnate or pectinate manner. Spines mostly indeterminate, occurring predominantly on the main axis and primary branches. Lateral branches occasionally arise from determinate spines. Branching mostly tertiary, and may be up to quaternary. Spines mostly pointed. Ramuli may be present in whorls
	Spinosum Id43	Srewe, Lombok	Cultivated	Green	26cm	
	Spinosum Id133	Makassar, Sulawesi	Cultivated	Brown	25cm	
	Spinosum Id152	Poto Tano, Sumbawa	Cultivated	Brown	21cm	

In summary, it is difficult to differentiate varieties within the *Kappaphycus* and *Eucheuma* genera, particularly so for *K. alvarezii*. However, differentiation at a species level appeared to be relatively easier and morphologically possible, provided the sample is intact, undamaged and sufficiently large.

4.3 PCR amplification

Both the *cox*1 and *cox*2-3 spacer molecular markers were easily amplified, with a respective size of approximately 350bp and 1500bp based on a 1kb ladder, as shown in Figure 4.6 and 4.7.

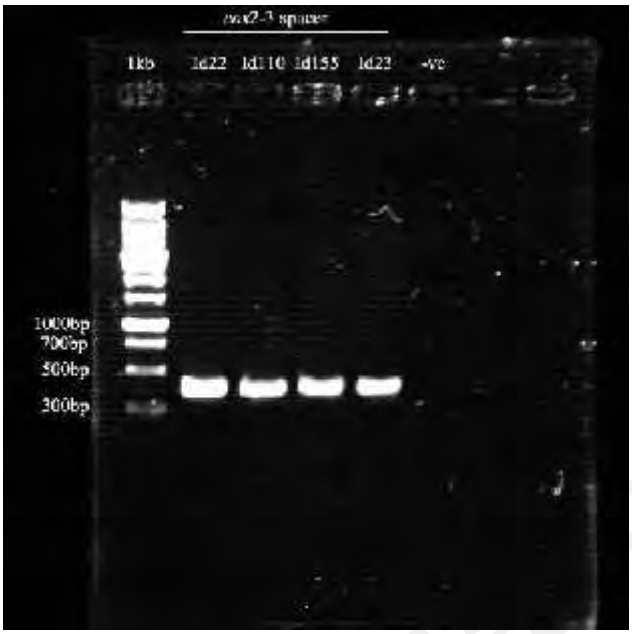

Figure 4.6 . PCR products of the *cox*2-3 spacer (~350bp) for *Kappaphycus alvarezii* (Id22 and Id110), *K. striatum* (Id155) and *Eucheuma denticulatum* (Id23). The negative control is denoted as -ve.

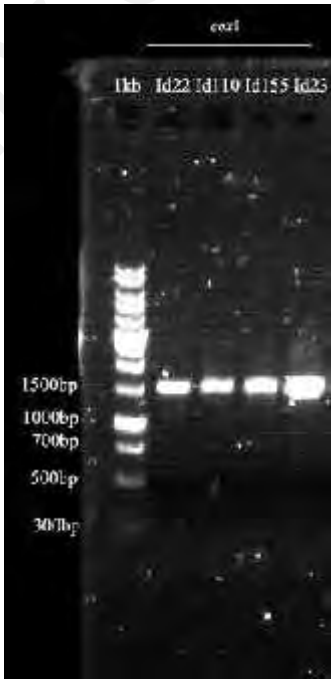

Figure 4.7. PCR products of the *cox1* gene (~1500bp) for *Kappaphycus alvarezii* (Id22 and Id110), *K. striatum* (Id155) and *Eucheuma denticulatum* (Id23).

4.4 Phylogenetic analyses

The total length of aligned sequences used for phylogenetic analyses for *cox*2-3 spacer sequences consisted of 341 bp while COI consisted of 577 bp. For aligned *cox*2-3 spacer sequences, 170 sites were constant, 28 sites were variable and 143 sites were phylogenetically informative. Meanwhile, the aligned partial sequences of COI, 398 sites were constant, 57 sites were variable and 122 sites were phylogenetically informative. The phylogenetic trees based Maximum likelihood (ML), Maximum Parsimony (MP), and Bayesian Inference (BI) are shown in Figure 4.8 for *cox*2-3 spacer and Figure 4.9 for *cox*1.

