PHYLOGENETIC ANALYSIS OF *KAPPAPHYCUS* AND *EUCHEUMA* SEAWEEDS BASED ON NUCLEAR, MITOCHONDRIAL AND CHLOROPLAST DNA MARKERS

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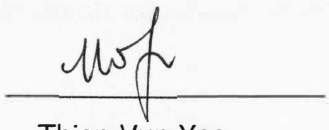

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ABSTRACT

PHYLOGENETIC ANALYSIS OF *KAPPAPHYCUS* AND *EUCHEUMA* SEAWEEDS BASED ON NUCLEAR, MITOCHONDRIAL AND CHLOROPLAST DNA MARKERS

Kappaphycus and *Eucheuma* seaweeds are economically important seaweed species in Sabah and currently are the most widely cultivated seaweed for commercial carrageenan production. The physical morphology of the seaweed is greatly influenced by its genetic make-up and environmental factors which has led to confusion in the taxonomy of the species. This study aims to resolve the phylogeny of *Kappaphycus* and *Eucheuma* seaweeds based on the nuclear, mitochondrial and chloroplast DNA, and to develop specific primers for the identification of *K. alvarezii, K. striatum* and *E. denticulatum*. A total of fifteen phenotypes of *Eucheuma* and *Kappaphycus* seaweeds were collected from Sebangkat Island, Semporna Sabah. Phylogenetic relationship of the mitochondrial encoded *cox*1 gene and *cox*2-3 spacer, the 18S and ITS regions of the nuclear ribosomal DNA and the plastid RuBisCo spacer of the seaweeds were successfully analyzed. The phylogenetic trees distinctly separated the three seaweed species viz. *K. alvarezii, K. striatum* and *E. denticulatum*. The results obtained showed some incongruence with the morphological characters data. It was determined that ITS region was the suitable locus for phylogenetic reconstruction and species differentiation for *Kappaphycus* sp. and *E. denticulatum*. Specific primers for ITS region were designed and developed for rapid and direct identification of *K. alvarezii*, *K. striatum* and *E. denticulatum* using multiplex PCR amplification which is based on species-specific amplicons ranging from 144-415 bp. The ITS region contains enough variation to generate unique identifiers at the species level for *Kappaphycus* sp. and *E. denticulatum*. The outcomes of this study will facilitate in the understanding of the phylogenetic relationship between *Kappaphycus* and *Eucheuma* seaweeds in Sabah.

ABSTRAK

Rumpai laut Kappaphycus dan Eucheuma adalah penting dari segi ekonomi di Sabah dan kini merupakan rumpai laut yang paling luas ditanam untuk pengeluaran karagenan. Morfologi fizikal rumpai laut amat dipengaruhi oleh faktor-faktor genetik dan persekitaran yang telah menyebabkan kekeliruan dalam taksonomi antara spesies. Kajian ini bertujuan untuk mencirikan rumpai laut Kappaphycus dan Eucheuma berdasarkan DNA nuklear, mitokondria dan kloroplas serta menghasilkan primer khusus bagi pengenalpastian K. alvarezii, K. striatum dan E. denticulatum. Sebanyak lima belas rumpai laut Kappaphycus dan Eucheuma yang berbeza dari segi fenotip telah dikumpul dari Pulau Sebangkat, Semporna Sabah. Hubungan filogenetik bagi gen cox1 dan cox2-3 spacer di mitokondria, rantau 18S dan ITS di ribosomal DNA nukleus dan pastid RuBisCo spacer di kloroplas dari rumpai laut telah berjaya dianalisis. Pokok-pokok filogenetik telah mengasingkan rumpai laut K. alvarezii, K. striatum dan E. denticulatum dengan jelas. Keputusan yang diperolehi menunjukkan beberapa ketidakkonsistenan dengan data perwatakan morfologi. Ia telah ditentukan bahawa rantau ITS adalah lokus yang sesuai untuk filogenetik semula dan pembezaan spesis Kapaphycus sp. dan E. denticulatum. Primer khusus berdasarkan rantau ITS telah direka berdasarkan jujukan yang diperolehi dan dihasilkan untuk mempercepatkan pengenalpastian K. alvarezii, K. striatum dan E. denticulatum dengan menggunakan amplifikasi PCR multipleks berdasarkan saiz khusus produk amplifikasi dalam lingkungan 144 – 415 bp. Rantau ITS mengandungi variasi yang cukup untuk menjana petunjuk unik di peringkat spesis untuk Kappaphycus sp. dan E. denticulatum. Hasil kajian ini akan memudahkan pemahaman hunbungan filogenetik antara rumpai laut Kappaphycus dan Eucheuma di Sabah.

