GENETIC DIVERSITY OF *GRACILARIA CHANGII* AND *GRACILARIA EDULIS* (GRACILARIACEAE, RHODOPHYTA) IN MALAYSIAN WATERS

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ABSTRACT

Gracilaria is the second largest genus of red algae that is widely distributed from the sub-boreal to tropical waters, and some are endemic to the Southeast Asian countries. *G. changii* (Xia et Abbott) Abbott, Zhang et Xia and *G. edulis* (Gmelin) Silva are potential species for commercialization in Malaysia as they have high yields of good quality agar with high gel strength for production of agarose and food grade agar. There is no cultivation of *Gracilaria changii* and *Gracilaria edulis* in Malaysia and regular sampling at various coastal areas in Peninsular Malaysia has indicated that the wild populations of these species are decreasing. Little is known about intraspecific genetic diversity and phylogeographic distribution of these economically important species despite its potential commercialization. The understanding of genetic diversity in both species will provide valuable information for conservation, plant breeding management and strain selection for cultivation. To assess suitable markers for inferring intraspecific genetic variation of the *G. changii*, the *cox*2-3 spacer and the *cox*1 gene were used on 40 specimens of *G. changii* from Peninsular Malaysia. Seven distinct mitochondrial haplotypes were identified with the *cox*1 gene and three mitochondrial haplotypes with the *cox*2-3 spacer. Intraspecific nucleotide differences ranged from 0-6 bp over 923 bp for the *cox*1 and 0-4 bp over 363 bp for the *cox*2-3 spacer, respectively. The results showed that the mitochondrial marker of the *cox*1 gene is a suitable intraspecific marker as it is more variable compared with the *cox*2-3 spacer. The *cox*1 gene was selected as the marker for the subsequent study which involved larger sampling areas and sampling size on the genetic diversity study of *G. changii* and *G. edulis.* This study presents the first attempt in assessing the intraspecific genetic diversity of *G. changii* and *G. edulis* from Malaysia, selected regions in Southeast Asian countries (Indonesia, Thailand, and Vietnam) and Japan using the mitochondrial marker of the *cox*1 gene. Eight

mitochondrial haplotypes (C1-C8) were revealed from 278 specimens of *G. changii* with nucleotide differences ranging from 0-6 bp (0-0.7%) over 923 bp. 15 mitochondrial haplotypes (E1-E15) were identified from 140 specimens of *G. edulis* varying from 0-20 bp (0-2.4%) over 846 bp. Results showed that haplotype C1 and haplotype E1 were the basal haplotypes of *G. changii* and *G. edulis*, respectively. Populations at Morib (Selangor) or Teluk Pelanduk (Negeri Sembilan) were suggested as probable ancestral populations of *G. changii*; Teluk Pelanduk (Negeri Sembilan), Batu Tengah (Malacca) or Kukup (Johore) as the ancestral populations of *G. edulis*. The specimens of *G. edulis* revealed a higher value of haplotype diversity, *Hd* (0.5291) and nucleotide diversity, *Pi* (0.00318) in comparison to *G. changii* (*Hd* = 0.3755; *Pi* = 0.00055). The lower haplotype diversity in *G. changii* relative to *G. edulis* in Malaysia shows either a more recent introduction or a higher rate of gene flow of *G. changii* relative to *G. edulis*. These species do not share similar histories. Their genetic variation and phylogeographic distributions may have been affected differently by the Malaysian continental drift, and recent colonization events through artificial introductions or natural distributions. The present study showed that the *cox*1 gene is a highly divergent genetic marker. This marker is applicable to resolve intraspecific genetic variation and phylogeographic structure for these ecologically and economically important species.

ABSTRAK

Gracilaria merupakan genus rumpai laut merah kedua terbesar dan mempunyai taburan yang meluas iaitu dari perairan 'sub-boreal' ke perairan tropika. *Gracilaria changii* (Xia et Abbott) Abbott, Zhang et Xia dan *Gracilaria edulis* (Gmelin) Silva berpotensi untuk dikomersilkan di Malaysia kerana pengeluaran hasil agar mereka bukan sahaja tinggi malah amat berkualiti dan mempunyai daya tahan gel yang tinggi. Namun begitu, tiada aktiviti penanaman *G. changii* dan *G. edulis* dilaksanakan di Malaysia. Penyampelan yang kerap di pelbagai kawasan pesisiran di Semenanjung Malaysia mendapati bahawa populasi semulajadi spesis-spesis tersebut sedang menyusut. Butiran mengenai kepelbagaian genetik intraspesifik bagi kedua-dua spesis tersebut masih pada peringkat permulaan. Pemahaman kepelbagaian genetik bagi kedua-dua spesis akan memberikan maklumat yang penting untuk pemuliharaan, pengurusan pembiakan dan pemilihan baka untuk penanaman. Kesesuaian gen *cox*2-3 "spacer" dan gen 'cytochrome oxidase subunit 1' (*cox*1) dalam menilai kepelbagaian genetik intraspesifik untuk 40 spesimen *G. changii* di Semenanjung Malaysia telah dijalankan. Tujuh haplotip *cox*1 dan tiga haplotip *cox*2-3 "spacer" telah dikesankan. Pembezaan nukleotida berjulat 0-6 bp daripada 923 bp bagi gen *cox*1 dan 0-4 bp daripada 363 bp bagi *cox*2-3 "spacer". Keputusan kajian menunjukkan bahawa gen *cox*1 adalah penanda intraspesifik yang sesuai dan lebih bervariasi berbanding *cox*2-3 "spacer". Gen *cox*1 telah dipilih sebagai penanda untuk kajian berikutnya yang melibatkan kawasan-kawasan sampel yang lebih banyak dan saiz sampel yang lebih besar kepada kajian kepelbagaian genetik *G. changii* dan *G. edulis.* Kajian ini membentangkan percubaan pertama dalam menilai kepelbagaian genetik intraspesies *G. changii* dan *G. edulis* dari Malaysia, kawasan-kawasan yang dipilih di negara-negara Asia Tenggara (Indonesia, Thailand, dan Vietnam) dan Jepun menggunakan penanda mitokondria gen *cox*1. Lapan haplotip *cox*1

(C1-C8) telah dikesan daripada 278 spesimen *G. changii* dengan perbezaan nukleotida berjulat 0-6 bp (0-0.7%) daripada 923bp. Sebanyak 15 haplotip *cox*1 (E1-E15) ditunjukkan daripada 140 spesimen *G. edulis* dengan perbezaan antara 0-20 bp (0-2.4%) daripada 846 bp. Keputusan menunjukkan bahawa haplotip C1 dan haplotip E1, masing-masing merupakan haplotip basal *G. changii* dan *G. edulis*. Populasi di Morib (Selangor) atau Teluk Pelanduk (Negeri Sembilan) telah dicadangkan sebagai populasi asal bagi *G. changii*; manakala Teluk Pelanduk (Negeri Sembilan), Batu Tengah, (Melaka) atau Kukup (Johor) sebagai populasi asal *G. edulis*. Spesimen *G. edulis* memberikan nilai kepelbagaian haplotip, *Hd* (0.5291) dan kepelbagaian nukleotida, *Pi* (0.00318) yang lebih tinggi berbanding dengan *G. changii* (*Hd* = 0.3755; *Pi* = 0.00055). Kepelbagaian haplotip *G. changii* yang lebih rendah berbanding *G. edulis* di Malaysia menunjukkan sama ada pengenalan yang lebih terkini atau kadar aliran gen *G. changii* yang lebih tinggi berbanding *G. edulis*. Spesis-spesis ini tidak berkongsi sejarah yang sama. Variasi genetik dan pengagihan filogeografi bagi kedua-dua spesis berkemungkinan dipengaruhi oleh hanyutan benua Malaysia, dan kejadian pengkolonian terkini melalui pengenalan tiruan atau pengagihan semula jadi. Kajian ini menunjukkan bahawa gen *cox*1 adalah penanda genetik yang mempunyai kadar evolusi yang tinggi. Penanda molekular ini berupaya mengungkaikan variasi genetik intraspesifik dan struktur filogeografi bagi spesis-spesis tersebut yang mempunyai kepentingan dalam aspek ekologi dan ekonomi.

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LIST OF SYMBOLS AND ABBREVIATIONS

°	Degree
°C	Degree Celsius
θ	Effective population size
π	Nucleotide diversity
%	Percent
cm	Centimeter
g	Gram
g cm^{-2}	Gram per square centimetre
GPS	Global Position System
H_0	Null hypothesis
H_1	Alternative hypothesis
ha	Hectare
kb	Kilobase-pair
km	Kilometer
mg	Milligram
mM	Milimolar
min	Minute
µg mL^{-1}	Microgram per mililiter
µl	Microliter
µm	Micrometer
nm	Nanometer
ng	Nanogram
pmol	Picomolar
Ppt	Parts per thousand
sec	Second
U	Unit

US$	Unites States Dollar
Wt	Weight
w v^{-1}	Weight per volume
A	Adenine
AFLP	Amplified fragment length polymorphism
BI	Bayesian Inference
bp	Base pair
C	Cytosine
*cox*1	Cytochrome oxidase subunit 1
*cox*2-3 spacer	Cytochrome oxidase subunit 2 and 3
*cox*3	Cytochrome oxidase subunit 3
cpDNA	Chloroplast deoxyribonucleic acid
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
G	Guanine
Hd	Haplotype diversity
ISSR	Inter-simple sequence repeat
ITS	Internal transcribed spacer
ML	Maximum Likelihood
mtDNA	Mitochondrial deoxyribonucleic acid
NCBI	National Center of Biotechnology Information
nDNA	Nuclear deoxyribonucleic acid
rDNA	Ribosomal deoxyribonucleic acid
OD	Optical density

PAUP	Phylogenetic Analysis Using Parsimony
PCR	Polymerase chain reaction
Pi	Nucleotide diversity
PP	Posterior probabilities
RAPD	Random amplified polymorphism DNA
*rbc*L	Large subunit of RuBisCO
rDNA	Ribosomal deoxyribonucleic acid
RNA	Ribonucleic acid
RNase	Ribonuclease
RFLP	Restriction fragment length polymorphism
SPN	Statistical parsimony networking
SRFA	Selective restriction fragment amplification
SSCP	Single-Stranded Conformation Polymorphism
SSR	Simple sequence repeats
T	Thymine
TAE	Tris-acetate-EDTA buffer
UHQ	Ultra-High Quality
UV	Ultraviolet

CHAPTER 1

INTRODUCTION

1.1 IMPORTANCE OF SEAWEEDS

Seaweed is a general term that is used to various types of algae that grow in marine waters. Seaweed is constructed fairly simply, a plant-like organism without actual roots, stems, vascular tissue, flowers and leaves. They are ecologically important to sustain the life on earth. Seaweeds are an essential contributor in marine primary production by contributing about 30-50% of the photosynthetic production on earth and form the basis of the ocean food chain, as habitat creators for shelters or protection and weather regulators (van den Hoek et al., 1995; Dhargalkar & Verlecar, 2009).

Seaweeds or marine benthic macroalgae are classified according to pigmentation, flagellar characteristics, cell wall composition and storage products. There are about 10,000 species of seaweed classified into three main groups on the basis of pigmentation; Rhodophyta (red algae, about 6,200 species), Phaeophyta (brown algae, about 1,800 species) and Chlorophyta (green algae, about 1,500 species) (de Almeida Mendes, 2012). All seaweeds contain chlorophyll *a* and possess a variety of accessory and protective photosynthetic pigments.

Seaweeds are valuable to man as a food source and raw material for industrial purposes. According to the evidence from archaeological of Chile, human use of seaweeds probably dates back more than 14,000 years (Karleskint et al., 2009), of which 107 genera and 493 species of seaweeds were used (Tseng, 1981). Coastal populations have used seaweeds as food, fertilizer for agriculture, fodder for livestock as well as in water treatment purpose.

Over 200 seaweed species make a substantial contribution to phycocolloids production and food consumption internationally (Zemke-White & Ohno, 1999) and

seaweed trade was valued at US$ 7.54 billion in 2007 (Hasan & Chakrabarti, 2009). Seaweeds are used in various way for human consumption as food ingredients, production of phycocolloids as additives in fast food preparation (Patarra et al., 2010), for medicinal, pharmaceutical and therapeutically applications, and in cosmetics industries.

In 1964, the "agaroidophyte" was first introduced to describe red algae that yield phycocolloids (agaroid) which are similar in chemical composition but have different physical properties (Diaz-Piferrer, 1964). Phycocolloids are polysaccharides deposit in seaweed cell walls of commercial importance and have been described with antioxidant, antiviral, anticoagulant and antitumoral activities (Mayer & Lehmann, 2001; Mayer & Hamann, 2004; Smit, 2004). Phycocolloids have been applied in the production of medicine for leg ulcers, cough, and in dental impression.

Agar gels have been used to produce sun cream, perfumed deodorant sticks, skin cream containing zinc oxide or penicillin in the cosmetic industries (De Roeck-Holtzhauer, 1991; McHugh, 2003). Agar is also used as laxatitive in the pharmaceutical industry (Armisen & Galatas, 1987; McHugh, 2003). For biotechnological application, agar is used as a culture medium, while agarose is used for electrophoresis of DNA and proteins (Van der Meer & Patwary, 1995).

Seaweeds are used as a fertilizer and soil conditioner since the nineteenth century and also as an animal feed additive for livestock (Indergaard & Østgaard, 1991). With new advancements in science and technology, algae are found to have additional uses for biotechnological applications (Graham & Wilcox, 2000). Some seaweeds possess an excellent capability to remove heavy metals from the water that they are exposed to. These seaweeds have also been applied in biomonitoring and bioremediation (Chandrkrachang et al., 1991; Hamdy, 2000; Alhakawati & Banks, 2004; Kumar et al., 2007; Marinho-Soriano, 2007; Ho et al., 2010; Luqman et al., 2012)

to assist in removing the dissolved inorganic nitrogen and phosphorus in fish farm. Nowadays, research of production of petroleum alternatives is on-going (Rodolfi, 2008; Vonshak, 2008). Large scales of seaweeds are cultivated in the ocean and the harvested seaweed has been fermented to generate methane gas (McHugh, 2003). Some species of *Gracilaria*, *Ulva* and kelps have been proposed as potential candidates for biofuel production (Adams et al., 2008; Hanisak, 2008; Kraan, 2008).

1.2 IMPORTANCE OF GENETIC DIVERSITY STUDIES OF SEAWEEDS

Genetic diversity is one of the implicit components and a fundamental source of biodiversity at all levels, along with species diversity and ecosystem diversity. Genetic diversity within a population has ecological effects on a population (in term of productivity, growth and stability), inter-specific interactions within communities and ecosystem-level processes (Hughes et al., 2008).

Advances in molecular biology provide the tools for measurement of genetic diversity. Measurement of genetic diversity will contribute important clues to understand the forces of nature that act on genetic variation, pattern and level of genetic variation. This information is useful in plant breeding, to determine the best strain to re-establish the exterminated populations and for development of strategies of ex situ conservation of genetic resources (Yow et al., 2011).

Molecular genetic analysis is a potential approach to assess genetic variation between or within a population of a species. It would provide insight into the processes influencing the geographic distribution and evolution of genealogical lineages within a species under study. Measurement of genetic diversity of seaweed with molecular tools has been accelerated with advances in molecular markers technologies. Adoption of

genetic markers for genetic variation studies provide valuable informative data for gene flow, population structure, phylogenetic relationships, phylogeographic studies, and parentage and relatedness analysis (Féral, 2002).

Conservation of genetic diversity is one of the three global conservation priorities, recognised by IUCN (premier international conservation body) and genetic diversity is required for a population in evolution and adaptation to cope the continuous process of environmental change. Distribution of genetic diversity among and within a species is uneven. The evolution potential rate of a population is proportionate to the amount of genetic variation, as genetic diversity represents slight differences in DNA sequences, and protein variation may result in functional biochemical and inextricably cause variation in reproductive rate and fitness, morphological or behaviour of population (Frankham et al., 2004).

Genetic diversity is a crucial component for each individual in a population to the long-term survival and provides considerable adaptation to varying environmental conditions. The development of molecular tools in genetic diversity study and the increase in commercial interest of seaweed drastically stimulates genetic diversity studies, to increase the viability of declining seaweed populations (Bagley et al., 2002).

Genetic diversity is correlated with reproductive fitness, ability to withstand in harsh conditions and survival success. This makes genetic diversity an important study for accurate identification of some commercially important characteristics that may be species-specific or strain-specific to increase yield and improve the strain of the seaweed. Hence, to fully utilize and conserve the seaweeds for economic importance, the cultivar-specific characteristics of seaweeds have to be elucidated using molecular approaches.

According to FAO (2006), in 2004, approximately 4 million wet tons of red algae have been cultivated worldwide with a market value of US$ 1.9 billion.

Gracilariaceae was listed as one of the important groups of seaweeds to be studied for their ecology and the present conditions in Southeast Asian countries by the Japan Society for the Promotion of Science (JSPS) on Coastal Marine Science during the years 2001-2010. *Gracilaria* is the second largest genus of red algae and many of which are ecological and economically important. More than 37,000 dry tons of *Gracilaria* has been harvested globally (Ye et al., 2006) and several species of *Gracilaria* have been documented as sources of food (e.g. *G. changii* and *G. tenuispitata*) for both humans and shellfish (abalone) and as a raw material for agar extraction. *Gracilaria* contains a notable amount of amino acids, fatty acids, bioactive compounds and antibiotic properties (Yow et al., 2011). Likewise, some of *Gracilaria* are useful for bioremediation of aquaculture wastes and as carbon dioxide sink (Muraoka, 2004; Turan, 2009). Some *Gracilaria* are currently proposed as potential bioethanol crops (Hanisak, 2008).

Gracilaria, is harvested from the wild populations of Malaysian waters. The surging global market demand for *Gracilaria* in agar production has increased and resulted in the over-harvesting of wild populations. Geographic distribution of seaweeds from Malaysian waters has been well documented by Du et al. (2008) using GIS. However, the marine biodiversity in Malaysia has been affected relentless from anthropogenic influences and caused the rapid change of marine environment, which lead to species richness decreases (Mazlan et al., 2005). Losses in seaweeds are also attributed to anthropogenic activities through pollution, habitat destruction, over-harvesting, coastal degradation, global climate change and transoceanic introduction of alien species and invasive species. In addition, ecological factors and natural evolutionary processes also affect a range of population, community and ecosystem responses that may generate changes in allele sequences or genotype frequency in seaweeds (Fussmann et al., 2007).

To date, there is no cultivation of *Gracilaria changii* and *Gracilaria edulis* in Malaysia. Several attempts have been made to cultivate *G. changii* on an experimental scale in Peninsular Malaysia, however no successful commercial cultivation scale has yet been implemented (Yow et al., 2013). The yield and quality of *Gracilaria* agar vary with the strains selected, seasonality, and growing conditions. Information on intraspecific genetic diversity of this economically important species is rudimentary (Yow et al., 2011 & Yow et al., 2013). Among the species of *Gracilaria* that are available in Malaysia, *G. changii* (Xia et Abbott) Abbott, Zhang et Xia and *G. edulis* (Gmelin) Silva have potential to be commercialised. The former is found to have high agar strength while *G. edulis* is a species that is widely cultivated in India (Jayasankar & Varghese, 2002). There is therefore an urgent need to study the genetic diversity of *G. changii* and *G. edulis* to provide information for selecting suitable strains (with potential features of high growth rate, high-yield of agar production and capable to survive in a harsh environments) for mariculture. This study might deepen our knowledge on conservation, restoration and agriculture, and improve our understanding of the origin and evolutionary relationship of *Gracilaria changii* and *Gracilaria edulis* from some of the Southeast Asia countries.

1.3 RESEARCH OBJECTIVES

The study is based on two objectives:

1. To compare the suitability of two mitochondrial encoded-markers, the cytochrome oxidase subunit 1 (*cox*1) and intergenic spacer between the cytochrome oxidase subunit 2 and 3 (*cox*2-3 spacer), to infer the genetic diversity variation of *Gracilaria changii* from Peninsular Malaysia.