4.4.1 *cox*2-3 spacer

The *cox*2-3 spacer has shown rather decent results in somewhat phylogenetically delineating members of the genera *Kappaphycus* and *Eucheuma* with the use of a *Solieria* outgroup.

Members of the genus *Kappaphycus* (Clade A- D) were inferred to be genetically distinct from *Eucheuma* (Clade E, F and G). The *K. alvaerzii* clade is divided into two subclades; a moderately supported Clade A1 (ML = 83.9%, MP = 76%, BI=65) which consists of specimens from Indonesia, Venezuela, Tanzania, Hawaii and Malaysia; and Clade A2 (ML = 72.9%, MP= 63%, BI= 94) which is composed of *K. alvarezii* from Africa. Several Hawaiian –*K. alvarezii*‖ specimens were shown by the *cox*2-3 spacer to lack affinity to both *K. alvarezii* and *K. striatum*, constituting a highly supported, but polytomy Clade A3 (ML = 99.6%, MP = 98%, BI = 1.00). All Indonesian

K. alvarezii samples in this study, regardless of the varieties and localities, were grouped in the monophyletic clade A1.

All cultivated *K. striatum* samples collected from Indonesia in this study were inferred to form a monophyletic clade B1 (ML = 79.6%, MP = 61%, BI = -) along with also cultivated *K. striatum* samples from Malaysia and the Philippines. Wild *K. striatum* samples from Malaysia and Indonesia reported respectively in Tan et al., (2012) and Zuccarello et al., (2006) collectively form a genetically distinct Clade B2 (ML = 85.3%, MP = 66%, BI = 0.87) with moderate support.

Kappaphycus sp. *Aring-aring* which were first reported in Malaysia (Tan et al.,2012) were clustered together as a sister taxa to all the aforementioned *Kappaphycus* (Clade C), with a high support of ML = 99.7%, MP = 94%, BI = 0.82. All these *Kappaphycus* species were then inferred to be sister to a lone *Kappaphycus cottonii* from the Philippines (ML = -, MP = 100%, BI = 1.00).

All five Indonesian *E. denticulatum* samples were grouped with another *E. denticulatum* E131 from Indonesia as well as *E. denticulatum –Spinosum*‖ from Malaysia, thus constituting Clade E1 (ML = 90.1%; MP = 84%; BI = 0.55). An *E. denticulatum* sample (E32) originating from Indonesia was also recorded along with *E. denticulatum* samples from Hawaii in the moderately supported Clade E2 (ML = 94.7%; MP = 70%; BI = 0.80). No *E. platycladum* and *Betaphycus philippinensis* were collected from Indonesia in the present study.

4.4.2 *cox*1

The *cox*1 phylogenetic tree was slightly simpler when compared to that of the *cox*2-3 spacer, but the main tree topology remained more or less similar. Again there is a somewhat clear separation between the genera *Kappaphycus* and *Eucheuma* (ML = 59.9%; MP = 76%; BI = 0.95). *Kappaphycus* was inferred to be monophyletic with moderate to high support (ML = 77.2%; MP = 73%; BI = 0.97), and the monophyly of *Eucheuma* strongly supported as well (ML = 100%; MP = 100%; BI = 1.00).

Clade annotations for the *cox*1 phylogenetic tree were synced with that of the *cox*2-3 spacer for easier referencing. Similar to that of the *cox*2-3 spacer, all twenty samples of *K. alvarezii* collected from Indonesia in this study formed a monophyletic group (ML = 93.1%; MP = 99%; BI = 1.00) with a *Kappaphycus* sample from Hawaii, and another one from the Philippines. Clade A3 consisted of *Kappaphycus* collected from the Hawaiian Islands (ML = 99.6%; MP = 100%; BI = 1.00), and was inferred to be sister to *K. alvarezii* in Clade A1.

K. striatum specimens were shown to be monophyletic in Clade B1 (ML = 99.5%; MP = 100%; BI = 0.57), clustering along with a possibly wrongly identified *Kappaphycus* sp. *Tambalang* from the Philippines. Much like that of the *cox*2-3 spacer tree, a lone Philippine sample of *K. cottonii* (AOL186gz) was indicated to be sister to all the other *Kappaphycus* species with moderately high support (ML = 77.2%; MP = 73%; BI = 0.97).