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LIST OF SYMBOLS AND ABBREVIATIONS

%	percentage
>	greater than
<	less than
≤	less than or equal to
≈	almost equal to
°C	degree Celsius
x	times
x g	times gravity
µg	microgram
µg/ml	microgram per milliliter
µl	microliter
µM	micromolar
bp	base pair
BS	bootstrap
cm	centimeter
cox	cytochrome oxidase
***cox*1**	cyctochrome oxidase subunit 1
***cox*2**	cyctochrome oxidase subunit 2
***cox*3**	cyctochrome oxidase subunit 3
cpDNA	chloroplast DNA
CTAB	cetyl trimethyl ammonium bromide
DNA	Deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
g	gram
ITS	internal transcribed spacer
kb	kilobase
M	molar
$MgCl_2$	magnesium chloride
ML	maximum likelihood
MP	maximum parsimony
mg	milligram
mg/µl	milligram per microliter
min	minute
ml	milliliter
mM	millimolar
mtDNA	mitochondrial DNA
NJ	Neighbor-joining
ng	nanogram
OTUs	operational taxonomic units
PCR	polymerase chain reaction
RFLP	restriction fragment length polymorphism
RuBisCo	Ribulose-1,5-bisphosphate carboxylase
r	ribosomal
***rbc*L**	large subunits of RuBisCo
***rbc*S**	small subunits of RuBisCo
rDNA	ribosomal DNA
sec	second

sp	species
spp	species
TE	tris-ethylenediaminetetraacetic acid buffer
U	unit
UPGMA	unweighted pair-group method using arithmetic mean
V	voltage
vs	versus

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Seaweeds are macroscopic algae with similar form to higher vascular plants, the only exception being that they do not have true roots, stem or leaves. Their vegetative plant body is called thallus that consists of the holdfast, stipe and blade. Like land plants, seaweeds are photosynthetic plants and in the presence of sunlight and nutrients from seawater, they photosynthesize and produce food. They live either in marine or brackish water environments. As primary producers, they play an essential role in the benthic food web providing habitats for nearshore benthic communities (Mann, 1973). Seaweeds fall into three divisions namely, Chlorophyta (green seaweeds), Phaeophyta (brown seaweeds) and Rhodophyta (red seaweeds) (Chan *et al.*, 2006).

Kappaphycus Doty and *Eucheuma* J. Agard are red seaweeds belonging to the Tribe Eucheumatoideae and are within the Phylum Rhodophyta, Class Rhodophyceae, Subclass Florideophycidae, Order Gigartinales and Family Areschougiaceae. The two genera, *Kappaphycus* and *Eucheuma*, are currently commercially grown for carrageenan (Zemke-White and Smith, 2006). Carrageenan are sulphated linear polysaccharides of D-galactose and 3,6-anhydro-D-galactose (Campo *et al.*, 2009). They have been extensively used in various industries such as food, pharmaceuticals, cosmetics, dairy products and textiles, mostly as gelling, stabilizing, and thickening agents. The total market for carrageenan has been estimated to be approximately US $300 million per year (McHugh, 2003).

Commercial cultivation of seaweed began in the Philippines during the latter half of the 1960s (Doty, 1977). Subsequently, the cultivation was expanded to other countries, most successfully in Tanzania, Vietnam, and some of the Pacific Islands such as Kiribati (McHugh, 2003). Seaweed cultivation was introduced in Sabah in 1978 and is carried out in Semporna, Kunak and Lahad Datu (Goh and

Lee, 2010). In West Malaysia, seaweed cultivation is conducted in places such as Lumut, Merlimau, Langkawi and Terengganu (Daily Mail, October 5, 2010). The seaweed is cultivated by tying young and healthy seedlings loosely with raffia prior to hanging them on the main rope or line. This cultivation method is known as the eco-friendly "tie-tie" method (Doty and Alvarez, 1975). The seaweed is ready to be harvested in 1.5 to 2 months time.