2. To study the genetic diversity of *Gracilaria changii* and *Gracilaria edulis* from various localities in Malaysia, selected regions in Southeast Asian and Japan using the *cox*1 gene.

The hypotheses to be examined are:

1. Hypotheses for *G. changii*:

   H_0: *G. changii* collected from various localities belong to the same haplotype.

   H_1: *G. changii* collected from various localities belong to different haplotypes.

2. Hypotheses for *G. edulis*:

   H_0: *G. edulis* collected from various localities belong to the same haplotype.

   H_1: *G. edulis* collected from various localities belong to different haplotypes.

CHAPTER 2

LITERATURE REVIEW

2.1 SEAWEED AND ITS COMMERCIAL IMPORTANCE

2.1.1 Potential Application of Seaweed as Food

Seaweeds contain abundant minerals, vitamins and protein, making them useful as a health food to provide nutrition for mankind and other organisms (Rebelloso et al., 2000; Patricia, 2003). Of 221 seaweed species (125 Rhodophtya, 64 Phaeophyta and 32 Chlorophyta) utilized worldwide, about 145 species are used for food consumption (Kaur & Ang, 2009). The red algae (Rhodophyta) with 6000 species distributed worldwide are one of the richest resources for industrial application and used extensively in food, cosmetic and pharmaceutical industries. The use of seaweed as food has been started in the early fourth century and sixth century in Japan and China, respectively. Japan has been reported as a largest consumer and producer (2,440 tonnes in 1984) by Coppens (1990). Xia & Abbott (1987) reported that the Chinese consume over 100 million pounds of fresh and dried seaweeds annually.

Traditionally, seaweeds have been used as a favourable source of human food with high nutritional value, especially in Pacific and Asian countries (e.g. *Porphyra* sp. (*nori*), *Laurencia* and *Osmundea* by Pattara et al., 2010); *Chondrus crispus* (*Irish moss*) in Ireland; *Palmaria palmate* (*dulse*) in England; *Porphyra* (*laver, nori*), *Undaria* (wakame), *Laminaria* (kombu), *Sargassum* and *Nostoc* (fa' tsoi) in Asia (Srimanobhas, 1979; Phang, 2006; Karleskint et al., 2009). Seaweeds are low calorie food with contain a notable amount of polysaccharides, minerals, proteins, vitamins, steroids and dietary fibers (Darcy-Vrillon, 1993; Lahaye, 1993; Dhargalkar & Verlecar, 2009). Utilisation of Malaysian seaweed as food has been well reported by several researchers. Some of the Rhodophyates such as *G. changii*, *G. tenuispitata*, *Gelidium* spp.,

Halymenia formosa, *Hypnea* spp., *Eucheuma cottonii* and *Kappaphycus* spp.; Chlorophytes such as *Caulerpa racemosa*, *Caulerpa lentillifera*, *Chaetomorpha antennina*, *Ulva lactuca*, *Ulva prolifera*; and Phaeophytes like *Sargassum* sp., *Turbinaria conoides* and *Dictyota* spp. are marketed as edible sea-vegetables and eaten either as raw or blanches in salads by Malaysian (Burkill, 1935; Ismail, 1987; Phang, 1987; Zemke-White & Ohno, 1999; Matanjun et al., 2009). In Southeast Asian countries, Indonesia is the largest consumer of seaweeds for food and herbal medicine application. The red algae of *G. edulis* and *G. crassa* have been cultivated in Myanmar for food (Phang, 2006). Besides, seaweeds can be as supplements and poultry feed to farm animals, abalone and fish feed (Mustafa & Nakagawa, 1995; Kaliaperumal, 1999; Schuenhoff et al., 2002). *G. changii* from Malaysia was found to contain high amount of fibres and minerals, with substantial amounts of lipids and proteins (Norziah & Chio, 2000). Seaweed-based food additives are also presently applied in the process of fast food preparation (Patarra et al., 2010).

McHugh (2003) reported that the use of seaweeds as human consumption had increased exponentially and resulted in the development of seaweed cultivation industries to support about 90% of the world market's demand. In addition of being source of food, seaweeds are playing a vital role in production of phycocolloids.

2.1.2 Potential Application of Seaweed as Phycocolloids Production

In 1964, the "agaroidophyte" was first introduced to describe red algae that yield phycocolloids (agaroid) which are similar in chemical composition but have different physical properties (Diaz-Piferrer, 1964). Seaweeds especially the red and brown seaweeds are major sources of phycocolloids. The main types of phycocolloids are agar (agarose), alginate (alginic acid) and carrageenan, which are a major source of industrially important seaweed products (Lewis et al., 1988). They are used as

thickening agents, gelling agents, laxative agent in medicines production, stabilizing agents in foods and cosmetic products, and for producing water-soluble films (Doty, 1973; Armisen, 1995; McHugh, 2003). The important commercial sources of agar are from the red algae of *Gracilaria, Gelidium, Pterocladia, Laurencia flexilis* and *Ahnfeltia*, while carrageenan is from the red algae of *Kappaphycus alvarezii*, *Eucheuma denticulatum, Solieria, Acanthophora spicifera* and *Hypnea*. Alginic acid is extracted from brown algae of *Sargassum, Laminaria, Fucus* and *Ascophyllum* (Abbott, 1996; Ohno & Largo, 1998; Wu, 1998; Guiry & Hesion, 1998; Merrill & Waaland, 1998; Zemke-White & Ohno, 1999; Meena et al., 2007; Villanueva et al., 2010). Carrageenan is mainly extracted from *Kappaphycus alvarezii* and *Eucheuma denticilatum*, which can be used as thickening and stabilizing agents in edible products. It is also used in numerous inedible products such as in toothpaste, cosmetics, lotions and textile paints (Stanley, 1987; Abbott, 1996; Prud'homme & Trono, 2001; Conklin et al., 2009). Alginates are commonly used as viscosifiers in frozen desserts, binders in animal feed products and pharmaceutical products, as stabiliser in paper production and to bind textile printing dyes (McHugh, 1987; Prud'homme & Trono, 2001). Of the seaweed phycocolloids, agar commands the highest price in the world market (Kain & Destombe, 1995) with high demand. The red algal genera principally used for agar extraction are *Gelidium* and *Gracilaria*. Agar is an accepted substitution for gelatine in the food, cosmetic, and pharmaceutical industries. In food for human consumption, agar can be found in confectionary products and used as clarifier in wines and vinegars. It has been suggested as an ideal gel-forming agent owning to has higher gelling temperature and melting point compared to gelatine.

Gracilaria (Gracilariales) is an important agarophyte genus and provides a main source of raw materials for agar production (Srimanobhas, 1979; Oliveira et al., 2000; Troell et al., 2003). *Gracilaria* agar has been reported as the first phycocolloid to be

used in human food production (Armisen, 1995). According to Abbott (1980), unlike *Gelidium*, *Gracilaria* offers considerable potential in vegetative cultivation due to fast growth rate and high yield of agar. *G. changii* has the highest potential for commercialization in Malaysia as it has high quality agar and agarose. Seasonal variation in agar yield and gel strength of *G. changii* from Malaysia was well studied with agar yield ranging from 12 to 25% dry weight and gel strength from 294 to 563 g cm^{-2} (Phang et al., 1996). Agarose yield and gel strength of *G. changii* is 16.3% and 950 g cm^{-2} respectively (Santos & Doty, 1983; Phang et al., 1996). *G. edulis* has been collected from Indonesia, transported and cultured in an outdoor tank in Japan. A high agar yield of 28.3% and gel strength of 580 g cm^{-2} were obtained from this species (Gerung et al., 1999). The extracted agar from *Gracilaria* has many uses. The lower grade agar is used in food products (e.g. frozen foods, fruit juices, dessert gel, and preservation purposes) and industrial applications as adhesives, textile printing, and paper sizing. The medium quality agar is used in biological culture media in pharmaceutical industries, bacteriology, public health laboratories and research laboratories (Gopal, 1979). The highly purified and upper market grades (neutral fractions of agar, called agarose) are used in gel electrophoresis, immunodiffusion and gel chromatography (Cardozo et al., 2006). Agar gels have been widely used in production of sun cream, perfumed deodorant sticks, skin cream containing zinc oxide or penicillin in the cosmetic industries. Agar has been recently employed in pharmaceutical and medical fields such as it can induce the apoptosis of cancer cells *in*-vitro in a therapy against cancer cells (Chen et al., 2004). The wide use of this compound has generated an increasing of scientific and commercial interest.

2.1.3 Potential Application of Seaweed for Medicinal and Pharmaceutical Uses

Prior to the 1950s, the uses of seaweeds for medicinal purpose were restricted (Lincoln et al., 1991). In the last three decades, the discovery of pharmacological properties from algae has increased dramatically (Qi et al., 2005). Many bioactive compounds from seaweeds are of commercial importance and have been described with antioxidant, antiviral, anticoagulant, antitumoral and anti-allergic properties (Mayer & Lehmann, 2001; Mayer & Hamann, 2004; Raman et al., 2004; Smit, 2004; Ye et al., 2008; Wang et al., 2009; O'Sullivan, 2010; de Almeida et al., 2011; Shelar et al., 2012).

According to Sekmokiene et al. (2007), the use of seaweeds has grown rapidly, as seaweeds are a potential ingredient of the health-promoting foods, many of which exhibited high nutritional benefits. Edible seaweeds contain a notable amount of dietary fiber and protein (MacArtain et al., 2007; Mamatha et al., 2007). Eight grams of serving intake of seaweeds may contain up to 12.5% of a person's daily fiber needs (MacArtian et al., 2007), as it is essential in prevention of a number of chronic diseases, e.g. heart disease, cancer, diabetes etc., (Guidel-Urbano & Goni, 2002), in reduction of blood cholesterol (Goni et al., 2000) and in the modulation of blood glucose (Brennan, 2005). Likewise, polysaturated fatty acids extracted from seaweeds (e.g. *Gracilaria* spp., *Palmaria palmata, Sargassum natans, Undaria pinnatifida* and *Laminaria digitata japonica*) have immense benefits for prevention of cardiovascular, inflammatory and nervous system disorders (Nada et al., 2004; van Ginneken et al., 2011; Abdullah, 2013). Bioactive compounds with cytostatic, antibacterial, antiviral, antifungal and antihelmintic properties have been found in red, brown and green algae (Newman et al., 2003; Pereira & Costa-Lotufo, 2012). The brown algae, *Fucus* species were discovered to have a considerable amount of antioxidant properties. Antioxidant compounds from seaweeds are an excellent source for treatment of a range of disorders and against aging processes (Shelar et al., 2012).

The sulphated polysaccharides from the red seaweeds of *Aghardhiella tenera* (Witvrouw et al., 1994), *Nothogenia fastigiata* (Damonte et al., 1994; Kolender et al., 1995), *Gracilaria corticata* (Mazumder et al., 2002), *Caulerpa* sp., *Corallina* sp., *Hypnea charoides*, *Padina arborescens* and *Sargassum patens* (Zhu et al., 2003) have potent antiviral effects against human infectious diseases: human immunodeficiencyvirus (HIV), respiratory syncytial virus (RSV) and herpes simplex virus (HSV) types 1 and 2. Meanwhile, several studies have been carried out to discover the potential antiviral activities from carrageenans (Carlucci et al., 1999; Cáceres et al., 2000) and a carrageenan-based vaginal microbicide called Carraguard has been shown to inhibit HIV and some of the sexually transmitted diseases efficiently (Smit, 2004).

Nutritional, biochemical and physiochemical properties of Malaysian seaweeds had generated an increased interest among researchers. Many of them been screened for mineral content (Krishnaiah et al., 2008), bioactive compounds, (Masuda et al., 2002; Varaippan, 2004; Nazefah, 2006; Wong et al., 2006), lipid and fatty acids (Norazmi, 2001; Chu et al., 2003; Matanjun et al., 2009; Shu et al., 2013). Tropical seaweeds of *Gracilaria* spp., *Hypnea musciformis*, *Dictyopteris* spp., *Eucheuma spinosum* and *Sargassum* spp. have been proven to contain antibiotic compounds. *Acetabularia major* has been used in treatment of gall stone, *Ulva lactuca* for antimicrobial, *Sargassum* for iodine production, *Corallina* and *Ulva pertusa* for antihelminthic (Burkill, 1935; Phang, 1987). *G. changii* from Malaysian waters has been proven to contain natural compounds of anti-ulcerogenic, anti-inflammatory and gastroprotective properties (Shu et al., 2013), as a promising free radical scavenger (Sreenivasan et al., 2007) and a potential chemotherapeutic agent in the treatment of breast cancers (Nazefah, 2006). In a short study reporting on the lipid and fatty acid content of selected Malaysian seaweeds by Norazmi (2001) and Chu et al. (2003), *Dictyota dichotoma* was discovered to contain the highest amount of lipids (17.6% ash-free dry weight), while all of the seaweeds

contained eicosapentanoic acid ranging from 2.4 to 10.7% total fatty acids, with *Gracilaria edulis* having the highest content. According to Abdullah (2013), *G. manilaensis* possesses almost all of the essential fatty acids and could be a potential resource for human consumption.

2.1.4 Potential Application of Seaweeds for Ecological Restoration and Other Uses

Marine macroalgae are promising bioremoval of heavy metals from metals-polluted marine waters due to industrial activities. Varieties of seaweeds have been studied for removal of heavy metals through bioremediation (Alhakawati & Banks, 2004; Hamdy, 2000; Ho et al., 2010; Kumar et al., 2007; Luqman et al., 2012; Marinho-Soriano, 2007). These toxic metals are hard to degrade and are known to affect marine ecosystem by interfere with photosynthetic process of plants and other autotrophs (Luqman et al., 2012). Likewise, the use of seaweeds for integrating aquaculture were discovered and executed worldwide recently. It has been reviewed by Turan (2009). Integrating algae production with the aquaculture is an ideal means in ecological restoration application by nutrient removal from waste water bioremediation. Seaweeds are potential candidate to culture in shrimp ponds to remove dissolved nutrients from the excess feed and to clean up the water (Phang et al., 1996). In southwestern Taiwan, *Gracilaria firma* is extensively polycultured with crab (*Scylla serrata*) and/or shrimp (*Penaeus monodon*) (Trono, 1988). In France, *Gracilaria* was also cultivated in oyster farms and removed 90% of ammonia excreted from the effluents (McHugh, 2003). Costanzo et al. (2001) investigated the potential use of *Gracilaria edulis* as a marine bioindicator to study the source and geographical extents of nutrients entering oligotrophic coastal waters. The study had been conducted to examine the potential of co-cultivation of native oyster (*Saccostrea commercialis*) with *Gracilaria edulis* to improve the water quality in shrimp farm by removing the waste nutrients that

otherwise will be discharged and could bring about eutrophication (Jones et al., 2002). The cultivation of *Gracilaria chilensis* in fish farms to remove at least 5% of dissolved inorganic nitrogen and 27% of dissolved phosphorus was documented (Troell et al., 1997). The use of *G. crassa* and *Ulva reticulata* as potential biofilters for removal of nutrients from fish-macroalgae integrated system has been studied by Msuya & Neori (2002).

The seaweeds of *Gracilaria*, *Acanthophora* and *Hypnea* can also be used as diets to oceanic economic species like abalone (Neori et al., 2000; Phang, 2006). Likewise, seaweeds can provide young larvae or juvenile of marine animals with protection from predators, e.g. *Gracilaria* and *Ulva* are providing a nursery habitat to juvenile of blue crabs (Epifanio et al., 2003; Mahalak, 2008).

In addition to these, seaweeds are estimated to contribute about 330 billion tones of atmospheric oxygen annually (Hall, 2011). A few species seaweeds have been suggested as the important carbon dioxide sink to produce oxygen and reduce the level of atmospheric carbon dioxide, thereby contributing to reduction of global warming simultaneously (Muraoka, 2004).

According to Lobban & Harrison (1994), seaweeds have also been suggested as potential solar-energy converters in the production of energy and nutrients for methane-producing bacteria. In recent years, the discovery of algae as a real potential source of biofuel has generated an increased interest among scientists and entrepreneurs (Rodolfi, 2008; Vonshak, 2008). There are a number of seaweeds have been proposed for biofuel production, e.g. *Gracilaria* species by Hanisak (2008), *Ulva* species by Robertson-Anderson (2008), and Kelp species by Adams et al. (2008) and Kraan (2008).

2.2 GRACILARIACEAE

The family Gracilariaceae belongs to the red algal phylum Rhodophyta. The Rhodophyta is an ancient lineage and contains rich species ranging from unicellular to multicellular species and ecologically as primary producers in the marine environment throughout the world (Robba et al., 2006). Red algae are mostly benthic and possess the greatest diversity of seaweeds as 98% of the 6,000 species are from the marine environment (Karleskint et al., 2009). Butterfield (2000) pointed out that red algae are the oldest known taxonomically resolved eukaryote dated at 1198 ± 24 million years old and marking the onset of major evolutionary eukaryotic radiation. Numerous red algae remain cryptic, possibly owing to high variation of morphology within species and between species, and the lack of conspicuous morphologically features, degree of phenotypic plasticity by environmental forces and complexities of life histories (Saunders, 2005; Robba et al., 2006).

The Gracilariaceae is one of the families as important source for agar production. The Gracilariaceae was previously placed under the order of Gigartinales Schmitz until Fredericq & Hommersand (1989) proposed that it should be placed under the new order Gracilariales based on a study of comparative cystocarp development. The main distinguishing feature of Gracilariaceae from the Gigartinales is the absence of auxiliary cells and connecting cells. According to Gurgel & Fredericq (2004), there are currently seven genera in Gracilariaceae that are recognized, namely *Gracilaria* Greville (1830), *Hydropuntia* Montagne (1842), *Melanthalia* Montagne (1843), *Curdiea* Harvey (1855), *Gracilariophila* Setchell et Wilson in Wilson (1910), *Gracilariopsis* Dawson (1949), and *Congracilaria* Yamamoto (1986). For the past several decades, reproductive structures had been used in the taxonomy of *Gracilaria*. The nutritive filaments which are also known as “traversing filaments” or “absorbing filaments” were

confirmed by Dawson (1949) to be a useful criteria in distinguishing the genus *Gracilariopsis* from *Gracilaria*.

Dawson (1949) discovered that there were three distinct types of spermatangia and stressed their importance in taxonomy. The three types of spermatangia include *chorda* type, which is a superficial layer of transformed outer cortical cells; *textorii* type which has shallow conceptacles in the upper cortex; and *verrucosa* type with the formation of pot-shaped or deep conceptacles. In 1984, Yamamoto examined the Gracilariaceae and proposed five types of spermatangial formation patterns. The carposporophytes of *Gracilaria* during the pre- and post-fertilisation development was studied in detail by Fredericq & Hommersand (1989). This genus was found to be characterized by the absence of an inner pericarp consisting of a prominent, the presence of tubular nutritive cells in the floor of the cystocarp and non-superficial spermatia. The multinucleate tubular nutritive cells undergo secondary fusions and fuse onto the cells in the floor of the cystocarp and also onto the cells of pericarp.