The monophyly of *E. denticulatum* specimens in Clade E1 and E2 were not as conspicuous as observed from *cox*2-3 spacer phylogeny. The former saw all five *E. denticulatum* specimens used in this study grouped together with an *E. denticulatum* (HRM15gz) *Spaghetti* originating from the Philippines; whereas the latter was constituted by an *E. denticulatum* (3953) from Hawaii, and an *E. denticulatum* (AOL053gz) *Endong* from the Philippines.

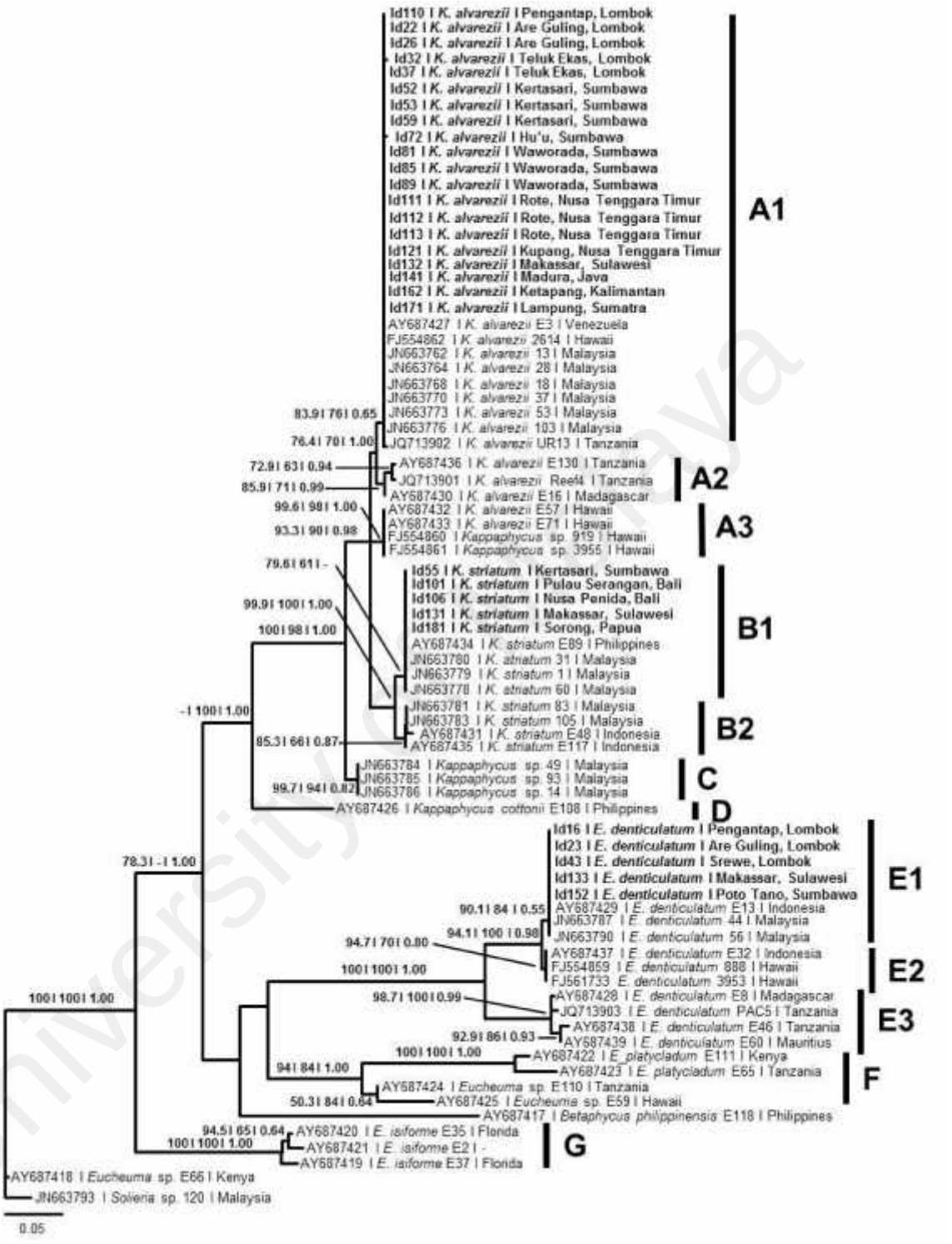

Figure 4.8. Maximum likelihood 50 % majority-rule consensus tree based on the *cox*2–3 spacer marker. -Ln likelihood score was 1,970.507. Substitution rate parameters: TC= 0.458408; TA= 0.020796; TG= 0.020796; CA= 0.020796; CG= 0.020796; AG=0.458408). Numbers at nodes are arranged according to ML bootstrap support/ MP bootstrap support/ Bayesian posterior probabilities. Samples are annotated in an order of sample no. / GenBank accession no. | Sample name | Origin.