Currently, there are two main species which are being cultivated in Sabah viz *Kappaphycus alvarezii* (Doty) Doty *ex* P. Silva and *Eucheuma denticulatum* N. L. Burman. During the past 10 years (1999 – 2009), seaweed farming in Sabah contributed 7.1 – 29.4 % by volume and 0.84 – 1.2 % wholesale value to the annual marine aquaculture production (Department of Fisheries, 2011). Under the 9th Malaysia Plan, Sabah was predicted to produce 250,000 metric tonnes seaweed by the year 2010. However, its current output is only about 50,000 metric tonnes per year. Therefore, in order for Sabah to develop the capacity to increase seaweed production, researchers are encouraged to apply modern biotechnological processes in order to develop novel technology for the production of high quality seaweed seedlings which feature high gel strength, faster growth rate and resistant to disease (Daily Express, March 28, 2007).

Identification of individual seaweed varieties is important as there are various species and varieties of seaweed that are morphologically similar. Large-scale farming of commercially important *Kappaphycus* and *Eucheuma* species and genetic engineering of desirable strains are ways to ensure a steady supply of quality raw materials to the seaweed industry. The characterization and accurate identification of marine species such as seaweed is an important approach to guarantee the quality of marine products. For species identification of edible seaweeds, approaches based on the recognition of specific protein patterns have been successfully applied. However, some processing method such as cooking can denature the proteins and the patterns obtained from native proteins are not suitable for species diagnosis (Joubert *et al.*, 2009).

The species diagnosis approach undertaken in this study was found is based on characterizing the nuclear ribosomal DNA (rDNA), mitochondrial DNA (mtDNA) and chloroplast DNA (cpDNA) regions of *Kappaphycus* and *Eucheuma* seaweeds. The phylogenetic relationship of *Kappaphycus* and *Eucheuma* seaweeds was resolved based on the DNA sequences obtained. In addition, specific primer pairs were developed to differentiate between the local *Kappaphycus* and *Eucheuma* seaweeds. Specific primer pairs were developed to serve as a diagnostic tool for commercial and wild strains of *Kappaphycus* and *Eucheuma* seaweeds.

1.2 Problem Statement

The taxonomy for *Kappaphycus* and *Eucheuma* are confusing as they are notoriously variable in form. They cannot be distinguished based on one specimen or a collection. Local seaweed farmers identify and describe the seaweeds mainly by observing the physical morphology of the seaweed, which is greatly influenced by both genetic make-up and environment factors. The seaweed farmers differentiate the seaweeds by examining the coloration, branch structure and other morphologies which are influenced by the environments in which they grow. Previous study carried out by Conklin *et al.* (2009) in Hawaii showed that the lack of diagnostic characters in the genus *Eucheuma* has led to the confusion of the distributions and spread of three eucheumatoid species, *K. alvarezii*, *K. striatum* and *E. denticulatum*.

1.3 Objectives

The research objectives of this research are as follows:

a) To characterize the nuclear ribosomal DNA (rDNA), mitochondrial DNA (mtDNA) and chloroplast DNA (cpDNA) loci of *Kappaphycus* and *Eucheuma* seaweeds in Sabah.

b) To determine the phylogenetic relationship of the overall *Kappaphycus* and *Eucheuma* seaweed population in Sabah, on the basis of rDNA, mtDNA and cpDNA loci.

c) To develop specific primer pairs of *Kappaphycus* and *Eucheuma* seaweeds based on rDNA locus and to apply these primers for distinguish between species.

1.4 Significance of Study

There is a lack of molecular taxonomy studies of Sabah seaweed. This is the first molecular data record of local *Kappaphycus* species and *Eucheuma* seaweeds. Molecular markers used in this study can assist in the identification and selection of desired seaweed strain based on DNA fingerprinting. The phylogenetic relationship of *K. alvarezii, K. striatum* and *E. denticulatum* can be resolved based on the characterization of nuclear, mitochondrial and chloroplast markers.

CHAPTER 2

LITERATURE REVIEW

2.1 *Kappaphycus* and *Eucheuma* Seaweeds

Kappaphycus and *Eucheuma* are classified under the Kingdom Plantae and sub-kingdom Biliphyta. They are defined as being members of the Tribe Eucheumatoidea within the Phylum Rhodophyta, Class Florideophyceae, Order Gigartinales and Family Solieriaceae. *Kappaphycus alvarezii, Kappaphycus striatum* and *Eucheuma denticulatum* belong to a group of commercially important species known as the "*Eucheuma* of commerce" (Santos, 1989) and are known in the trade as 'cottonii' (*Kappaphycus* sp.) and 'spinosum' (*E. denticulatum*).