The taxonomic classification of *Gracilaria* according to Gurgel & Fredericq (2004) based on small subunit rDNA and internal transcribed spacer (ITS) sequences is as follows:

Rank	**Name**
Kingdom	Plantae
Phylum	Rhodophyta
Class	Rhodophyceae
Subclass	Florideophyceae
Order	Gracilariales
Family	Gracilariaceae
Genus	*Gracilaria* Greville synonym *Hydropuntia* Montagne

In traditional classification, species of *Gracilaria* are identified based on morphological structure; the external forms of the fronds, the branching modes, the grade of constriction at the bases, whether the basal parts of branches are constricted, as well as the shape of the frond (cylindrical, compressed or flattened) (Lim, 2003). However, these characteristics are not stable and are always subject to the influence of external conditions such as ecology, weather and habitat. Hence the identification procedure has been improved by including techniques in hybridization (Bird & McLachlan, 1982; Yamamoto & Sasaki, 1988), molecular biology (Goff & Coleman, 1988; Carroll, 1989), characterization of cell wall compounds (Craigie et al., 1984), chromosomal characteristics (Gargiulo et al., 1987; Yabu & Yamamoto, 1989; Bird & Rice, 1990; Bird et al., 1992; Godin et al., 1993; Kapraun, 1993; Kapraun et al., 1993) and breeding of different cultured strains (Bird & McLachlan, 1982; Yamamoto & Sasaki, 1988; Bird & Rice, 1990; Rice & Bird, 1990; Plastino & Oliveira, 1990, 1997). Despite the availability of various taxonomic tools, the taxonomy of *Gracilaria* remains a problem (Bird, 1995) and subjected to taxonomic and nomenclatural changes (Silva et al., 1996).

The taxonomy of this commercially important *Gracilaria* is still problematic as there is a limitation of distinct morphological characteristics (Bird & McLachchan, 1982; Bird, 1995). Due to the economic importance of *Gracilaria,* several studies on their taxonomy have resulted in taxonomic revisions and nomenclature changes (Silva et al., 1996). The high degree of morphological variation in seaweeds is due to their plasticity which causes difficulties and confusions in the taxonomy of *Gracilaria* species (Bird & McLachlan, 1982) and species identification based on morphological characters alone is unreliable (Bellorin et al., 2008). Hence, it is essential to include molecular techniques for taxonomic studies.

2.2.1 *Gracilaria* Greville Genus

Gracilaria is the second largest genus of the red algae (Brodie & Zuccarello, 2007) comprising more than 150 species distributed worldwide (Byrne et al., 2002; Guiry & Guiry, 2013) where more than 110 species from tropical shores (Rueness, 2005). This genus is the third largest farmed algal group worldwide (Zemke-White & Ohno, 1999). *Gracilaria* is diverse in its distribution between 50° North latitude and 50° South latitude. It is widely distributed from the sub-boreal to tropical waters, and can be found abundantly in the Southeast Asian countries. It is successfully cultivated in commercial scale in China (Ren et al., 1984), Taiwan (Shang, 1976; Yang, 1982), Chile (Santelices & Ugarte, 1987), Philippines, the Republic of Korea, Indonesia, Namibia, Vietnam and Argentina (McHugh, 2003). Of the agarophytic *Gracilaria* genus, only *G. edulis* (Gmelin) Silva, *G. tenuistipitata* Chang et Xia, *G. crass* Harvey ex. J. Agargh and *G. verrucosa* are being cultivated in Asia (Phang et al., 1996; Phang, 2006). This genus is now considered synonymous with *Hydropuntia* (Gurgel & Fredericq, 2004).

The species of *Gracilaria* in Malaysia and Singapore have been described by a number of researchers (Xia & Abbott, 1987; Abbott et al., 1991; Phang, 1994; Lim & Phang, 2004). In Malaysia, a total of 20 species of *Gracilaria* have been recorded, namely: *G. cacalia* (Agardh) Dawson; *G. canaliculata* (Kutzing) Sonder; *G. changii* (Xia & Abbott) Abbott, Zhang & Xia; *G. confervoides* Wigger) Greville; *G. coronopifolia* J. Agardh; *G. crassa* Harvey ex. J. Agargh; *G. cylindrical* Boergesen; *G. dura* Agargh; *G. edulis* (Gmelin) Silva; *G. firma* Chang et Xia; *G. foliifera* (Forskaal) Boergesen; *G. lichenoides* (Lamouroux) Greville; *G. minor* (Sonder) Chang & Xia; *G. multifurcata* Boersegen; *G. salicornia* (C. Agardh) Dawson; *G. subtilis* (Xia & Abbott) Abbott; *G. taenoides* Agardh; *G. tenuistipitata* Chang et Xia; *G. textorii* (Suringer) De Toni; and *G. urvillei* (Montagne) Xia & Abbott. The genus *Gracilaria* is the most abundant agarophytic seaweed found in Malaysia. *G. changii* and *G. edulis* have shown

its potential applicability for food consumption, phycocolloids production, sources of biopharmaceutical products, biofuel production and in ecological restoration. To fully utilise the Malaysian species of *Gracilaria*, it is important to understand their biochemical composition, ecology, taxonomic status, more importantly their intraspecific genetic diversity and phylogeographic pattern. This study is mainly focus on the genetic diversity of *G. changii* and *G. edulis* in Malaysian waters.

2.2.1.1 *Gracilaria changii* (Xia et Abbott), Abbott, Zhang et Xia, 1991

Basionym: *Polycavernosa changii* Xia et Abbott, 1987

Synonym: *Hydropuntia changii* (Xia et Abbott) Wynne, 1989

Gracilaria changii is one of the most abundant agarophytic seaweeds widely distributed in Malaysia (Phang, 1994; Tseng & Xia, 1999; Wong et al., 2006; Teo et al., 2007; Teo et al., 2009), Thailand (Lewmanomont, 1994; Tseng & Xia, 1999), Vietnam (Ohno et al., 1999), Myanmar (Burma) (Silva et al., 1996), Philippines (Abbott, 1994; Tseng & Xia, 1999; Kraft et al., 1999), and Singapore (Phang, 1994; Pham et al., 2011). The type species *G. changii* from Middle Banks, Penang, Malaysia was originally described by Xia and Abbott (1987) and it can be found abundantly in the mangrove areas fringing the west coast of Peninsular Malaysia (Phang et al., 1996; Lim & Phang, 2004). For many years, *G. changii* was confused with *G. cylindrica and G. blodgetti* because of the constrictions at the branch bases (Phang, 1994). *G. changii* grows naturally on intertidal mudflats and generally grow as epiphytic algae on mangrove roots, shells, pebbles and fish cages. It is capable of adapting to the harsh silted mangrove (Phang et al., 1996) and grows together with a range of epiphytes in nature. *G. changii* contains a notable amount of protein, fatty acids (Chu et al., 2003) and bioactive compounds (Nafezah, 2006; Wong et al., 2006). The wide use of high quality

agar and agarose with good gel strength extracted from *G. changii* (Abbott, 1980; Phang et al., 1996) has generated an increased interest among researchers in various aspects of RAPD molecular markers for differentiation of different life stages (Sim et al., 2007), tissue and protoplast culture (Yeong et al., 2008), functional genomics and proteomics (Wong et al., 2006; Teo et al., 2007; Ho et al., 2009; Teo et al., 2009; Siow et al., 2012), genetic transformation (Gan et al., 2003), in bioremediation (Ho et al., 2010) and biomedical (Shu et al., 2013).

2.2.1.2 *Gracilaria edulis* (Gmelin) Silva

Basionym: *Fucus edulis* Gmelin 1768

Synonym: *Hydropuntia edulis* (S.G. Gmelin) Gurgel et Fredericq 2004

Hydropuntia fastigiata (Chang et Xia) Wynne 1989

Polycavernosa fastigiata Chang et Xia 1963

Fucus coralloides Poiret

Fucus lichenoides Greville

Fucus lichenoides Turner

Sphaerococcus lichenoides C. Agardh

Ceramianthemum lichenoides Kuntze

This species is diverse in its distribution, as it found in Malaysia, Singapore, Indonesia, Thailand, Vietnam, Mynmar, the Philippines (Ohno et al., 1999), Taiwan (Lin, 2009), Pacific Islands (Tsuda, 1985), Australia (Millar & Xia, 1997), Africa (Silva et al., 1996) and India (Rao, 1972). It grows naturally on intertidal mud flats, rocks, mangrove roots and on fish cages. According to Millar & Xia (1997), *Hydropuntia edulis* (Gmelin) Gurgel et Fredericq (2004) was described for the first time from Indonesia as *Fucus edulis* by Gmelin (1768). Based on the *rbc*L sequences obtained by

Gurgel & Fredericq (2004), *G. edulis* has been transferred to the genus of *Hydropuntia* as *Hydropuntia edulis* (S. G. Gmelin) Gurgel et Fredericq (2004). However, for consistency and ease of references, we retain it as *Gracilaria edulis* in this study. Occasionally, *G. edulis* can be found in close association with *G. changii*, sharing the same intertidal muddy substrate. They can be distinguished by their morphological features, whereby the former is bushier and dark red while the latter is reddish (Phang, 1994). This species contains a high amount of fatty acids and has been the subject of interest among researches in aspects of pharmaceutical in drug development (Murugan & Iyer, 2012). It has been also used as food, source of commercially important phycocolloids of agar, as animal fodder, as marine bioindicator (Costanzo et al. 2001; Jones et al., 2002) and bioremediator (Luqman et al., 2012). Quite a number of studies have been done on the growth of *G. edulis* (Raikar et al., 2001; Ganesan et al., 2011; Yu et al., 2013). *G. edulis* has been successfully cultivated in India with vegetative cultivation (Siraimeetan & Selvaraj, 1999) and spore cultivation (Jayasankar & Varghese, 2002) to cope the surging global market demand of *G. edulis* for agar production.

2.3 SEAWEED RESOURCES AND SEAWEED CULTIVATION IN MALAYSIA

The high value of seaweeds and their products have made them economically important natural resources in Malaysia. Geographic distributions of seaweeds and species richness in Malaysia have been well documented by Ooi et al. (2006), Phang et al. (2006) and Du et al. (2009). To date, a total of 386 specific and intraspecific taxa have been recorded in Malaysia namely: 27 families and 182 taxa of Rhodophyta; 13 families and 102 taxa of Chlorophyta; 8 families and 85 taxa of Phaeophyta; and 8

families and 17 taxa of Cyanophyta (Phang et al., 2007). Malaysia has a coastline of 4,800 km: 2,100 km for Peninsular Malaysia and 2,700 km for East Malaysia. Biogeographically, Malaysia can be divided into two regions: (i) Peninsular Malaysia is bounded by the Straits of Malacca on the west, Tebrau Straits on the south and the South China Sea on the east; (ii) East Malaysia is bounded by the South of China Sea on the west, Sulu Sea on the North-West and Celebes Sea on the south. The seas surrounding Malaysia are rich in biological diversity and support a variety range of marine habitats such as coral reefs, mangrove swamps, seagrass beds, rocky shores and sandy beaches (Mazlan et al., 2005). Phang (1998) reported that the sheltered coastline of the west coast of Peninsular Malaysia is dominated by mangrove swamps and experiences semi-diurnal tides. The east coast of Peninsular Malaysia is characterised by sandy and rocky shores and has a mixed tidal system. The coastlines of Sabah and Sarawak are characterised by sandy and rocky beaches with coral reefs and have a mixed tidal system. The surface water temperatures range between 27 to 29°C with salinity of 28 to 34 ppt (parts per thousand).

In Malaysia, seaweed cultivation was established in Sabah since 1978 using cultivation methods of raft and long line systems. The east coast of Sabah is a conducive sheltered area for seaweed cultivation with salinity ranging between 30-35 ppt and dominated by sandy and coral seabed. *Kappaphycus alvarezii* and *Eucheuma denticulatum* are widely cultivated in the upper part of the sublittoral zone using monofilament or long line system for carrageenan production. According to the report by the Department of Fisheries Malaysia (2011), about 111,300 tonnes of seaweeds were harvested locally in 2008, 138,855 tonnes in 2009 and it increased to 138,897 tonnes (RM 28.2 million) in 2010. Small scale basis cultivation of agarophytes species like *Gracilaria* spp. also found in Sabah. According to Doty & Fisher (1987), the first attempt at experimental cultivation of *G. edulis* utilizing tetraspores was implemented in

the Penang Straits in 1983-1984. *G. changii* has been cultivated experimentally using the monofilament method for shrimp ponds and mangrove ponds cultivation at Ban Merbuk, Kedah by Department of Fisheries Malaysia (Phang, 1998). However, so far there is no commercial scale cultivation of *Gracilaria* in Malaysia.

In 2012, the Farmers Organisation Authority (FOA) proposed to extend cultivation of this economically important red seaweed throughout Peninsular Malaysia and it has been implemented in Kuala Muda, Kedah. (Bernama News, 11st June 2012). Cultivation of *G. manilaensis* in brackish earth ponds has been executed in Kuala Muda, Kedah (Ismail et al., 2012).

2.4 GENETIC DIVERSITY OF SEAWEEDS

Genetic diversity, a key component of biodiversity, is defined as any measure that quantifies the magnitude of genetic variability with a population. Genetic diversity is the diversity within species and refers to any variation either in its most primary level of nucleotides, genes, chromosomes, or whole genomes of an individual (Wright, 1920; Fisher, 1930). Measurement of genetic diversity contributes important clues to an understanding of the nature of forces acting on genetic variation, pattern, and level of genetic variation, evolutionary history and adaptation of an organism. Genetic diversity within a population also has ecological effects on productivity, growth and sustainability, as well as inter-specific interactions within communities and ecosystem-level processes (Hughes et al., 2008).

According to Futyuma (1986), interest in study of genetic diversity is mainly focused on its origin and maintenance, its role in the evolution of sexual reproduction and how the genetic variance influences the rate of evolutionary change within

populations. Level of genetic diversity of each population is varying and depends mainly on variation in the size of populations, pattern and abilities of dispersal among populations, and evolutionary relationships. Factors of chemical, physical and biological caused the constantly changing of environments over short or long timeframes. To cope with altering environments, genetic diversity of a species provides raw material upon which natural selection acts to cause evolution. The potential rate of evolution in a population is proportionate to the amount of genetic variation in a population. Generally, larger populations will have greater allele diversity, possibly indicating greater capacity for evolutionary adaptation to survive in a range of environmental conditions. In contrast, individuals in small populations are less able to adapt themselves in diverse environmental conditions as they are likely to be homogenous in terms of genetic, anatomy and physiology (Bagley et al., 2002). Loss of genetic diversity may reduce evolutionary potential and reproductive fitness of a population to endurance in stressful environments (Frankham et al., 2004).

Ongoing natural processes of natural selection, mutation, migration and genetic drift determine the current level of genetic diversity within a species. Natural selection is a primary force to remove "unfit" individuals from a population for increasing the average fitness. Mutation is an ultimate source of evolutionary change, happening when there are occasional errors in DNA replication or induced by environmental factors such as ultraviolet or damage of DNA caused by chemical radiation. Mutations are occurring relatively slow but continuously. Migration or gene flow is the exchange of individuals between populations. The rate of migration could correlate to the frequency of reproduction and distance dispersal of an individual. Generally, migration is expected to enhance the genetic diversity within populations and to homogenize genetic variation among populations. Genetic drift represented the random changes in gene frequencies of different alleles in every generation due to limited number of breeding adults. In term of

population genetics theory, genetic drift may become one of the major sources of genetic variation between populations (Halliday, 1993). It may decrease genetic diversity within populations but increase among populations (Bagley et al., 2002).

Genetic diversity is most often characterized using data that depict variation in either discrete allelic states or continuously distributed (i.e. quantitative) characters, which lead to different possible metrics of genetic diversity: allelic diversity, allelic richness, genotypic richness, mutational diversity and effective population size (θ), nucleotide diversity (π), coefficient of genetic variance (CV) and genetic varianc(V_G). The variation of allelic states or continuous traits may either be neutral (have no effect on ecology) and non-neutral with respect to fitness consequences (Hughes et al., 2008). Molecular markers such as Amplified Fragment Length Polymorphism (AFLP), microsatellite, protein polymorphisms and direct DNA sequence (Avise, 2004), typically represent discrete allelic states that are assumed to be neutral.

According to Lankau & Strauss (2007), genetic variation in one species might allow it for coexistence with competitors; meanwhile, competitor species diversity maintains this genetic diversity. A variety of additional reciprocal effects between ecological factors and genetic diversity, as genetic diversity and evolutionary processed can influence a range of population, community and ecosystem responses that may alter the allelle sequences or genotypes frequency (Fussmann et al., 2007).

Genetic variation is composed of non-genetic-based variation (due to environmental affects) and genetic-based variation (due to dissimilarities in alleles and heterozygosity at loci). Genetic diversity can be gauged using morphological characterisation (quantitative variation), molecular approaches (the visible direct effects of deleterious alleles, direct measurement of variation in DNA sequences), and biochemically (variation of protein). Theoretically the highest level of genetic diversity in DNA sequences is observed in sequences with little functional significance. In

contrast, the lowest level of genetic variation is commonly observed for functionally important regions of molecule (Frankham et al., 2004).

According to Jump et al (2008), anthropogenic environmental changes lead to changes in genetic diversity within and among populations. The change in the global environment originating from human activities or global climate change is one of the factors contributed to genetic variation of an organism, especially marine organisms, which play pivotal roles in many biochemical processes to sustain our biosphere by providing food, assimilation of waste and regulation of the dispersed effects of global climate change. Adaptation to climate change may threaten species diversity through loss of hidden genetic diversity within an individual organism.

Transoceanic introductions of marine organisms as an impact of the globalization of ship transport systems and fisheries aggravate the threat to coastal ecosystems (Uwai et al., 2006a). More than one hundred seaweed species have been widely dispersed across their native ranges due to anthropogenic activities (Farnham & Irvine, 1973; Rueness, 1989; Curiel et al., 1998; Fletcher & Farell, 1999; Rueness & Rueness, 2000; Boudouresque & Verlaque, 2002; Smith et al., 2002; Kim et al., 2004). According to Tseng (2001), *Saccharina japonica* in Japan was accidentally introduced to the coastline of China around 1927. Losses in seaweeds are also attributed to human activities through habitat destruction, over-harvesting, water pollution, development of coastal areas for tourism, global climate change and introduction of alien species. Buschmann et al. (2001) reported there was a major shift of the algal industry. In the 1980s there was a shift from obtaining raw material from harvesting natural beds to predominantly mariculture-based production, as a result of decline in wild populations due to over-exploitation. Expansion of mariculture also led to introductions of marine algae from one geographic region to another as has been documented by a number of researchers (Nelson & Maggs, 1996; McIvor et al., 2001; Uwai et al., 2006a).

According to a review of global seaweed introduction by Williams & Smith (2007), a total of 277 introduced seaweed species have been recorded worldwide and they are introduced to non-native populations around the world by means of aquaculture, aquarium introduction, ballast and hull fouling. Aquaculture and fouling of vessel hulls are the most significant vectors for seaweed introductions.

A number of networking approaches have been developed lately for estimating within-species relationships: statistical parsimony, statistical geometry, split decomposition, median-joining networks and molecular-variance parsimony (refer to Posada & Crandall, 2001). In the present study, a distance method of the statistical parsimony network is adopted to analyse all the sequences data. The statistical parsimony method of Templeton, Crandall and Sing (1992) (TCS) program is used to create a statistical parsimony network (Clement, 2000) in this present study to elucidate genetic diversity distribution and allows tracking of common ancestral haplotype by calculate the minimal number of mutational steps. This software is working based on the coalescent assumptions of allele geneology. The traditional methods of phylogeny reconstruction assuming a bifurcating tree count not precisely infer the intraspecies phylogeny and ancestral haplotypes are not longer available in population; in contrast, ancestral haplotypes will be the highest frequent sequences in a population level study in coalescent theory (Watterson & Guess, 1977; Crandall & Templeton, 1993). This approach has been applied with traditional methods to infer the relationships among organism with wide range of divergence (Crandall & Fitzpatrick, 1996; Benabib et al., 1997) and in discovery of phylogeographic history of a diversity of leatherside chub (Johnson & Jordon, 2000) and freshwater mussel (Turner et al., 2000). According to Castelloe & Templeton (1994), estimation of haplotype outgroup probabilities from frequencies of haplotype is positively correlated to haplotype age. Differences of

haplotypes could correlate to genotypic or phenotypic adaptations to the environmental changes.