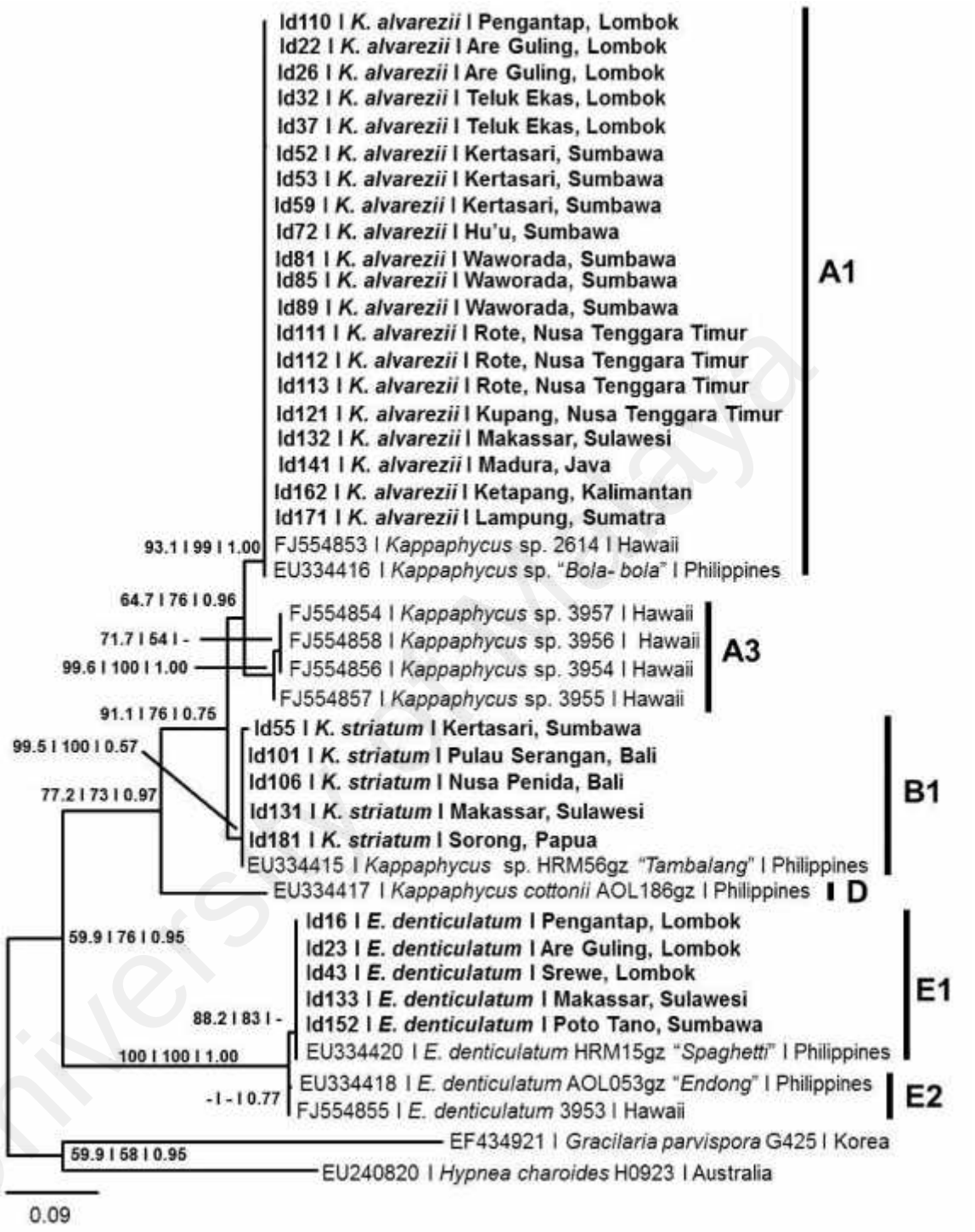

Figure 4.9. Maximum likelihood 50% majority-rule consensus tree based on the *cox*1 marker. -Ln likelihood score was 1,992.384. Substitution rate parameters: TC= 0.489987; TA= 0.067173; TG= 0.012399; CA= 0.067173; CG= 0.012399; AG=0.3508678). Numbers at nodes are arranged according to ML bootstrap support/ MP bootstrap support/ Bayesian posterior probabilities. Samples are annotated in an order of sample no. / GenBank accession no. | Sample name | Origin.

CHAPTER 5: DISCUSSION

5.1 Taxonomic studies based on morphology

The morphology and color variations of the *Kappaphycus* (Doty) Doty ex P.C Silva and *Eucheuma* J. Agardh collected corresponded to the locality. However, intraspecific local varieties were hard, if not impossible to differentiate from one another. Local varieties were shown to be invalid by molecular data (to be discussed later). Despite the challenges in distinguishing local varieties (within species), results from the present study have shown that differentiation at species level was relatively easier. This is especially true when the plants in question are in undamaged conditions. *K. striatum* is relatively compact in size and has angular, denser lateral branches which form a dorsally symmetrical flower-like structure; and can be easily differentiated from *K. alvarezii* which tend to have sympodial, long and open branching patterns, often with relatively lengthier, flexuous and slender terminal branches. *Kappaphycus* can be morphologically distinguished from *Eucheuma* based on the presence of spines as well as cystocarps associated with lateral branches throughout the thalli of *Euchuema*. However the relatively extensive taxa of *Eucheuma* mean that these characters may not always be 100% accurate. The morphological observations in this study were in line with those described on *Kappaphycus* and *Eucheuma* by Doty (1988). The shortcomings of morphological identification entices the application of molecular methods in offering faster and more accurate ways in identifying members of these commercially important carrageenophytes.