The three species are the main source of *kappa-* and *iota*-carrageenan (McHugh, 2003) and are responsible for about 88 % of worldwide raw materials. *Kappa*-carrageenan is predominantly obtained by extraction of *K. alvarezii* (Rudolph, 2000). Meanwhile, *E. denticulatum* is the main species for the production of *iota*-carrageenan (Campo *et al.*, 2009). The commercial value of these seaweeds is related to the characteristics of the biopolymers that they synthesize, the infrared absorption by their gels has come to be a measure of differences among genera and species (Santos, 1989). Hurtado *et al.* (2008) showed that the carrageenan content and molecular weight of *K. striatum* are affected by its stocking density, culture period and water depth.

Commercial farms are located mainly in the Philippines, China, Indonesia, Malaysia, Tanzania and Kiribati (FAO, 2011) with a total market volume of more than 140,000 tonnes per annum of commercially dried seaweed, with a value of over US $ 70 million in 2008 (Guiry, 2008). Commercial cultivation of *Kappaphycus* and *Eucheuma* only reproduces by vegetative fragmentation. It has not been observed to reproduce sexually in cultivation or in the wild (Smith *et al.*, 2002). Instead, at the tip of each branch is a cluster of apical cells potentially high in

regenerative capabilities that are able to regenerate a new thallus after breaking off. A broken tip can grow into full-sized thalli in a short period of time.

Kappaphycus and *Eucheuma* seaweeds occur naturally throughout the Indo-Pacific region from eastern Africa to Guam, in waters of China and Japan and mostly in algal reef of islands in Southeast Asia (Doty, 1987). They have been introduced to numerous parts of the world for the development of seaweed farming. These introductions of cultivated varieties, primarily from the Philippines or originating in the Philippines, have occurred both inside and outside the native range of the commercial eucheumoids (Ask *et al.*, 2003). According to Zemke-White and Smith (2006), the genus *Kappaphycus* was introduced in 19 tropical countries, whereas *Eucheuma* was introduced into at least 13 tropical countries.

2.1.1 Morphology

K. alvarezii, K. striatum and *E. denticulatum* are tough, fleshy and firm marine seaweeds. Their thallus is not differentiated into root, stem or leaf. *K. alvarezii* and *E. denticulatum* have formed a range of green, red and brown variants (Trono and Lluisma, 1992; Dawes, 1992) but little is known about possible causes of colour differences where as for *K. alvarezii*, Azanza-Corrales (1990) noted that the colours are retained brown, green and red probably vary with the season ambient light.

Branch diameter, branching patterns and thalli texture are the three main differentiating criteria for *Kappaphycus* and *Eucheuma* seaweeds. Despite the irregular branching patterns, *K. alvarezii* tend to exhibit the largest branch diameter, followed by *K. striatum* and *E. denticulatum*. Branching frequency is the highest in *K. striatum*, where the degree of branching may be quinary or more and the length of branches seldom exceeding 2 cm, thus giving a compact, bunch-like overall appearance (Tan *et al.*, 2012).

K. alvarezii and *K. striatum* are morphologically plastic and have variable forms. They have bushy thallus consisting of numerous round branches. They are frequently and irregularly branched, with major branches relatively straight, with lacking or no secondary branches near the tips. The surface can be both rough and

smooth with shiny green to brown or reddish in colors. The normal size is around 20 to 30 cm in diameter. The large plants can grow up to one meter in size.

The difference between *K. alvarezii* and *K. striatum* is that *K. alvarezii* is characterized by its long and cylindrical thallus and sparse branches with sharp pointed tips (Figure 2.1) while *K. striatum* is characterized by stubby and thick cylindrical branches with blunt and forked tips, which resemble a cauliflower shape (Figure 2.2) (Hurtado *et al.*, 2008).

Figure 2.1: ***Kappaphycus alvarezii.*** (a) Brown form of *K. alvarezii* from Mobassa, Kenya; (b) Introduced *K. alvarezii* from Mobassa, Kenya

Source: www.algaebase.org