2.5 MOLECULAR APPROACHES IN SEAWEED STUDIES

Algae can have highly variable morphology within species and between species. The lack of definitive characters and highly convergent morphology may lead to misidentification of species. The uncertainties of identification in red algal taxonomy can be overcome by the adoption of molecular techniques in the 1980s (Robba et al., 2006). Applications of molecular tools in species identification have come to rely heavily by algal systematists for close to two decades (Harper & Saunders, 2001). Genetic information renders a superior tool for studying the genetic variation, diversity, evolution, phylogeny, phylogeography and history of algae (Zuccarello & West, 2002). Hebert et al. (2004) commented the DNA barcoding is an extremely powerful tool especially when incorporated with traditional taxonomic tools. Saunders et al. (1996) have proven the use of morphological, reproductive and molecular evidence to discriminate between species of the Ceramiceae. Avise (1994) and Hillis et al. (1996) demonstrated the molecular approaches for the studies on evolution and population genetics are seeing increasing interest in biology. Hybridization is a crucial component in improving the potential of evolutionary of organism (Rieseberg, 1997) and being demonstrated by Lewis & Neushul (1995) for marine algae.

Measurement of genetic diversity with molecular tools has been accelerated with advances in molecular markers technologies. In recent years, application of molecular techniques has become important in species identification (Conklin et al., 2009). Adoption of molecular techniques for genetic diversity study provided valuable

information of the amount of diversity present at intraspecific and interspecific levels, diversity structure within and among population in space and over time, gene flow, identity of individual, genetic distance, linkage disequilibria and the inter-locus correlation of allelic variation and evolutionary history (Karp, 2002). Molecular tools derived different informational content. It is crucial to understand the requirements of information may be needed to address the issues on genetic diversity and to adopt the most appropriate tool.

Several molecular tools are available and being widely used to elucidate genetic variability of marine macroalgae, such as DNA markers used in allozyme (Intasuwan et al., 1993), restriction fragment length polymorphism (RFLP; Kamikawa et al., 2007), intersimple sequence repeat (ISSR; Wang et al., 2008; Zhao et al, 2008), microsatellite marker (Wattier et al., 1997; Guillemin et al., 2005; Zhang et al., 2009a; Song et al., 2013), random amplified polymorphism DNA (RAPD; Zhao et al., 2008), amplified fragment length polymorphism (AFLP; Donaldson et al., 2000; Pang et al., 2010) and DNA sequencing (Gargiulo et al., 2006; Bellorin et al., 2008).

2.5.1 Deoxyribonucleic Acid (DNA) Isolation and Purification

Isolation of genomic DNA from algae is greatly impeded due to abundance of polysaccharide content, acidic proteins and secondary metabolites in the cellulosic walls (Chakraborty et al., 2008; Yi et al., 2010). According to Khanuja et al. (1999), there are three major factors influencing the quality of isolated genomic DNA, which are i) DNA degradation due to endonucleases, ii) co-isolation of inhibitor compounds and secondary metabolites, and iii) and co-isolation of high molecular weight polysaccharides. High quality DNA is required for PCR or PCR-based techniques, which is a critical factor to ensure the success of amplification with reproducible results (Amani et al., 2011). Several DNA isolation protocols have been developed by a

number of researchers in obtaining quality DNA from seaweeds (refer to Joubert & Fleurence, 2005; Chakraborty et al., 2008). Commercially available kits for DNA isolation is one of the rapid and efficient protocols for DNA isolation from small amount of sample used.

2.5.2 Amplified Fragment Length Polymorphism (AFLP)

AFLP approach is a powerful PCR-based technique based on the detection of genomic restriction fragments by PCR amplification with hemi-specific primers (Vos et al., 1995; Zabeau & Vos, 1993). This technique also known as selective restriction fragment amplification (SRFA). In this technique, the genomic DNA is first digested with one or more restriction enzymes. Restriction half-site specific adaptors are ligated to the cut ends and followed by two rounds of PCR amplification continuously. At first, the primers are based on the adaptor and restriction site specific sequences with one additional nucleotide (act as selective bases). In the second round, between two or three additional nucleotides are added. The fragments with complementary bases internal to the adaptors and restriction site will be amplified to generate multi-locus profile.

The overriding advantageous of AFLP is it able to detect high level of polymorphism rapidly, no priori sequence information is needed, has high repeatability of multi-locus profile (Féral, 2002; Meudt and Clarke, 2007; Yi et al., 2010), generates high marker densities for positional cloning, high resolution and repeatability. AFLP markers can be acted as genetic and physical DNA markers, allowing the integration of genetic and physical maps. This technique has been exponentially used in a variety of applications: paternity analyses (Krauss, 1999), map expansion (Castiglioni et al., 1998), genetic diversity (*Undaria pinnatifida*, Zhang et al., 2009b & Li et al., 2013; *Laminaria japonica*, Yi et al., 2010 & Shan et al., 2011; *Gracilariopsis lemaneiformis*, Pang et al., 2010) and species identification. As dominant markers, homozygous and

heterozygous individuals cannot be distinguished by this analysis, resulting in a decrease of the genetic information available. In addition, problem of size homoplasy (Bonin et al., 2007; Caballero et al., 2008) and development of locus-specific markers from individual fragments can be difficult. According to Vekemans et al. (2002), the effect of size homoplasy can be minimized by selection of appropriate number of selective nucleotides in primer sequence and by scoring larger size of bands in preference.

2.5.3 Random Amplified Polymorphic DNA (RAPD)

Random Amplified Polymorphic DNA (RAPD) is first developed in 1990 by two groups of researches (Welsh & McClelland, 1990; Williams et al., 1990) and as the first PCR-based molecular marker extensively used for genetic variation analyses. RAPD analysis is one of the most common DNA fingerprinting techniques. The use of a single short random oligonucleotide primer in PCR reaction to amplify the DNA fragments from 10-50 discrete regions in the genome to obtain a fingerprint of multiple bands and polymorphism. This approach is a robust, rapid and minimal preliminary work is required (Molnar et al., 2000; Huh et al., 2006). RAPD markers are suggested to be useful for large-scale biogeographic populations analysis with hundreds to thousands kilometers apart (Bi et al., 2011). The major limitation of this technique is that this dominant nature RAPD marker does not able to discriminate between homozygote and heterozygote loci and the profiling is varying between laboratories (Mondini et al., 2009).

There are a number of studies in classification and taxonomic at the levels of genus and species have been reported using random amplified polymorphic DNA (RAPD), for example, *Gracilaria* species (Gonzalez et al., 1996; Meneses, 1996; Lim, 2001); *Gelidium* species (Patwary et al., 1993; Bouza et al., 2006); *Porphyra* (Dutcher

& Kapraun, 1994) and *Sargussum* species (Ho et al., 1995a,b; Engelen et al., 2001; Wong et al., 2004). Likewise, this assay has been used to assess the allozyme diversity and population structure of wild radish (Huh and Ohishi, 2001). This approach is also applicable in some genetic diversity studies of *Sargassum* species (Noormohammadi et al., 2011), *Ulva* species (Prasad et al., 2009) and the red algal *Phycodrys rubens* (Van Oppen et al., 1996) and on the genus of *Porphyra* in Korea (Huh et al., 2006).

A study by Lim et al. (2001) concluded RAPD is a fast and useful technique to understand the genetic relationship between Malaysian *Gracilaria salicornia* and it closely related species using the *rbc*L gene and the ITS region. The use of RAPD markers in the study of sex and life stage of *G. gracilis* (Martinez et al., 1999) and *G. changii* (Sim et al., 2007) have been undertaken and provided reliable results.

2.5.4 Restriction Fragment Length Polymorphism (RFLP)

RFLPs, co-dominant markers (Botstein et al., 1980) as the first shot in the genome revolution (Dodgson et al., 1997), in which DNA is digested with highly specific restriction enzymes to compares their restriction sites in different individual. The number of restriction fragments may vary for different individuals. The restriction fragments are separated by electrophoresis, and the presence and absence of the restriction fragments of a given length are acted as character states. In 1993, Stiller & Waaland reported the small subunit ribosomal RNA (SSU rRNA) in RFLP analysis has been used to distinguish between the species of *Porphyra* from the Pacific coast of North America. RFLP is one of the major tools used to investigate genetic diversity within and between species (Old & Primrose, 1998; Pierce et al., 2000). However, RFLP has been shown to have drawbacks such as laborious, hazardous and time-consuming for examination of different kind of restriction enzyme and lengthy technical

procedure, and large quantities of high-quality DNA required for restriction digestion and Southern blotting (Sharma et al., 2008).

2.5.5 Single-Stranded Conformation Polymorphism (SSCP)

This molecular method was first described by Orita et al. (1989). The principle of SSCP to distinguish between mutants DNA fragments is based on the molecular conformation of single-stranded DNA. The single-stranded DNA is nucleotide-sequence specific and subject to alter by substitution, insertion or deletion, then affects the migration rate of molecule through polyacrylamide gel. This molecular method involves no specific equipment and applied rapidly, easily and non-radioactively.

Zuccarello et al. (1999) reported that the SSCP method was applicable in detection of plastid variation of just a single base pair difference within a population of the mangrove red algal *Caloglossa leprieurii* from Australia. Stach-Cairns et al. (1997) believed this method could also be employed in Rubisco spacer of brown algae as this method is fast, robust and extremely sensitive in detection of low level of genetic variation.

2.5.6 Microsatellite

Microsatellite, also called simple sequence repeats (SSR), consists of multiple copies of tandemly repeats of short DNA motifs ranging from 1 to 6 base pairs (Litt & Luty, 1989; Tautz, 1989; Weber & May, 1989) with extremely high degree of polymorphism. This co-dominant marker has demonstrated high rates of genetic polymorphism in higher plants and marine animals (Guo et al., 2009; Zhang et al., 2008). The utilisation of single-loci nuclear microsatellite by a few researches in population studies in red (Engel et al., 2002), brown (Coyer et al., 2003) and green (van der Strate et al., 2002) seaweeds have provided information of paternity, kinship and

gene flow. Microsatellite is one of the methods in measure of genetic diversity studies (Guillemin et al., 2005). This technique is being currently favoured owing to the high rate of variation and genome individual can be inferred directly. Moreover, the data generated from microsatellite are amenable for input and storage in database formats and can be exchanged between laboratories. These advantages have been increasingly outweighed. Waittier et al. (1997) reported the use of single-locus microsatellite on study of the population genetic of *Gracilaria gracilis* due to the high variability of microsatellite loci.

However, genetic homogeneity and low rate of SSR polymorphism was reported on Rhodophyta of *Gracilariopsis lemaneiformis* (Zhang et al., 2009a), *Porphyra* species (Pang et al., 2010) and *Chondrus crispus* (Provan et al., 2012) between different geographical areas. The development of microsatellite in seaweed biogeographic studies is laborious due to homoplasy, limited number of polymorphic loci and relatively low genomic frequencies. Time consuming- where a new primers must be developed for each species is also place a limit on the potential gains from this technique.

2.5.7 Gene Sequencing

Advances in molecular biology provide essential tools in measurement of genetic diversity by relating to the evolution, biogeography and systematics of red algae by exploiting genetic variation in the entire genome of an organism.

DNA-based molecular markers provide a pivotal role in the assessment of genetic diversity for a highly plastic genus such as *Gracilaria* (Bird & McLachlan, 1982). DNA sequencing is extensively used to study the phylogenetic relationships of the species from the family Gracilariaceae (e.g. Destombe & Douglas, 1991; Bellorin et al., 2002; Gurgel & Fredericq, 2004; Liao & Hommersand, 2003; Iyer et al., 2005, Gargiulo et al., 2006; Lin, 2006; Bellorin et al., 2008; Kim et al., 2008a). Zuccarello et

al. (2006a) pointed out that molecular markers have been proven applicable to assess red algal systematics but also in revealing genetic variation within red algal species. A large set of DNA markers that have been employed for studying phylogenetic and genetic diversity of seaweeds such as the *cox*1 gene, (Robba et al., 2006; Yang et al., 2008; Sherwood, 2008; Kim et al., 2010; Yow et al., 2011; Yow et al., 2013), mitochondrial cytochrome oxidase subunit 3, *cox*3, (Steel et al., 2000; Coyer et al., 2004; Uwai et al., 2006b); the mitochondrial-encoded *cox*2-3 spacer (Zuccarello et al., 1999; Byrne et al., 2002; Zuccarello & West, 2002; Cohen et al., 2004; Rueness, 2005; Zuccarello et al., 2006b; Chiasson et al., 2007; Vidal, 2008; Teasdale & Klein, 2010; Vis et al., 2010), nuclear-encoded internal transcribed spacers of the ribosomal cistrons, ITS1 and ITS2, (Bellorin et al., 2002; Marston & Villalard-Bohnsack, 2002; Cho et al., 2007; Lindstrom, 2008; Hu et al., 2009; Moniz & Kaczmarska, 2010), the plastid-encoded RuBisCo spacer (Byrne et al., 2002; Zuccarello et al., 2006a), and the *rbc*L gene (Nam et al., 2000; McIvor et al., 2001; Gurgel & Fredericq, 2004; Yang et al., 2013).

Gene sequences are an alternative approach to multi-locus profiling and are applicable in evolutionary history and diversity studies from species to population levels. In seaweed, there are three sources of genes: nuclear (nDNA), chloroplast (cpDNA) and mitochondrial (mtDNA) genomes which are differ in their mode of recombination, inheritance and evolutionary rates.

2.5.7.1 Mitochondrial Genome

Application of mitochondrial DNA in animal on population studies, phylogenetic reconstruction and phylogeographic studies has increased prevalent owing to the rapid evolution, lack of recombinant, uniparental inheritance and extensive intraspecific polymorphism of this genome (Avise, 1994). The success of the

mitochondrial marker cytochrome oxidase subunit I (*cox*1) in animals led to the assessment of this marker for application in DNA barcoding in red algae (Saunders, 2005).

To date, there are a number of mitochondrial genomes of the Rhodophyta and Phaeophyta have been completely sequenced: *Porphyra purpurea (Wahlenberg)* C. Agarhd (Burger et al., 1996), *Chondrus crispus* Stackhouse (LeBlanc et al., 1995), *Gracilariopsis lemaneiformis* (Zhang et al., 2012) and *Saccharina* species (*S. japonica*; *S. longipedalis*; *S. angustata*; and *S. coriacea* by Yotsukura et al., 2010; *S. longissima* and *S. hyperborean* by Zhang et al., 2012). Detection of conserved gene orders Gracilariales, Bonnemaisoniales and Ceramiales to yield highly variable DNA sequence data is possible with these sequences (Zuccarello et al., 1999).

Mitochondrial DNA region has been reported provides plausible resolution results for species identification in red macroalgae (Hebert et al., 2003; Saunders, 2005; Robba et al., 2006; Lane et al., 2007; Hu et al., 2009). The mitochondrial cytochrome oxidase subunit 1 (abbreviated *cox*1 or COI) gene coded an enzyme involved in the last step of electron transport chain. The gene of *cox*1 will be used for this study to understand the genetic diversity of *Gracilaria changii* and *Gracilaria edulis* and this gene to be an ideal marker for DNA barcoding in red algae (Geraldino et al., 2006; Robba et al., 2006; Yang et al., 2008; Le Gall & Saunders, 2010; Kim et al., 2010; Yang et al., 2013) as well as useful for revealing informative species relationship, population structure, cryptic diversity (Robba et al., 2006), phylogeography and the hidden diversity of red algae (Yang et al., 2008; Sherwood et al., 2011). The usefulness of the mitochondrial markers, *cox*1 (e.g. Robba et al., 2006; Yang et al., 2008; Yow et al., 2011) and the *cox*2-3 spacer (e.g. Zuccarello & West, 2002; Yow et al., 2013) as molecular markers in the measurement of genetic diversity have been reported. Furthermore, the data from the *cox*1 is also extremely valuable in determination of

genetic complement within a studied region, which enables the huge advances in conservation management (Robba et al., 2006). Hebert et al. (2003) suggested the nucleotide arrangement of 5' end of *cox*1 gene is applicable for cataloging biodiversity. According to the first paper study of haplotype analysis of the *cox*1 coding gene in red algae by Yang et al. (2008), commented that the *cox*1 gene is a reliable molecular marker for the study within species of *Gracilaria vermiculophylla* from Korea and Japan. The fairly low number of variable sites within species paired with high variation among species facilities the *cox*1 gene as an ideal marker for barcoding. In contrast, the high variation in this gene may hamper its utility for the interspecific relationship study. However, the present resolution of the genus *Gracilaria* could be improved using first and second codon or amino acid data of the *cox*1 (Yang et al., 2008). Current research on freshwater and marine red algae proved that the *cox*1 is a potential candidate marker for phylogeographic studies (House et al., 2008; Sherwood et al., 2008; Hu et al., 2010). Based on a phylogenetic study of *Gracilaria manilaensis* using the mitochondrial marker of the *cox*1 gene with the unpublished data by colleagues in Algae Laboratories, University Malaya, there was a clear genetic distinction between *G. manilaensis* and *G. changii*.

Likewise, the *cox*2-3 spacer is an intergenic spacer separating the conserved mitochondrial gene of *cox*2 and *cox*3 and it has been proved as a potential universal marker for non-coding DNA (Gabrielsen et al., 2002; Zuccarello & West, 2002; Zuccarello et al., 2002b). This mitochondrial marker was shown to be useful for intra- and inter-specific studies. The size of this spacer is about 350 bp and appears to contribute sufficient DNA sequence variability for population and phylogeographic studies in red algae (Zuccarello et al., 1999) even with single base pair changes on indication of reproductively isolated cryptic species (Zuccarello & West, 2003). The *cox*2-3 spacer has been proven as a warranted marker on phylogeographical study for

the Rhodophytes (Zuccarello et al. 1999; Zuccarello & West, 2002, 2003; Chiasson et al., 2003; Zuccarello et al., 2006a; Andreakis et al., 2007; Vis et al., 2008; Halling et al., 2013). This region was reported variable within population of the red algae *Caulacanthus ustulatus* (Zuccarello et al., 2002b). According to Andreakis et al. (2007), the *cox*2-3 spacer has higher mutation rate which evolved four and 14 times faster in comparison to the plastid *rbc* spacer and the nuclear LSU region respectively in red algae *Asparagopsis* spp.. The introduction of *G. vermiculophylla* from the Pacific Ocean to the northeastern Atlantic Ocean by anthropogenic has been study by Rueness (2005) using the *rbc*L gene and the *cox*2-3 spacers.

2.5.7.2 Plastid Genome

The fairly limited complete plastid genome sequences of the Rhodophyta is *Porphyra purpurea* (Reith & Munholland, 1995) and the Chlorophyte is *Chorella vulgaris* (Wakasugi et al., 1997).

Both the large subunit of ribulose biphosphate carboxylase/oxygenase gene (*rcb*L) and the small subunit of ribulose biphosphate carboxylase/oxygenase gene (*rcb*S) co-transcribed in plastid of the algae from Rhodophyta and Phaeophyta (Kostrzewa et al., 1990; Fujiwara et al., 1993; Bhattachary & Medlin, 1995). According to Freshwater et al. (1994), the *rbc*L gene codes for an enzyme involved in the process of carbon fixation. In Rhodophyta, this gene is usually applied as species-level marker for phylogenetic study owning to its high level of mutational rate (Yang et al., 2012) but this region shown low intraspecific variation in many taxa compared to the *cox*2-3 spacer (House et al., 2008). In green seaweed and land plants, the *rbc*S is encoded in nucleus. Quite a number of studies agreed the usefulness of a short intergenic spacer region namely, Rubicso spacer, on algal inter- and intra-species phylogenetic reconstruction (Goff et al., 1994; Stache-Crain et al., 1997; Zuccarello & West, 1997;

Kamiya et al., 1998). Large and small subunit of Rubisco (ribulose-1, 5-bisphosphate carboxylase/oxygenase), *rbc*L-*rbc*S spacer, are components of the plastid genome. Differences nucleotide sequences of these components have been employed to discriminate between several isolates of *Gracilaria* and *Gracilariopsis* (Goff et al., 1994). Based on several taxonomic studies on the Gracilariaceae by Gurgel and his coworkers, the *rbc*L is a potential marker for the classification within species level (Gurgel et al., 2003, 2004; Gurgel & Fredeqicq, 2004). Brodie et al. (1996 & 1998) has also proven the potential of Rubisco spacer to discriminate between *Porphyra* species in Britian and surrounding waters.