5.2 Molecular analyses

5.2.1 DNA isolation and amplification using polymerase chain reaction (PCR)

The *Kappaphycus* and *Eucheuma* samples preserved in silica gel were used for molecular analysis. DNA isolation was first conducted in order to obtain the pure genomic DNA of each sample, omitting other foreign matters which might interfere with DNA amplification.

DNA isolated using the i-genomic Plant DNA Extraction Mini Kit (iNtRON Biotechnology, Korea) were good in DNA yield and gave good PCR amplifications. However DNA isolated directly from herbarium specimens were relatively poorer in terms of concentration, resulting in poor PCR amplifications. This can be ascribed to the degradation of DNA when kept under room conditions. Additionally, *Kappaphycus* and *Eucheuma* herbariums have the tendency to excrete water gradually, thereby increasing the risk of fungal contaminations if herbarium sheets are not maintained dry.

The primer sets for *cox*2-3 spacer and *cox*1 were amplified without much problem as these two DNA regions have been used for the study on *Kappaphycus* and *Eucheuma* (Zuccarello et al., 2006; Conklin et al., 2006).

5.3 Phylogenetic analyses

5.3.1 *cox*2-3 spacer

The DNA marker *cox*2-3 spacer was reported to be effective in molecular systematics due to several important traits, which includes short-lengthand easy amplification, without much compromise on resolution power (Conklin et al., 2009; Tan et al., 2012; Zuccarello et al., 2006). Only the *cox*2-3 spacer was used in the present study instead of the plastid- encoded RuBisCO spacer due to the better resolving capabilities for *Kappaphycus* and *Eucheuma* (Tan et al., 2012; Zuccarello et al., 2006). Resulting phylogenetic trees have shown the *Solieria* outgroup to be the better outgroup as compared to *Betaphycus philippinensis*, offering clearer delineation of *Kappaphycus* and *Eucheuma* taxa (Tan et al., 2012).