2.5.7.3 Nuclear Ribosomal DNA

Nuclear rDNA is an extranuclear genome and it is available in all living organisms in a number of copies that are distributed over the genome (Harmsen & Karch, 2004). In most cases, coding 18S and 28S rDNAs have been applied for phylogenetic inference studies at higher taxon levels. Wattier & Maggs (2001) reviewed most of the phylogeographic studies were conducted using the internal transcribed spacer (ITS) in the ribosomal cistron of the nuclear genome. Ribosomal internal transcribed spacer (ITS) regions have been employed in studies of biogeographical, intra-specific, dispersal direction, post-glacial recolonization, species identification and phylogenetic in Rhodophyta (Kunimoto et al., 1999; Broom et al., 2002; Marston & Villard-Bohnsack, 2002; Hu et al., 2009) owning to its rapid evolution rate and less subjected to functional constraints (Jung et al., 2010; Teasdale & Klein, 2010). The advantages of the regions were contributed the relatively high rate of nucleotide substitution, readily PCR- amplified and sequenced with conserved primers. Hu et al. (2009) shows that nrDNA ITS is a potentially applicable DNA barcoding marker for the level of species identification of Rhodophyta. Spacer of ITS1 and ITS2 are highly

variable and usable for ordinal and familiar identification, while conserved 5.8S locus for generic and species identification. Step of cloning prior to sequencing is necessary owing to its high level of intra-individual ITS variation (Famà et al., 2000; Lange et al., 2002; Uwai et al., 2006b).

The application of different molecular approaches for genetic diversity and phylogeographic studies of seaweed are listed in Table 2.1.

Table 2.1 A summary of molecular approaches used in genetic diversity and phylogeographic studies of seaweeds.

No	Seaweed	Molecular Analyses	Author(s)
	Rhodophyta		
1	*Acanthophora spicifera*	*Cox*2-3 spacer, nuclear LSU, ISSR	O' Doberty & Sherwood, 2007
2	*Asparagopsis taxiformis*	*Cox*1	Sherwood, 2008
3	*Amansia glomerata*	*Cox*1 & Universal plastid amplicon	Sherwood et al., 2011
4	*Batrachospermum gelatinosum*	*Cox*1 & *rbc*L	House et al., 2010
5	*Bostrychia radicans & B. moritziana*	*Cox*2-3 spacer & Rubisco spacer	Zuccarello et al., 2006b
6	*Caloglossa leprieurii*	Rubisco spacer	Zuccarello et al., 1999
7	*Caloglossa leprieurii*	Rubisco spacer	Kamiya, 2004
8	*Chantransia pygmaea*	*Cox*2-3 spacer & *rbc*L	Chiasson et al., 2007
9	*Chondrus crispus*	ISSR	Wang et al., 2008
10	*Chondrus crispus*	*Cox*1 & ITS2	Hu et al., 2010
11	*Gracilaria changii*	*Cox*1	Yow et al., 2011
12	*Gracilaria changii*	*Cox*1 & *cox*2-3 spacer	Yow et al., 2013
13	*Gracilaria chilensis*	Microsatellite DNA markers	Guillemin et al., 2005
14	*Gracilaria vermiculophylla*	*Cox*2-3 spacer & *rbc*L	Rueness, 2005
15	*Gracilaria vermiculophylla*	*Cox*1 & *rbc*L	Yang et al., 2007
16	*Gracilariopsis lemaneiformis*	SSR & AFLP	Pang et al., 2010
17	*Grateloupia doryphora*	ITS, *cox*2-3 spacer & RAPD	Marston & Villard-Bohnsack, 2002
18	*Kappaphycus* & *Eucheuma*	*Cox*2-3 spacer & Rubisco spacer	Zuccarello et al., 2006a
19	*Kappaphycus* & *Eucheuma*	RAPD	Dang et al., 2008
20	*Kappaphycus* & *Eucheuma*	*Cox*2-3 spacer	Halling et al., 2013
21	*Mastocarpus papillatus*	ITS 1 & ITS 2	Lindstrom, 2008
22	*Murrayella periclados*	*Cox*2-3 spacer	Zuccarello et al., 2002a
23	*Palmaria palmata*	*Cox*2-3 spacer	Provan et al., 2005
24	*Polysiphonia harveyi*	*rbc*L gene	McIvor et al., 2001
25	*Porphyra* spp.	RAPD	Huh et al., 2006

Table 2.1 (continued).

26	*Porphyra spiralis*	Group I introns *cox*2-3 and *rbc*L-S regions	Milstein et al., 2008
27	*Porphyra umbilicalis*	ITS , ribosomal DNA group-I intron, cox2-3 spacer	Teasdale & Klein, 2010
28	Red algae	*Cox*2-3 spacer	Zuccarello et al., 1999
29	Red algae: *Bangiales, Ceramiales, Corallinales, Gigartinales, Gracilariales* & *Rhodymeniales*	*Cox*1	Robba et al., 2006
30	*Spongites*	*Cox*2-3 spacer	Vidal, 2008
	Phaeophyta		
31	*Fucus serratus*	*Cox*3	Coyer et al., 2004
32	*Laminaria japonica*	AFLP	Yi et al., 2010
33	*Scytosiphon lomentaria*	ITS 1 & ITS 2	Cho et al., 2007
34	*Sargassum hemiphyllum*	ITS2, rubisco spacer, mitochondrial intergenic *Trn*W_I spacer	Cheang et al., 2010
35	*Sargassum muticum*	Mitochondrial spacer (*Trn*W_1), rubisco spacer and ITS2	Cheang et al., 2010
36	*Sargassum muticum*	RAPD & ISSR markers	Zhao et al., 2008
37	*Sargassum polyceratium*	RAPD	Engelen et al., 2001
38	*Sargassum* sp.	ISSR & RAPD	Noormohammadi et al., 2011
39	*Undaria pinnatifida*	*Cox* 3 & ITS	Uwai et al., 2006b
40	*Undaria pinnatifida*	AFLP	Zhang et al., 2009b
41	*Undaria pinnatifida*	AFLP	Li et al., 2013
	Chloraphyta		
42	*Ulva* sp.	RAPD	Prasad et al., 2009
43	*Ulva prolifera*	ISSR	Zhao et al., 2011

CHAPTER 3

MATERIALS AND METHODS

3.1 FIELD COLLECTION AND SPECIMEN PROCESSING

Healthy samples of *Gracilaria changii* and *Gracilaria edulis* (Gracilariaceae, Rhodopyhta) were identified and collected randomly in the field from various localities around Malaysia, Thailand, Vietnam and Japan. Photographs show the sampling collection sites in Malaysia (Fig. 3.1- 3.8).

The collected specimens were kept in an ice-box while transported back to the laboratory. The specimens were stored at -20ºC immediately upon arrival at lab before further processing. The collected specimens were cleaned with seawater and distilled water. Mud or dirt, entangling epiphytes, epizoites, fungi and extraneous materials were removed by successive washing in seawater with the final rinse in distilled water to remove excessive salt. The cleaned specimens were blotted and left to dry in a clean air-conditioned room. Seaweed samples which were used for the morphological study were preserved in 3-5% of formalin-seawater or pressed on herbarium sheets. Herbarium vouchers of both species were deposited in University of Malaya Seaweeds and Seagrasses Herbarium, Malaysia. Specimens for DNA isolation were desiccated with silica gel. The dried samples were ground to a powder form with liquid nitrogen and stored at -20ºC immediately before DNA isolation.

(a)

(b)

Figure 3.1 (a & b) Collection site of *Gracilaria* species in sandy-soil area at Middle Banks, Penang.

(a)

(b)

Figure 3.2 (a) Collection site of *G. changii, G. edulis and G. salicornia*; **(b)** *G. changii* attached to the roots of *Avicennia*, a mangrove tree at Morib, Selangor.

(a)

(b)

Figure 3.3 (a) Collection site of *G. changii* and *G. edulis*; **(b)** *G. changii* attached to a pebble at Teluk Pelanduk, Negeri Sembilan.

(a)

(b)

Figure 3.4 (a) Collection site of *G. changii* and *G. edulis*; **(b)** *G. edulis* attached to the roots of *Avicennia*, a mangrove tree at Pantai Dickson, Negeri Sembilan.

(a)

(b)

Figure 3.5 (a) Collection site of *G. changii* and *G. edulis*; **(b)** *G. edulis* attached to a pebble at Batu Besar, Malacca.

(a)

(b)

Figure 3.6 Collection sites of *G. edulis* **(a)** in Sungai Kong Kong, Johore and *G. changii* **(b)** in Kuala Sungai Merbok, Kedah from fish cages.

(a)

(b)

Figure 3.7 (a & b) Collection site of *G. changii* in muddy area at Pulau Nunuyan Laut, Sabah.

(a)

(b)

Figure 3.8 (a & b): Collection site of *G. changii* in muddy area at Kampung Dandulit, Sabah.

3.2 MORPHOLOGICAL STUDIES OF *GRACILARIA* SPECIES

For morphological examination, the specimens in herbarium sheet were observed for the morphological appearance and were classified based on the morphological descriptions from Xia & Abbott (1987) and Lim & Phang (2004). For morphological studies, the reproductive structures were identified and hand sections were conducted using a sharp blade to obtain the thinnest sections. Sections were then placed on a clean glass slide and stained with 0.05% of aniline blue solution and subsequently mounted in 40% karo syrup. The prepared slides were observed under light microscope (Olympus BX 41).

3.3 DEOXYRIBONUCLEIC ACID (DNA) ISOLATION WITH DNEASY PLANT MINI KIT

Genomic DNA of *Gracilaria* species were extracted from dried tips ground in liquid nitrogen with the DNeasy Plant Mini Kit (Qiagen, Germany). The DNeasy Plant Mini Kit is designed for extracting DNA from plant tissue. All the isolation steps were carried out according to the instructions of manufacturer with minor modifications. The maximum amount of starting materials for DNA isolation using this kit was 100 mg for wet specimens and 20 mg for dried specimens.

The seaweed materials were mechanically ground into fine powder in liquid nitrogen by micropestle. The cells were chemically lysed with the addition of lysis buffer (AP1) and RNase was added together with lysis buffer to digest the RNA in the specimen, further disrupted by 20 minutes sonication using Powersonic 405 Sonicator (Hwashin Technology Co.) at room temperature, and followed by 25 minutes incubation

in water bath at 65ºC to break the cells and release the cellular content. Precipitation buffer (AP2) was added to the mixture and incubated for 5 minutes on ice. Proteins, detergent and polysaccharides were precipitated by salt precipitation upon cell lysis. The cell debris and other precipitation were removed in a single step of filtration and homogenization via brief spinning of QIAshredder Mini spin column (a unique filtration and homogenization). The cleared lysate was transferred to a new tube to which binding buffer with ethanol (AP3/E). The mixture was transferred to DNeasy Mini spin column and followed by centrifugation for binding of DNA on the membrane. Further washing step by washing buffer (AW) in removing contaminants such as polysaccharides and proteins, and finally the DNA bound to the membrane was eluted in 100 µl elution buffer (AE).

3.4 SPECTROPHOTOMETRIC DETERMINATION OF PURITY AND CONCENTRATION OF DNA

Quantification of the isolated DNA and purity was measured using BioPhotometer (Eppendorf, Germany) with 1 µl of DNA to 49 µl of elution buffer in 50X dilution. The ratio of optical density (OD) absorbance at 260 nm and 280 nm (OD_{260}/ OD_{280}) reflects the purity of DNA. Normally, a pure DNA yields a ratio of 1.8 -2.0. For DNA contaminated by protein or carbohydrate, the reading will be significantly less than reading of 1.8; while a ratio more than 1.8 suggests that the isolated DNA sample is contaminated by RNA. For concentration measurement, at 260 nm, the OD reading of 1 is approximately equivalent to 50 µg mL^{-1} of double-stranded DNA.

3.5 GENE SEQUENCING

Comparison of the use of two mitochondrial encoded markers, the cytochrome oxidase subunit 1 (*cox*1) and the intergenic spacer between the cytochrome oxidase subunit 2 and 3 (*cox*2-3 spacer) on inferring intraspecific genetic variation of *Gracilaria changii* from Peninsular Malaysia were carried out. The marker with higher resolution and more variable will be selected and employed in the present study to reveal genetic diversity of *Gracilaria changii* and *Gracilaria edulis* from Malaysian waters, Southeast Asian countries and Japan.

3.5.1 Polymerase Chain Reaction (PCR) of the *rbc*L Gene, *cox*2-3 Spacer and *cox*1 Gene

The isolated genomic DNAs were used as template in PCR amplification. The amplifications of DNAs were performed in a final volume of 25 µl containing 2.5 µl 10X buffer, 0.2 mM of each dNTP (dATP, dTTP, dCTP, and dGTP), 10 pmol of each forward and reverse primer, 1.5 U *Taq* polymerase and 20-50 ng of genomic DNA. Ultra-High Quality (UHQ) water was added to make up the final total volume of 25 µl. Negative control was prepared with the same constituents but without genomic DNA as an indicator of contamination of buffer and reagents used in PCR.

Amplification was carried out by GeneAmp® PCR system 2700 (Applied Biosystem) Thermal cycle or Eppendorf Thermal Cycler (Eppendorf, Germany) with an initial denaturation at 94ºC for 10 minutes to denature the double-stranded DNA, followed by 35 cycles of amplification (denaturing at 94ºC for 30 seconds, annealing at 50ºC for 30 seconds and extension at 72ºC for 2 minutes) with a final extension for 10 minutes at 72ºC. Modification of annealing temperature and concentration of DNA were carried out to obtain high yield product.

3.5.1.1 Large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (RUBISCO), *rbc*L Gene

For taxonomic purpose, the plastid-encoded large subunit of *rubisco* gene (*rbc*L) was used for phylogenetic study to identify *G. changii* with morphological differences. The large subunit of *rubisco* (*rbc*L) gene was amplified by PCR using one set of primers: F57 and R*rbc*S Start R. The amplified DNA was sequenced using three primers, which were and F57, F753 and R*rbc*S Start R, adapted from Freshwater & Reuness (1994) (Table 3.1).

Table 3.1 Synthetic primers used for sequencing of the *rbc*L gene.

Primer	**Direction**	**Sequence**
F57	Forward	5’ - GTAATTCGATATGCTAAAATGGG- 3’
F753	Forward	5’ - GGAAGATATGTATGAAAGAGC- 3’
R*rbc*S Start R	Reverse	5’ -GTT CTT TGT GTT AAT CTC AC- 3’

The parameters applied for amplification of the *rbc*L gene is summarised as followed:

Step	**Temperature (ºC)**	**Duration (Time)**	**Cycle**
Initial denaturation	94	10 min	
Denature	94	30 second	35
Annealing	50	30 second	
Extend	72	2 min	
Extension	72	10 min	
Storage	4	infinity	

3.5.1.2 Mitochondiral Cytochrome Oxidase Subunit 2 and 3 (*cox*2-3 spacer)

Primer pairs of *cox*2F and *cox*3R was used for PCR amplifications. The listed primers and amplification conditions were adapted from Zuccarello et al. (1999) (Table 3.2). Optimisation of PCR parameters on annealing temperature were modified from 45ºC to 55ºC.

Table 3.2 Synthetic primers used for sequencing of the *cox*2-*cox*3 spacer.

Primer	**Direction**	**Sequence**
*Cox*2F	Forward	5' - GTACCWTCTTTDRGRRKDAAATGTGATGC - 3'
*Cox*3R	Reverse	5' – GGATCTACWAGATGRAAWGGATGTC - 3'

The parameters applied for amplification of the *cox*2-*cox*3 spacer:

Step	**Temperature (ºC)**	**Duration (Time)**	**Cycle**
Initial denaturation	94	4 min	
Denature	94	1 min	5
Annealing	45	1 min	
Extension	72	1 min	
Denature	93	30 second	30
Annealing	55	30 second	
Extend	72	30 second	
Extension	72	5 min	
Storage	4	infinity	

3.5.1.3 Mitochondial Cytochrome c Oxidase Subunit I Gene (*cox*1)

Two combinations of primers were used to amplify the mitochondrial *cox*1 region: *cox*1 43F and *cox*1 1549R (Geraldino et al., 2006), and GazF1 and GazR1 (Saunders, 2005), respectively. The sequences of the primers are shown in Table 3.3. Optimisations of PCR parameter on annealing temperature were modified from 45ºC to

55ºC. Primers of C622F and C880R (Yang et al., 2008) were employed for sequencing purposes.

Table 3.3 Synthetic primers used for sequencing of the *cox*1 gene.

Primer	**Direction**	**Sequence**
*cox*1 43F	Forward	5'- TCAACAAATCATAAAGATATTGGWACT -3'
*cox*1 1549R	Reserve	5'- AGGCATTTCTTCAAANGTATGATA -3'
Gaz F1	Forward	5' – TCAACAAATCATAAAGATATTGG -3'
Gaz R1	Reserve	5'- ACTTCTGGATGTCCAAAAAAYCA -3'

The parameters applied for amplification of the *cox*1 gene is summarised as followed:

Step	**Temperature (ºC)**	**Duration (Time)**	**Cycle**
Initial denaturation	94	10 min	
Denature	94	30 second	35
Annealing	(depend on primer used)	30 second	
Extend	72	2 min	
Extension	72	10 min	
Storage	4	infinity	

3.5.2 Determination of the Yield and Quality of Amplified Product

The yield and quality of amplicon was examine by running electrophoresis on 1% (w v^{-1}) TAE agarose gel pre-stained with ethidium bromide in TAE buffer (40 mM Tris-acetate, 2 mM EDTA). 2 µl 1kb commercial DNA ladder was used to estimate the quantity and the correct size (bp) of amplified DNA region. The gel was run for 30 minutes at 100 V, and viewed under UV illuminator using AlphaImager 2200 (Alpha Innotech) right after electrophoresis.

3.5.3 Purification of Amplified Product and DNA Sequencing

Two methods of purification: gel extraction and direct purification were carried out. Gel extraction will be performed if multiple bands were observed on single lane. The remaining products were loaded into agarose gel and a desired size of band was excised then purified with the QIaquick Gel Extraction Kit (Qiagen, Germany), according to the instructions of manufacturer.

For amplified product with single band on gel electrophoresis, purification of the remaining PCR amplified products using QIAquick PCR Purification Kit (Qiagen, Germany) was carried out according to the instructions of manufacturer. The purified products from both methods were examined on 1% (w v^{-1}) TAE agarose gel pre-stained with ethidium bromide before sent for sequencing commercial companies: First Base Laboratory or MyTACG Bioscience Enterprise.

For First Base Laboratory, samples were sequenced using BigDye® Terminator v3.1 Sequencing Kit and analyzed with ABI PRISM® 377 Genetic Analyzer. While for MyTACG, samples were sequenced using BigDye® Terminator v1.1, v3.0 and v3.1 Sequencing Kit and analyzed with Applied Biosystems 3730xl DNA Analyzer.

Cycle sequencing conditions were as follows: 25 cycles of 96ºC for 10 seconds, 50ºC for 5 seconds and 60ºC for 4 minutes at rapid thermal ramp of 1ºC/ sec. The control DNA sequence used was the M13F (-29) control primer with pGEM® - 3Zf (+) control template.

3.6 Phylogenetic Analysis of the *rbc*L Gene for identification of *Gracilaria changii*

Sequences for the *rbc*L gene of the Gracilariaceae were downloaded from the GenBank sequence database provided by National Center for Biotechnology

Information (NCBI). The generated partial *rbc*L gene of *G. changii* from selected localities together with the *rbc*L sequences downloaded from GenBank were used for the phylogenetic analysis. *Melanthalia abscissa (*New Zealand) and *Curdiea crassa* (Bongin Bay, North of Sydney, NSW Australia) were used as the outgroup taxa for the phylogenetic analysis. The generated sequences together with sequences downloaded from the GenBank as listed in Table 3.4.

Sequencing data were analysed and edited using ChromasPro version 1.42 (2003-2008 Technelysium Pty Ltd) and BioEdit Sequence Alignment Editor version 7.0.9.0 (Hall, 1999) software. Edited sequences were aligned by CLUSTAL X program (Thompson et al., 1997) and further refined using naked eyes before subjected to the phylogenetic analyses. The phylogenetic trees were constructed using the maximum likelihood (ML) and Bayesian Inference (BI) analyses, as implemented in PAUP*4.0b10 (Swofford, 2003). The program Kakusan version 3 (Tanabe, 2007) was used to find out best fit models for (i) maximum likelihood (ML) analysis performed using Treefinder version of October 2008 (Jobb et al., 2004); and (ii) Bayesian Inference (BI) analysis performed using Mr. Bayes 3.1.2 (Huelsenback et al., 2001). Cladogram of ML was constructed by program of TreeFinder and with 1,000 ML bootstrap replicates. The method of four Markov chains over two independent runs of 2 X 10^6 generations was employed for BI analysis. Only nodes with Bayesian posterior probabilities (PP) greater than 50% as a validity supported clade for the construction of a 50% majority rule consensus tree. All phylogenetic trees were viewed with Treev32.

Table 3.4 List of the species identification, collection locality and the *rbc*L GenBank accession number used for construction of Maximum Likelihood and Bayesian trees.

Species	Collection Locality	GenBank Accession Number
Outgroups (Gracilariales)		
Curdiea crassa Millar	Bongin Bay, North of Sydney, NSW Australia	AY049427
Melanthalia abscissa (Turner) Hooker & Harvey	New Zealand; coll. Gurgel, C.F. and Fredericq, S	AY049428
Ingroup (Gracilariales)		
Gracilaria beckeri (J. Agardh) Papenfuss	Sharks Bay, Port Alfred, South Africa; coll. M. H. Hommersand	AY049377
Gracilaria capensis Schmitz ex Mazza	Sharks Bay, Port Alfred, South Africa; coll. M. H. Hommersand	AY049378
Gracilaria huangii	The Bluff, Durban, KwaZulu-Natal, South Africa; coll. O. De Clerck	DQ296121
Gracilaria viellardii Silva	Taiwan; coll. S.M.Lin	AY049394
Gracilaria spinulosa	Little Yeliu, Taitung, Taiwan; coll. S.M. Lin & F.K. Huang	AY737444
Gracilaria sp.	Keelung, Taiwan; coll. S.M. Lin	AY737440
Gracilaria vieilardii	Bulusan, Taiwan; coll. Allen Liu	AY737445
Gracilaria incurvata	Chiba: Shimoda, Japan	DQ095792
Gracilaria textorii (Suringar) De Toni	Tongyong 4, South Korea	DQ095810
Gracilaria flabelliforme (Crouan & Crouan) Gurgel & Fredericq	Walton Rocks, Florida, USA; coll. C. F. Gurgel & S. Fredericq	AY049354
Gracilaria occidentalis (Børgensen) Bodard	Offshore LA, USA; coll. C. F. Gurgel & S. Fredericq	AY049322
Gracilaria ornate Areschoug	Fort Randolph, Colon city, Panama; coll. B. Wysor	AY049318
Gracilaria cylindrica	North Carolina, Onslow Bay,USA; coll. Freshwater & Johns	EU605706
Gracilaria apiculata J. Agardh	Mexico	AY049352
Gracilaria domingensis Sonder ex Kützing	Praia Rasa, Bu´zios city, Rio de Janeiro State, Brazil; coll. C. F. Gurgel	AY049371
Gracilaria cervicornis (Turner) J.Agardh	Key West, Florida, USA	AY049368
Gracilaria cuneata Areschoug	Brazil; coll. C. F. Gurgel	EU380717
Gracilaria curtissiae J. Agardh	-	AY049340
Gracilaria armata (C. A. Agardh) Greville	Grotta della Regina, Bari, Italy	AY651044
Gracilaria longa	Napoli, Rivafiorita, Italy	AY651050
Gracilaria bursa-pastoris (S. Gmelin) Silva	Clew Bay, Co. Mayo, Ireland	AY651049

Table 3.4 (continued).

Gracilaria lacinulata (Vahl) Howe	La Encrujijada, Venezuela	AY049332
Gracilaria tikvahiae McLachla	Aregood, Tampa Bay, Florida, USA	AY049440
Gracilaria cuneifolia	Tongyong, South Korea	DQ095789
Gracilaria damaecornis J. Agardh	HBOI, Florida, USA	AY049326
Gracilaria parvispora	Hawaii, USA	EF434945
Gracilaria intermedia J. Agardh	Puerto Escondido, Peninsula Paraguana, Venezuela; coll. C. F. Gurgel	AY049336
Gracilaria yoneshigueana Gurgel, Fredericq et J. N. Norris	Prainha beach, Arraial do Cabo City, Rio de Janeiro State, Brazil; coll. A. Taouil	AY049327
Gracilaria venezuelensis W.R. Taylor 1942	Florida, Tampa Bay, close to the mouth of Cockroach Bay, USA; coll. Clinton Dawes	DQ239487
Gracilaria oliveirarum Gurgel, Fredericq et J.N. Norris	La Vela de Coro, Falcon State, Venezuela; coll. C.F. Gurgel	AY049330
Gracilaria smithsoniensis Gurgel, Fredericq et J.N. Norris	Galeta Point, Atlantic Panama; coll. B. Wysor	AY049321
Gracilaria hayi Gurgel, Fredericq et J.N. Norris	Hutchinson Is. beach close to Fort Pierce jetty, Fort Pierce, FL, USA; coll. C. F. Gurgel	AY049319
Gracilaria galetensis Gurgel, Fredericq et J.N. Norris	Galeta Point, Atlantic Panama; coll. B. Wysor	AY049320
Gracilaria mammillaris (Montagne) Howe in Britton	Offshore LA, USA; coll. C. F. Gurgel & S. Frdericq	AY049323
Gracilaria arcuata Zanardini	Hilutangdu, Cebu, Philippines; coll. S. M. Lin	AY049383
Gracilaria salicornia (C. Agardh) Dawson	Sulpa, Cebu, Philippines; coll. S. M. Lin	AY049385
Gracilaria longa Gargiulo, De Masi et Tripodi	Lake Ganzirri, Messina, Italy	AY651053
Gracilaria pacifica Abbott	Indian Island, WA, USA; coll. M. H. Hommersand	AY049398
51 *Gracilaria changii*	Middle Banks, Penang	-
7 *Gracilaria changii*	Gua Tanah, Malacca	-
322 *Gracilaria changii*	Selalang, Sarikei, Sarawak	-
293 *Gracilaria changii*	Kampung Gong Batu, Terengganu	-
42 *Gracilaria changii*	Middle Banks, Penang	-
T1 *Gracilaria changii*	Ao Cho, Trat, Thailand	-
V19 *Gracilaria changii*	Phu Quoc, Kien Giang, Vietnam	-
1 *Gracilaria changii*	Morib, Selangor	-
64 *Gracilaria changii*	Batu Tengah, Malacca	-
374 *Gracilaria changii*	Pulau Nunuyan Laut, Sandakan, Sabah	-
375 *Gracilaria changii*	Pulau Nunuyan Laut, Sandakan, Sarawak	-

Table 3.4 (continued).

377 *Gracilaria changii*	Kampung Dandulit, Sandakan, Sabah	-
Gracilaria changii (Xia & Abbott) Abbott, Zhang & Xia	-	AY049388
Hydropuntia dura	Taranto, Mar Piccolo, Italy	AY651058
Hydropuntia sp	Kibbutz Maagan Mihail, Israel	DQ241571
Hydropuntia crassissima P. et H. Crouan in Maze´ et Schramm	Fort Randolph, Colon City, Panama; coll. B. Wysor	AY049351
Hydropuntia usneoides (C. Agardh) Gurgel et Fredericq comb. nov.	Walton Rocks, Florida, USA	AY049363
Hydropuntia cornea J. Agardh	Puerto Escondido, Peninsula Paraguana, Falcon State, Venezuela; coll. C. F. Gurgel	AY049338
Hydropuntia caudata	Vero Beach, USA	AY049359
Hydropuntia secunda Crouan & Crouan	HBOI, Florida, USA	AY049361
Hydropuntia punctata	Five Caves, Orchid Island, Taiwan; coll. S.M. Lin	AY737448
Hydropuntia edulis (Gmelin) Silva	Malaysia	AY049391
Hydropuntia edulis	Batac 1, Philippines	EF434914
Hydropuntia preissiana (Sonder) Womersley	Cervantes, Australia; coll. M. H. & F. H. Hommersand	AY049403
Hydropuntia rangiferina (Ku¨tzing) Piccone	La Vista del Mar, upper Calarian, Zamboanga City, Philippines; coll. S.M. Lin	AY049380
Hydropuntia eucheumatoides Harvey	Tambuli, Cebu, Philippines; coll.: S. M. Lin	AY049389
Hydropuntia urvilleii (Montagne)	Lee Point, Darwin, Australia; coll. M. H. Hommersand	AY049402
Gracilaria tenuistipita Chang et Xia	Hog Island Bay, Eastern Shore, VA, USA; coll. C. Tyler	AY049312
Gracilaria vermiculophylla	Belon estuary, France	AY725172
Gracilaria tenuistipitata Chang et Xia	Tokawa, Japan; coll. M. Yoshizaka	AY049324
Gracilaria chilensis	De los Lagos: Lachangua, Chile	DQ095784
Gracilariopsis longissima	Roscoff, France	DQ095786
Gracilariopsis megaspora	Lake Butler, Robe, Australia; coll. H. B. S. Womersley	AY049422
Gracilariopsis funicularis	Senegal	EU158087
Gracilariopsis cata-luziana Gurgel, Fredericq & Norris	Vera Cruz area, Mexico; coll. C. F. Gurgel	AY049406
Gracilariopsis tenuifrons	Araya Pennesula, Sucre, Venezuela	U04171
Gracilariopsis sjoestedtii (Kylin) (Kylin)	-	AY049413
Gracilariopsis costaricensis Dawson	South end, Playa Tamarindo, Nicoya Penninsula, Guanacaste, Costa Rica; coll. D. T. Talbot & D. W. Freshwater	AY049423

Table 3.4 (continued).

Gracilariopsis lemaneiformis (Bory) Dawson, Acleto et Foldvik	Yacilla, Paita, Piura, Peru; coll. C. Acleto & R. Zuniga	AY049415
Gracilariopsis carolinensis Liao & Hommersand in Gurgel, Liao, Fredericq & Hommersand	Kure Beach, Fort Fisher, NC, USA; coll. D. W. Freshwater	AY049412
Gracilariopsis hommersandii Gurgel, Fredericq & Norris	Falcon State, Peninsula Paraguana, La Encrucijada, Venezuela	AY049407
Gracilaria chorda	Daeryeon, China	EF434904
Gracilariopsis chorda	Jindo, Hoidong, South Korea	DQ095785
Gracilariopsis changii	Sail Rock, Kenting National Park, Taiwan; coll. S. M. Lin	DQ119746
Gracilariopsis sp	Kenting National Park, Southern Taiwan; coll. S. M. Lin	FJ235529
Gracilariopsis silvana	Falcon State, La Vela de Coro, Venezuela; coll. C.F.D. Gurgel	AY049309
Gracilariopsis mclachlanii	Pangamkungu, Tanzania	EU158092
Gracilariopsis sp mclachlanii	Sazadongo, Tanzania	EU168333
Gracilariopsis heteroclada (Zhang et Xia) Zhang et Xia in Abbott	Dapdap, Bulusan, Luzon, Philippines; coll. S. M. Lin	AY049411
Gracilariopsis persica	Iran	EU158094

3.7 Haplotype Analysis

Sequencing data were analysed and edited using ChromasPro version 1.42 (2003-2008 Technelysium Pty Ltd) and BioEdit Sequence Alignment Editor Version 7.0.9.0 (Hall, 1999) software. Edited sequences were aligned by CLUSTAL X program (Thompson et al., 1997) and further refined using naked eyes before subjected to genetic diversity analyses and intraspecific phylogenetic analysis.

3.7.1 Statistical Parsimony Network

In intraspecific genetic diversity analyses among the mitochondrial haplotypes of *G. changii* and *G. edulis* were inferred using the statistical parsimony networking (SPN) approach implement in TCS version 1.21 (Clement et al., 2000). This program able to calculate the minimum number of mutational steps by which the sequences could be joined with >95% confidence. The haplotype diversity (*Hd*) and nucleotide diversity (*Pi*) were determined by DNA sp program (Rozas & Rozas, 2000). Estimation of genetic distance of between the *cox*1 haplotypes for *G. changii* and *G. edulis* were calculated in PAUP* v4.0b10 (Swofford, 2003).

3.7.2 Intraspecific Phylogenetic Analysis

The intraspecific phylogenetic relationship among the *cox*1 haplotypes for *G. changii* and *G. edulis* were inferred using Maximum Likelihood (ML) and Bayesian Inference (BI) analyses. The program Kakusan version 3 (Tanabe, 2007) was used to find out best fit models for (i) maximum likelihood (ML) analysis performed using Treefinder version of October 2008 (Jobb et al., 2004); and (ii) Bayesian Inference (BI) analysis performed using Mr. Bayes 3.1.2 (Huelsenback et al., 2001). Cladogram of ML was constructed by program of TreeFinder and with 1,000 ML bootstrap replicates. The

method of four Markov chains over two independent runs of 2 X 10^6 generations was employed for BI analysis. Only nodes with Bayesian posterior probabilities (PP) greater than 50% as a validity supported clade for the construction of a 50% majority rule consensus tree. All phylogenetic trees were viewed with Treev32 (Page, 1996). For all of the analyses, the *cox*1 sequences of *Gracilariopsis longis* (EF434940) and *Gracilariopsis chorda* (EU567363) were downloaded from the GenBank sequence database and used as outgroups. One representative sequence of each haplotype for *G. changii* and *G. edulis* were used for phylogenetic analyses (Table 3.5). Part of the seven *cox*1 haplotypes data of *G. changii* were published in Yow et al. (2011 & 2013) (GenBank accession number: C1=GU645769, C2= GU645777, C3=GU645763, C4=GU645771, C5=GU645770, C6=GU645786, C7=JX228063).

Table 3.5 List of representative *cox*1 haplotypes of *G. changii* and *G. edulis* used for construction of Maximum Likelihood and Bayesian trees.

Species	**Haplotype**	**GenBank Accession No.**	**Abbreviation**
G. changii	C1	GU645769	GU645769 (C1)
	C2	GU645777	GU645777 (C2)
	C3	GU645763	GU645763 (C3)
	C4	GU645771	GU645771 (C4)
	C5	GU645770	GU645770 (C5)
	C6	GU645786	GU645786 (C6)
	C7	JX228063	JX228063 (C7)
	C8	-	Sarawak (C8)
G. edulis	E1	-	Morib (E1)
	E2	-	MiddleBanks (E2)
	E3	-	TmnSeriKuala (E3)
	E4	-	BatuTengah (E4)
	E5	-	GuaTanah (E5)
	E6	-	BatuTengah (E6)
	E7	-	Kukup (E7)
	E8	-	KongKong (E8)
	E9	-	Kukup (E9)
	E10	-	Nunuyan (E10)
	E11	-	GreenView (E11)
	E12	-	TelukPelanduk (E12)
	E13	-	Indonesia (E13)
	E14	-	Indonesia (E14)
	E15	-	Japan (E15)

CHAPTER 4

RESULTS

4.1 MORPHOLOGY AND REPRODUCTIVE STRUCTURES STUDIES ON *GRACILARIA* SPECIES

4.1.1 *Gracilaria changii* (Xia et Abbott) Abbott, Zhang et Xia

Healthy samples of *Gracilaria changii* were collected in the field from a variety of habitats in Malaysia, Thailand and Vietnam. Table 4.1 summarises the list of collection sites, habitats and Global Position System (GPS) coordinates of *G. changii* that were collected.

Table 4.1 Detailed information of the sampling points and geographical information for *Gracilaria changii* collected from Malaysia, Thailand and Vietnam.

COUNTRY	STATE	LOCATION	HABITAT	GPS READING
Malaysia	Kedah	Kuala Sungai Merbok	Attached on fish cages	N 05° 40.553' E 100° 22.722'
	Penang	Middle Banks	Sandy soil area	N 05° 21.897' E 100° 19.574'
	Perak	Kuala Sepetang	Fish cages	N 04.86217' E 100.57339'
	Selangor	Morib	Mangrove	N 02°45.739' E 101°26.070'
	Negeri Sembilan	Teluk Pelanduk	Sandy and rocky beach	N 02° 25' 08.08' E 101° 53' 07.20'
		Pantai Dickson	Rocky and mangrove	N 02° 25' 02.23' E 101° 53' 41.73'
	Malacca	Batu Besar	Sandy and rocky	N 02° 20.758' E 102° 02.682'
		Batu Tengah	Sandy and rocky	N 02° 20.936' E 102° 02.352'
		Gua Tanah	Sandy and rocky	N 02° 13.862' E 102° 10.948'
	Johore	Sungai Pulai	Fish cages	N 01° 26.117' E 103° 32.850'
		Sungai Kong Kong	Fish cages	N 01° 31.072' E 193° 59.911'
		Teluk Sari	Mangrove	N 02° 37'26.9 E 103° 45'27.9
	Terengganu	Kampung Gong Batu	Fish cages	N 05° 41.221' E 102° 42.465'

Table 4.1 (continued).

COUNTRY	STATE	LOCATION	HABITAT	GPS READING
Malaysia	Sarawak	Selalang	Fish cages	N 01° 58.444' E 111° 26.729'
	Sabah	Kawasan Perumahan Green View	Sandy and rocky	N 05° 51.909' E 118° 07.247'
		Kampung Dandulit	Attached on coarse rocks, muddy area	N 05° 59.694' E 117° 54.555'
		Pulau Nunuyan Laut	Muddy area	N 05°54.811' E 118°05.451'
Thailand		Ao Cho, Trat	N/A	N 12° 58.516' E 102° 33.442'
Vietnam		Phu Quoc, Kien Giang	N/A	N 10°20.000' E 103°59.300'

*N/A: Not Available

Based on the sampling collections, *G. changii* has been collected from various localities in Malaysia: Kedah, Penang, Perak, Selangor, Negeri Sembilan, Malacca, Johor, Terengganu, Sabah and Sarawak. *G. changii* from Thailand and Vietnam were also collected and examined. *G. changii* is naturally found to grow as attached epiphytes to roots of mangrove plants (e.g. *Avicennia* trees), sandy beaches, rocks, coral, muddy areas and attached on fish cages. Photographs show the specimens collected from different natural habitats (Fig. 3.1-3.8). *G. changii* collected were no significant morphological variation and regardless of the locations. However, the specimens from Selalang (Sarawak) and Pulau Nunuyan Laut (Sabah) were phenotypically dissimilar.

Gracilaria changii (Xia et Abbott) Abbott, Zhang et Xia was first described by Xia & Abbott (1987) based on the type specimen from Middle Banks, Penang. *G. changii* collected from different localities showed general features: appeared brownish to dark brown in fresh state and black when dry; has a discoid holdfast with turgid and cylindrical thallus ranging from five to 25 cm in length; branching is irregular, secund or alternate in three to four orders. One of the distinctive characteristics of *G. changii* is that each branch is abruptly constricted at the base forming a slender stipe, swollen distal end of stipe, and gradually tapering towards the apex of the plant. Figure 4.1

shows the fresh specimen of *G. changii* collected from type locality, Middle Banks, Penang. Vegetative cells in transverse section encompass large and rounded medullar cells with thick walls of 8.2 to 21.3 µm and two to three layers of cortical cell and the transition abrupt.