Most of the Indonesian specimens were grouped together in the *Kappaphycus alvarezii* clade (Clade A1) along with samples from Columbia, Hawaii, Indonesia, Malaysia, Panama, Philippines, Africa and Vietnam (Conklin et al., 2009; Halling et al., 2012; Tan et al., 2012; Zuccarello et al., 2006). This has shown that there is little genetic distinction between members of Clade A1, which were said to have been originally propagated from the Philippines. No significant association was observed between Indonesian *K. alvarezii* with those constituting Clade A2 and Clade A3, which are to date unique to South Africa and Hawaii respectively. As mentioned by Tan and co-workers (2012), there are no indications as to whether these *K. alvarezii* are valid as different species, particularly so for those residing within Clade A3. Solid morphological studies

would be required in order to delineate a clear species boundary between these taxa.

Even though all *K. striatum* specimens collected in this study were clustered in Clade B1 with samples from Malaysia and Philippines; Zuccarello and co-workers (2006) earlier recorded two genetically different *K. striatum*- isolate E48 and E117 from Kodingareng Keke Island (South West Sulawesi) and Maratua Island (East Kalimantan) respectively. The monophyly of these two samples, along with three wild *K. striatum* (83, 98 and 105) from Malaysia were strongly supported as Clade B2. Although cultivars of this particular genotype are yet to be reported, it appeared that wild populations are common along the Makassar Strait, right up to the Eastern Sabah coastlines. It is possible that the Malaysian reported *Kappaphycus* sp. *Aring-aring* would also be found along the Makkassar Strait due to the sharing of a somewhat similar ecological niche. No *Kappaphycus cottonii* were collected in this study, thereby leaving the taxonomic position of this particular seaweed uncertain.

Eucheuma denticulatum sampled from Indonesia in the present study were all grouped together in Clade E1 along with earlier specimens from Indonesia, Malaysia, Philippines and Africa (Halling et al., 2012; Tan et al., 2012; Zuccarello et al., 2006). Eucheumoids from Clade E1 generally exhibit brittle thalli with pinnate to pectinate branches (also termed spines, where local name *Spinosum* was originally derived), and are different from *E. denticulatum* specimens of Clade E2 which includes several Indonesian samples, amongst others of Hawaiian and Philippine origin. According to Ganzon-Fortes and co-workers (2011), members of Clade E2 are infrequently branched, with smooth, slender terete axes and infrequent whorls of spinous ramuli at

terminal branches. This particular morphotype, which is often called *Endong* in the Philippines, have been described as a rare variety with support by *cox*1 molecular data (Montes et al., 2008), henceforth named *E. denticulatum* (Burman) Collins and Hervey var. endong Trono and Ganzon-Fortes var. nov. Members of both Clade E1 and E2 were inferred to be genetically distinct to the *E. denticulatum* found exclusively in Africa (Clade E3). Additionally, there are no apparent patterns on the distribution of these two *E. Denticulatum* although both can be found around the Lombok, Sumbawa and Sulawesi islands. No other *Eucheuma* species were recorded in this study due to limited samples.

5.3.2 *cox*1

The mitochondrial cytochrome *c* oxidase subunit I was originally used for DNA barcoding in animals. The idea of DNA barcoding was then transferred to plants, fungi or seaweed taxa. Saunders et al., (2005) have shown that the mitochondrial *cox*1 marker can be used to identify species of red algae, which encouraged the establishments of DNA barcode libraries for the rhodophytes. The length of the *cox*1 molecular marker used in this study is approximately 1407bp and was reported to display great potential in picking up closely related species (Brodie et al., 1996, 1998; Brodie & Nielsen, 2005). In this study the *cox*1 dataset were rooted using a *Gracilaria parvispora* EF434921 and *Hypnea charoides* EU240820 outgroup which were deemed sufficiently closely related to the genera *Kappaphycus* and *Eucheuma*.