The spermatangial receptacles range from *verrucosa* to *polycavernosa* type. The cystocarps were constricted at bases, semi-globose or conical in sharp, 0.2 to 0.6µm; pericarps consisted of two types of cells: inner part with seven to nine compressed cells, outer part with five to six rows of rounded to oval cells; gonimoblast filaments consist many small cells, carposporangia rounded or ovoid in shape. The tetrasporangia were ovoid or sometimes elongate, found densely scattered over the surface of the thallus.

Figure 4.1 Herbarium of *G. changii* from type locality, Middle Banks, Penang, Malaysia.

Based on the morphological observations, *Gracilaria changii* collected from Selalang (Sarawak) and Pulau Nunuyan Laut (Sabah) were different compared to *G. changii* from other populations. The specimens from Selalang, Sarawak, were congruent with morphological descriptions of *G. changii* from Xia & Abbott (1987) and Lim & Phang (2004), but these specimens had discoid holdfast with no main axis (Fig. 4.2). For *G. changii* from Pulau Nunuyan Laut, Sabah, they grow in habitat of muddy area with seawater salinity of 30 ppt. The specimen adheres imperfectly to the herbarium paper upon drying. The plants are brownish red in colour and ranged from 35-42 cm in height (Fig. 4.3). They had discoid holdfast with no main axis, the branching was irregular, alternate or secund in mostly three orders with conspicuous constriction at the base (Fig. 4.4), and rounded to blunt branching apex with new branches formed from blunt branching apex (Fig. 4.5). Vegetative cells in transverse section encompass large and rounded medullar cells and two to three layers of cortical cell and the transition abrupt, where outermost cells elongated to spherical (Fig. 4.6). The cystocarps were prominently protruding, slightly rostrate, not constricted at the base, 291-748 µm by 396-1208 µm; pericarps consisted 11-12 layers of cells, 53-102 µm thick; gonimoblast consists many small cells, carposporangia rounded or ovoid in shape, and of 13-20 by 3-11 µm in size; presence of nutritive filaments (Fig. 4.7). The tetrasporangia were ovoid or sometimes elongate, densely scattered over the surface of frond (Fig. 4.8). Table 4.2 shows the comparison of morphological and anatomical characters for *Gracilaria changii* from other localities and *G. changii* from Pulau Nunuyan Laut, Sabah. Further identification with molecular approach using the plastid-encoded large subunit of rubisco gene (*rbc*L) for *G. changii* from Selalang and Pulau Nunuyan Laut was carried out.

Table 4.2 Comparison of morphological and anatomical characters for *Gracilaria changii* from other localities and *G. changii* from Pulau Nunuyan Laut, Sabah.

Character	***Gracilaria changii* (Xia et Abbott) Abbott, Zhang et Xia**	***Gracilaria changii* from Pulau Nunuyan Laut**
Color	Brownish to dark brown	Brownish red
Discoid holdfast	Present	Present
Branching pattern	Irregular, alternate or secund; abrupt constriction at the base	Irregular, alternate or secund, constricted at the base
Branching order	3- 4 orders	Mostly 3 orders
Branch apex	Tapering towards apex	Blunt
Main axis	Present	Absent
Cortex & medullar	2-3 layers of cortical cells; rounded medullary cells, and the transition abrupt	2-3 layers of cortical cells; rounded medullary cells, and the transition abrupt
Cystocarp	Prominently protruding, slightly rostrate, constricted at the base	Prominently protruding, slightly rostrate, not constricted at the base
Nutritive filament	Present	Present
Gonimoblast filament	Consisting of many small cells, carposporangia rounded or ovoid in shape	Consisting of many small cells, carposporangia rounded or ovoid in shape
Tetrasporangia	Tetrasporangia scattered over the surface of frond	Tetrasporangia scattered over the surface of frond
Reference	Xia & Abbott , 1987; Lim & Phang , 2004	-

Figure 4.2 Fresh specimen of *G. changii* from Selalang, Sarawak, East Malaysia.

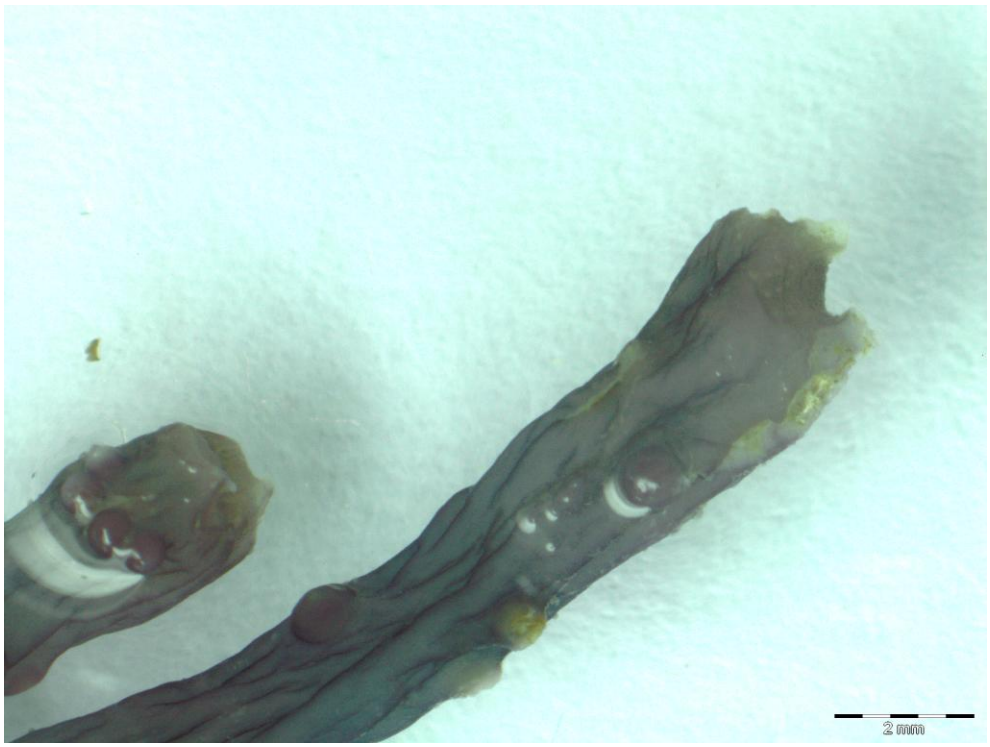
Figure 4.3 Fresh specimen of *G. changii* from Pulau Nunuyan Laut, Sandakan, Sabah, East Malaysia.

(a)

(b)

Figure 4.4 *G. changii* from Pulau Nunuyan Laut **(a)** Fresh female thallus showing the discoid holdfast with no main axis (arrow); **(b)** Branching mode was irregular, alternate or secund in mostly three orders with conspicuous constriction at the base (arrows).

(a)



(b)

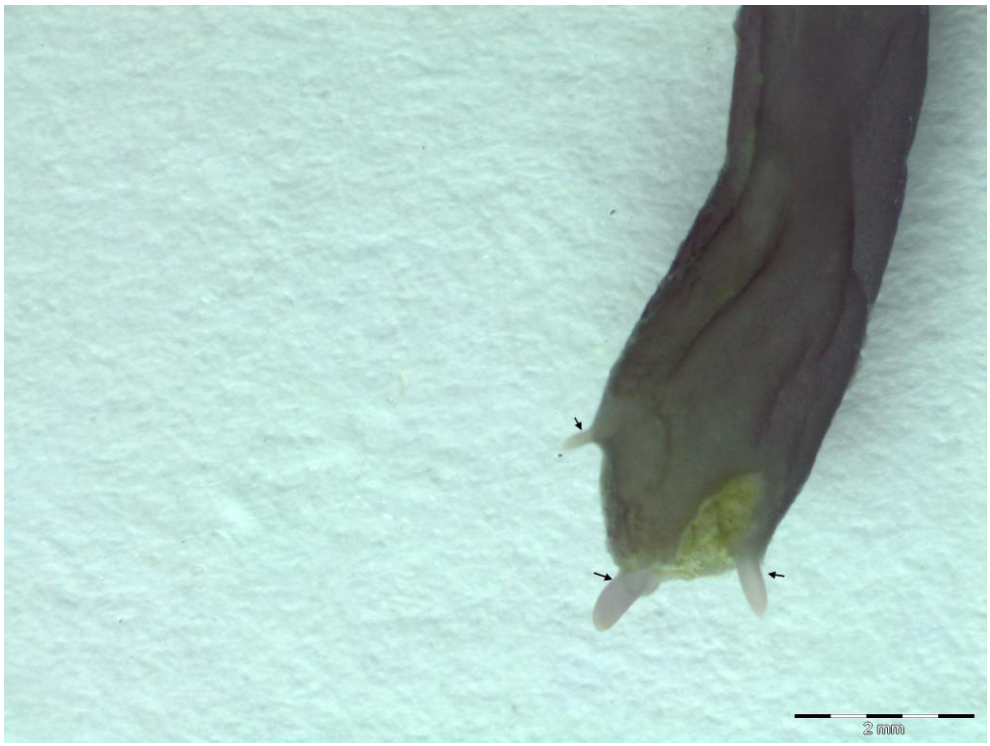
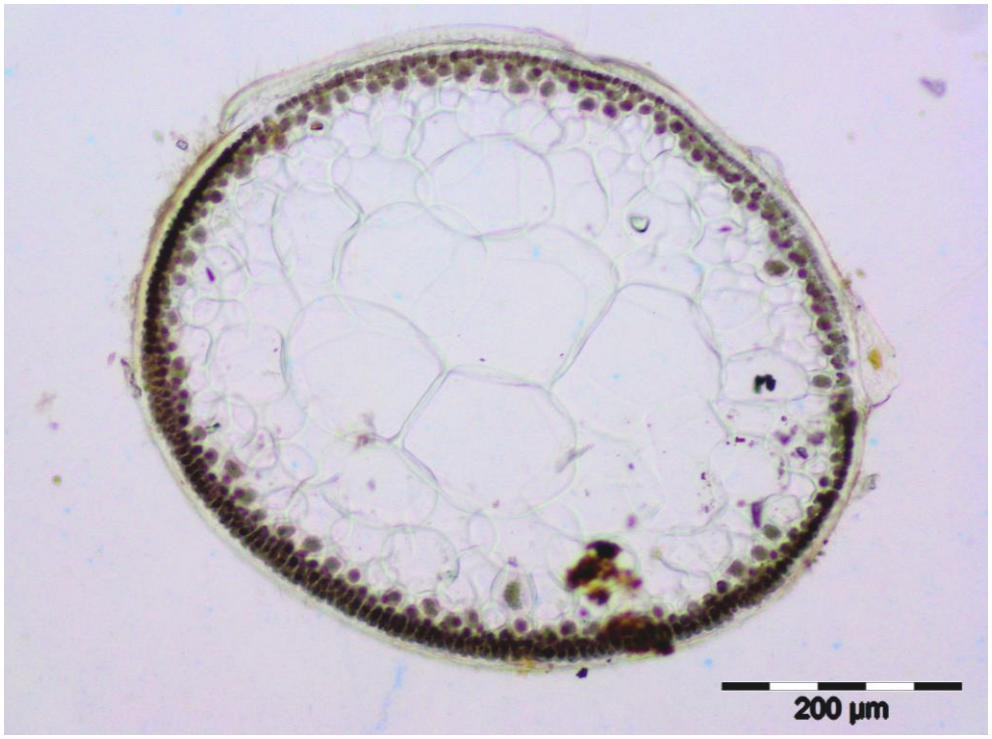


Figure 4.5 Dried specimen of *G. changii* from Pulau Nunuyan Laut showed **(a)** Rounded to blunt branching apices; **(b)** New branches formed from blunt branching apex (arrows).

(a)



(b)

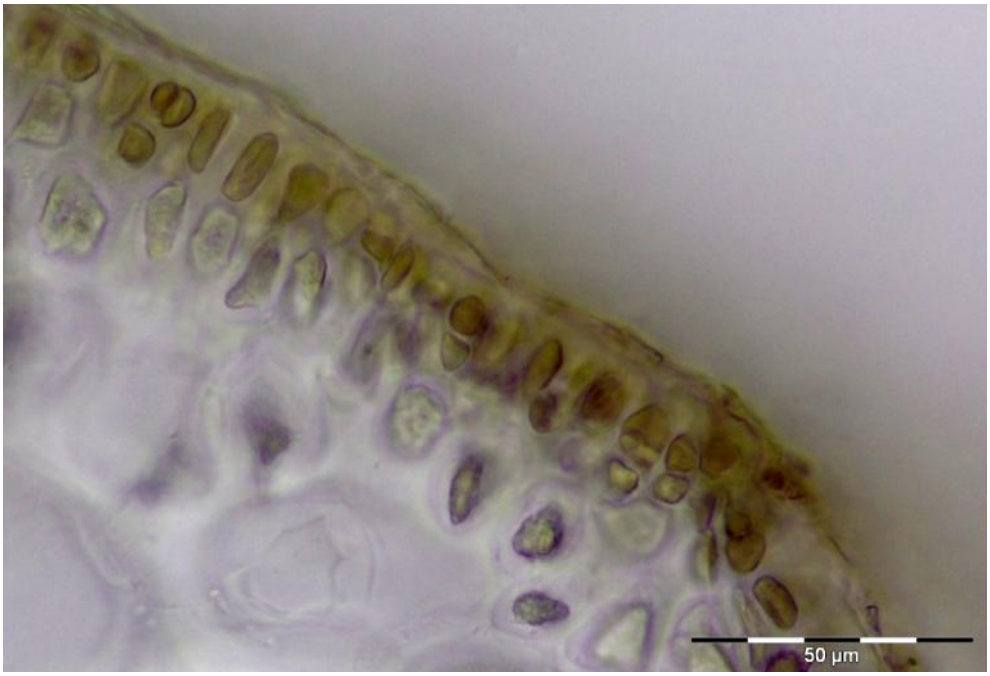
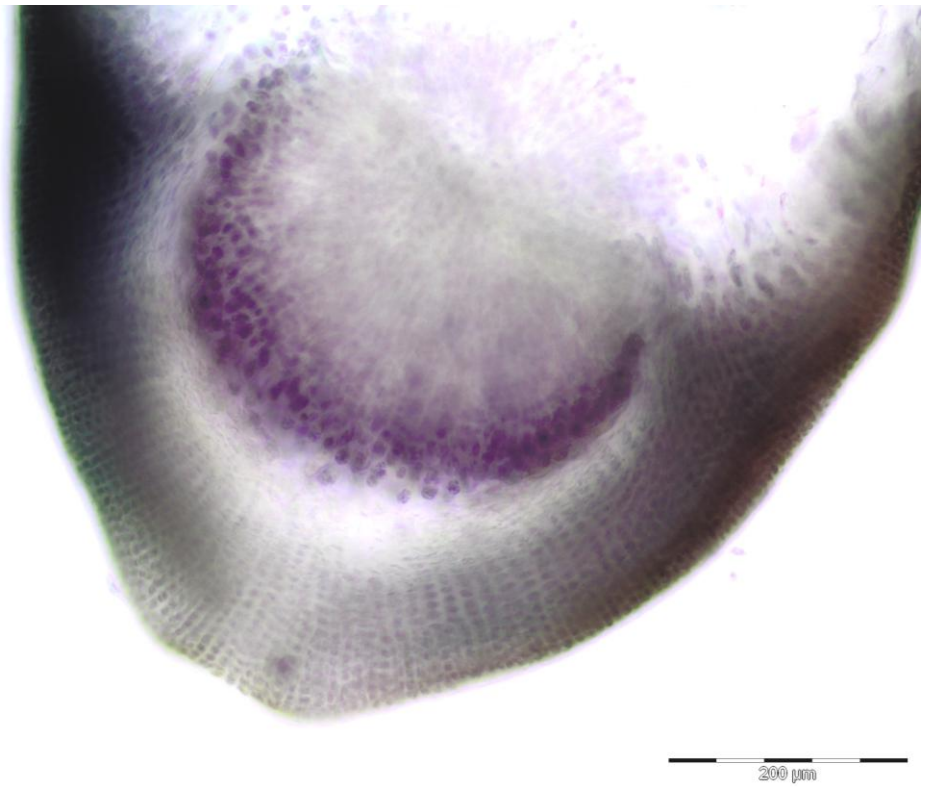


Figure 4.6 *G. changii* **(a)** Photomicrograph of cross-section of a thallus showing a transition of medulla cells from medulla to cortex abrupt; **(b)** Photomicrograph showing the cortex consisting of 2-3 cell layers and outermost cells are elongated to spherical.

(a)



(b)

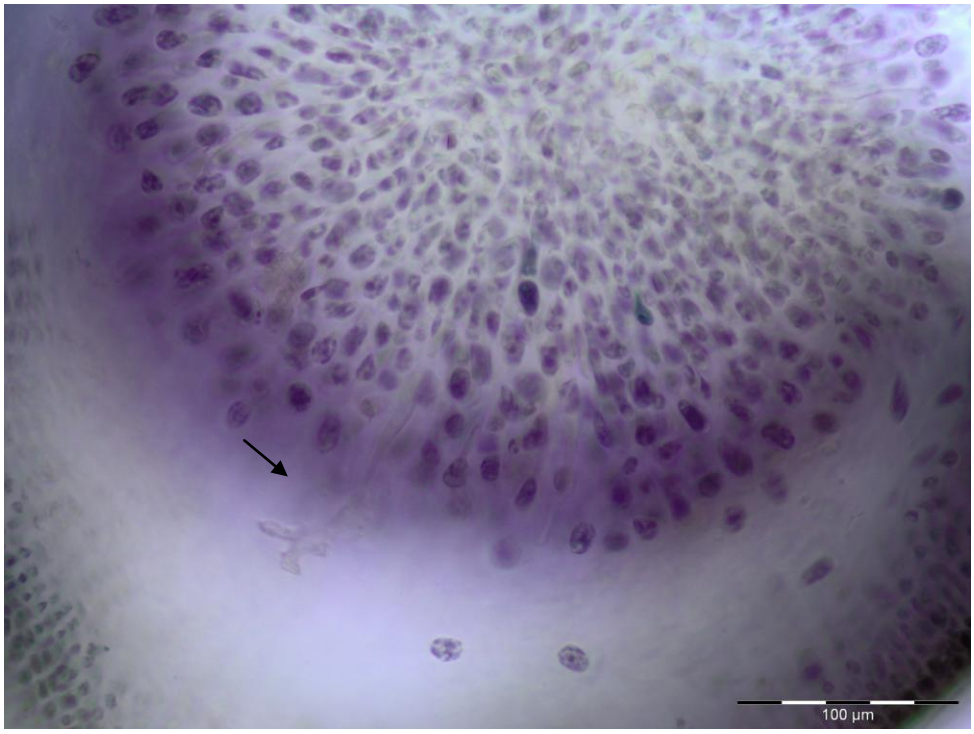
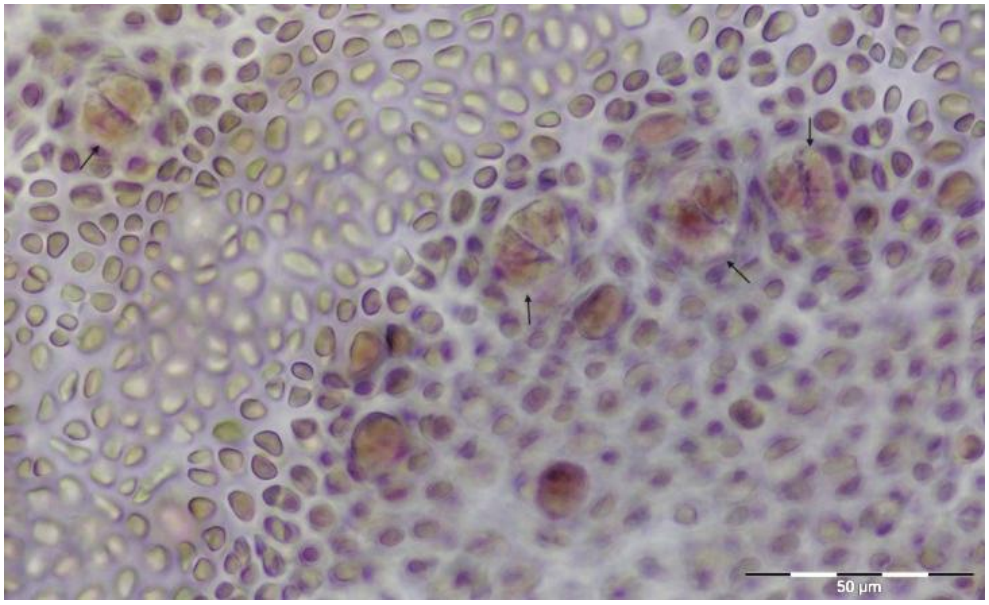


Figure 4.7 *G. changii* **(a)** Photomicrograph showing transverse section of a mature cystocarp where pericarps consisted 11-12 layers of cells, 53-102 µm thick; gonimoblasts consisting of small cells, carposporangia rounded or ovoid in shape, and of 13-20 by 3-11 µm in size; **(b)** transverse section of a cystocarp showing a presence of nutritive filament (arrow).