Due to the relatively smaller dataset of the *cox*1 marker in this context, it is merely used as supplemental data for the *cox*2-3 spacer dataset. However, it is interesting to note that for the current dataset, the Hawaiian –*K. alvarezii*‖ (Clade A3) was inferred with moderate support (ML= 64.7%; MP= 76%; BI= 0.96) to be sister to *K. alvarezii*. This result supported the combined *cox*2-3 spacer and RuBisCO spacer dataset that these Hawaiian specimens were more closely related *to K. alvarezii* rather than *K. striatum* (Zuccarello et al., 2006). The lone *Kappaphycus cottonii* AOL186gz was also shown to be sister to both *K. alvarezii* and *K. striatum*, with moderate to high support via ML, MP and BI analyses (ML= 77.2%; MP= 73%; BI= 0.97); also in agreement with the combined *cox*2-3 spacer and RuBisCO spacer tree by Zuccarello and co-workers (2006). Nevertheless, the current *cox*1 tree topology is expected to change with increment in taxa which will be the main focus in subsequent work to genetically map Indonesian *Kappaphcyus* and *Eucheuma* populations. Although the resolving power of the *cox*1 gene is incompletely assessed at this point due to limited representative species within the dataset, its potential as a phylogenetic marker and DNA barcode is definitely worth looking into in the future.

CHAPTER 6: CONCLUSION

6.1 Conclusion

Results from this study coincide with earlier reports that molecular systematics is an effective means in supplementing morphological data, accurately discriminating between species of *Kappaphycus* (Doty) Doty ex P.C Silva and *Eucheuma* J. Agardh. Despite the relatively small datasets used, but this study included samples from most of the main island in Indonesia namely, Jawa, Sumatra, Lombok, Kalimatan, Sulawesi, Bali, Papua, Nusa Tenggara Barat and Nusa Tenggara Timur while in previous study by Zuccarello *et al.*, 2006 only included mainly around Bali and a few specimens from Sulawesi, Lombok and Maratua Island. This study provides the overview on the cultivated species of *Kappaphycus* and *Eucheuma* in Indonesia. Both the *cox*2-3 spacer and *cox*1 markers displayed satisfactory resolving power in terms of phylogeny, and may be useful as DNA barcodes specific to *Kappaphycus* and *Eucheuma*. However, the present data showed that both the *cox*2-3 spacer and *cox*1 genetic markers were not variable enough to differentiate among varieties, as reported by Zuccarello et al., (2006) and Tan et al., (2012). Further examination using more variable markers is needed to verify the link between genetics and morphological plasticity.

Based on the current study, the following conclusions could be made:

a) The species of *Kappahycus* and *Eucheuma* cultivated in Indonesia are *K. alvarezii*, *K. striatum* and *E. denticulatum*. The local names which were based on morphological features were not supported by molecular analyses.

b) Mitochondrial *cox*2-3 spacer and *cox*1 are suitable for differentiating *Kappaphycus* and *Eucheuma* up to species level and not at variety level.

6.2 Appraisal of the study and areas for future research

This study serves as a preliminary effort in mapping the biodiversity and distribution of cultivated *Kappaphycus* and *Eucheuma* in Indonesia. Even though sampling coverage was rather limited, and mostly involved cultivated species, valuable insights were revealed, particularly significant the limited gene pool of farmed *K. alvarezii*, *K. striatum* and *E. denticulatum*, at least for those cultivated around the Bali, Lombok and Sumbawa islands. However, considering the large amount of cultivated samples collected in this study, additional data on the carrageenan yield and quality of each variety would significantly increase the value of this research, also serving as guidelines for better farming efficiency. Better strains should also be maintained as seedlings, and subjected to further research, with emphasis on improving growth and carrageenan yield.

Albeit the potential taxing challenges, particularly in terms of funding, facilities and logistics; upcoming research should prioritize on sampling of wild specimens around the Indonesian islands. Considering the strategic location of some of the Indonesian islands within the confines of the Coral Triangle, discovery of new species or haplotypes is all but certain. Introduction of these newly discovered carrageenophytes, preferably those with good productivity and robustness would not only increase the overall carrageenan yield of the country, but would also contribute to a larger genetic variation

of farmed species; thus generating higher income for Indonesia and at the same time insuring farmed *Kappaphycus* and *Eucheuma* from loss of fertility or potential disease or pest outbreaks.

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