(a)



(b)

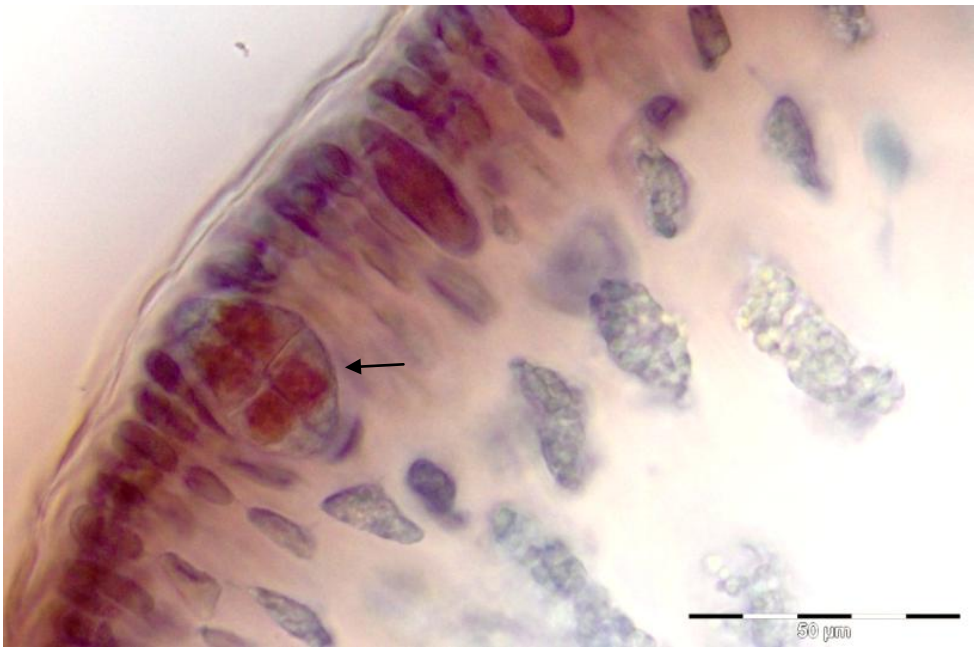


Figure 4.8 *G. changii* **(a)** The tetrasporangia are densely scattered over the entire surface of frond in surface view (arrows); **(b)** Cross-section of a tetrasporangia-bearing branch showing mature tetrasporangium (arrow) and young ones.

4.1.2 *Gracilaria edulis* (Gmelin) Silva

Healthy samples of *Gracilaria edulis* were collected randomly in field from a variety of habitats in countries of Malaysia, Thailand, Indonesia and Japan. Table 4.3 summarises the list of collection sites, habitats and Global Position System (GPS) coordinates of *G. edulis* that were collected.

Table 4.3 Detailed information of the sampling points and geographical information for *Gracilaria edulis* collected from Malaysia, Thailand, Indonesia and Japan.

COUNTRY	STATE	LOCATION	HABITAT	GPS READING
Malaysia	Kedah	Pulau Sayak	Muddy area	N 05° 39'38.78" E 100°20' 11.10"
	Penang	Batu Feringgi	Attached on fish cages	N 05° 28.522' E 100.249'
		Middle Banks	Sandy beach	N 05° 21.897 E 100° 19.574'
	Selangor	Morib	Mangrove swamp	N 02°45.739' E 101°26.070'
	Negeri Sembilan	Teluk Pelanduk	Sandy and rocky	N 02° 25' 08.08' E 101° 53' 07.20'
	Malacca	Batu Besar	Sandy and rocky	N 02° 20.758' E 102° 02.682'
		Batu Tengah	Sandy and rocky	N 02° 20.936' E 102° 02.352'
		Gua Tanah	Sandy and rocky	N 02° 13.862' E 102° 10.948'
	Johore	Kukup	Fish cages	N 01° 19.692' E 103° 26.455'
		Merambung shore	Fish cages	N 01° 21'08.83" E 104° 01.03'
		Pulau Che Kamat	Sandy and rocky	N 01° 31.072' E 193° 59.911'
		Sungai Kong Kong	Fish cages	N 01° 31.072' E 193° 59.911'
		Teluk Ramunia	Sandy and rocky	N 02° 37'26.9 E 103° 45'27.9
	Sabah	Kawasan Perumahan Green View	Sandy and rocky	N 05° 51.909' E 118° 07.247'
		Pulau Nunuyan Laut	Muddy area	N 05°54.811' E 118°05.451'
Thailand		Prachuap Khiri Khan	N/A	N 11°49.000' E 99°48.000'
Indonesia	Lombok	Landangluar	Attached on rocks that covered in mud	S 08°27.719' E 116°02.164'
		Gili Meno	Sandy & rocky beach	S 08°21.299' E 116°03.199'

Table 4.3 (continued).

COUNTRY	STATE	LOCATION	HABITAT	GPS READING
Indonesia	Lombok	Gili Genting	Attached on rocks	S 08°43.834' E 115°57.937'
		Batu Kijok	Attached on rocks	S 08°44.510' E 116°01.381'
		Rambang	Attached on rocks	S 08°43.893' E 116°33.103'
Japan	Okinawa	Bise, Motobu, Okinawa	Intertidal pool, attached to rock	N 26° 42.075' E 127°52.747'

*N/A: Not Available

Gracilaria edulis (Gmelin) Silva for this study were collected from a range of localities in Malaysia: Kedah, Penang, Perak, Selangor, Negeri Sembilan, Malacca, Johore, Terengganu, Sabah and Sarawak. *G. edulis* from Indonesia and Japan were also included for better understanding of the genetic variation of this agarophyte. *G. edulis* is normally found in close association with *Gracilaria changii* in the same stream habitats. Photograph show the specimens collected from Batu Tengah, Malacca, Peninsular Malaysia (Fig. 4.9). The specimens that were identified based on the morphological descriptions from Lim & Phang (2004). *G. edulis* collected were similar and regardless of the locations.

The plants appeared dark red in fresh state, and black when dried. Thalli of *G. edulis* arises from a discoid holdfast, plant is much branched and wiry than *G. changii*, form entangled masses branching of dichotomous or trichotomous up to maximum of seven orders. The branches are cylindrical with spinelike ultimate apices and slightly constricted or not at base. Vegetative cells in transverse section encompass large and rounded medullar cells with the transition abrupt, where outermost cells elongated to spherical (Fig. 4.10a). Figure 4.10 (b) is the surface view showing the present of hair cells. The cystocarps are globose, constricted at bases with rostrate tips (Fig. 4.11a). The pericarps were thick and consist of nine to fourteen rows of cells, with robust basal absorbing filaments having many branches (Fig. 4.11b). The spermatangial receptacles

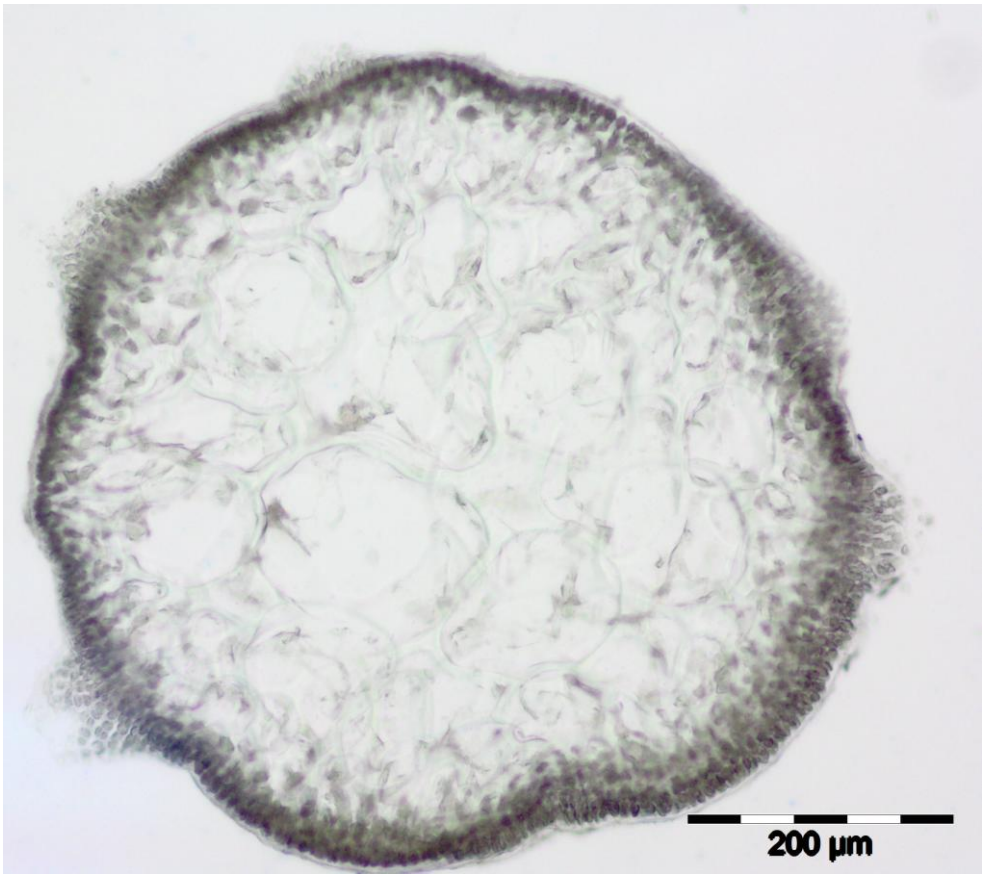
range from *verrucosa* to *polycavernosa* type (Fig. 4.12). The tetrasporangia were ovoid to oblong, found scattered over the surface of the thalli. Table 4.4 shows the morphological and anatomical characters for *Gracilaria edulis* in this study.

Table 4.4 Morphological and anatomical characters for *G. edulis.*

Character	***Gracilaria edulis* (Gmelin) Silva**
Color	Dark red
Holdfast	Discoid
Branching pattern	Dichotomous or trichotomous, slightly constricted or not at the base
Branching order	Up to seven orders
Branch apex	Apices pointed
Main axis	Present
Cortex & medullar	1-2 layers of cortical cells; rounded medullary cells, and the transition abrupt
Cystocarp	Globose with rostrate tips, constricted at the base
Spermatangia	Range from the *verrucosa* and *polycavernosa*-type
Nutritive filament	Extending into pericarp
Gonimoblast filament	Consisting of many small cells, carpospores rounded or ovoid in shape
Tetrasporangia	Tetrasporangia densely scattered over the surface of frond

Figure 4.9 Fresh specimen of *G. edulis* collected from Batu Tengah, Malacca, Peninsular Malaysia.

(a)



(b)

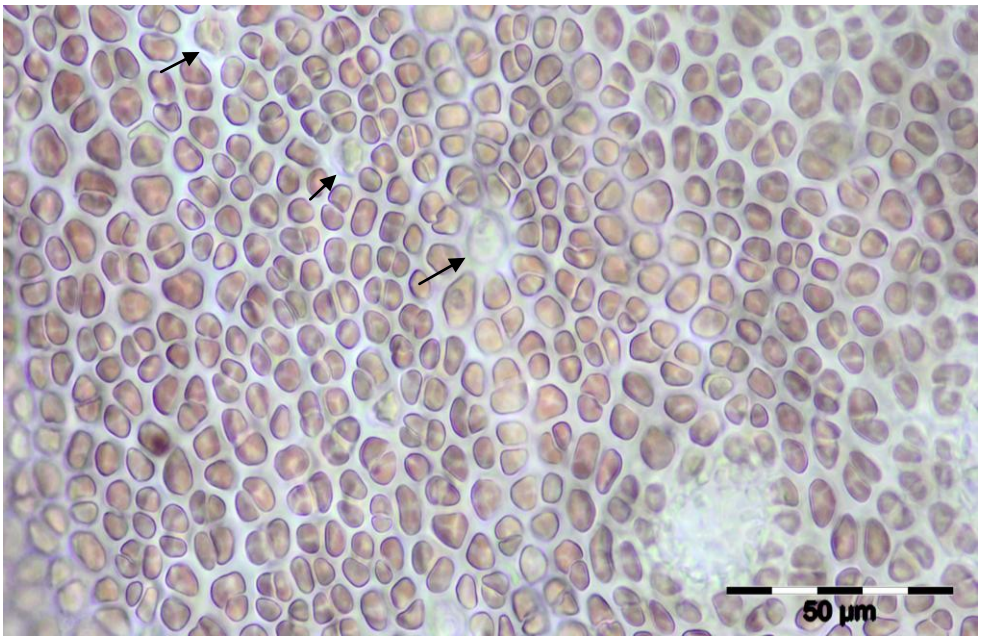
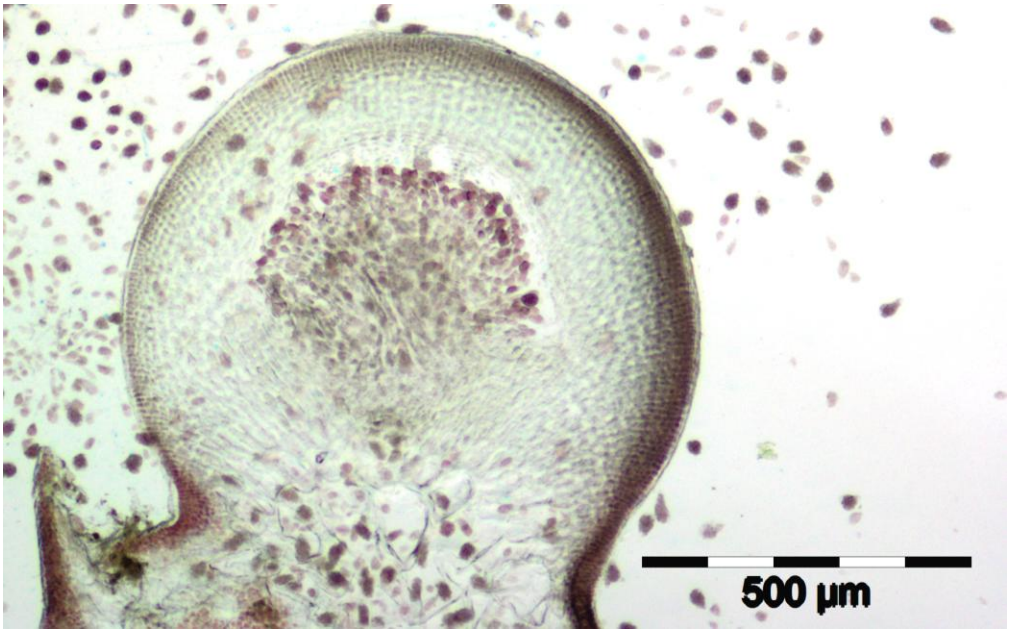


Figure 4.10 *G. edulis* **(a)** Photomicrograph showing a transition of medulla cells from medulla to cortex abrupt; **(b)** Surface view of hair cells (arrows).

(a)



(b)

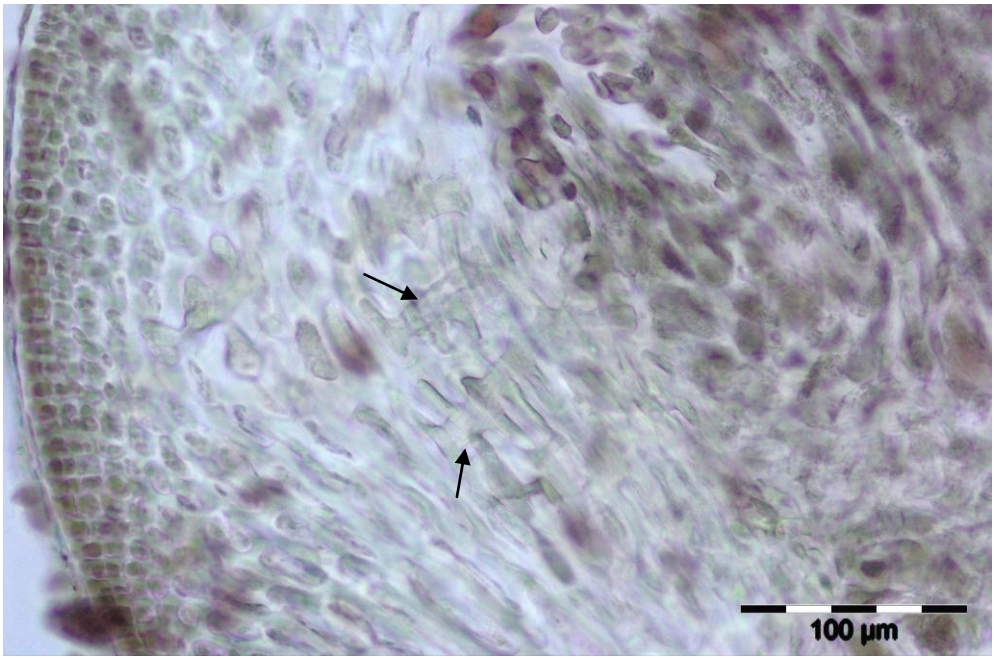
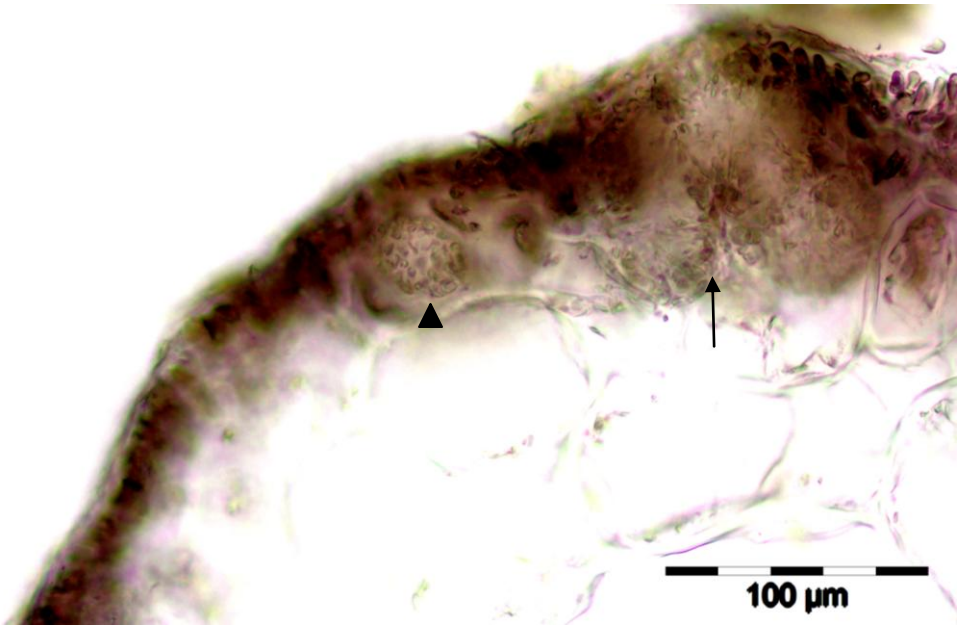


Figure 4.11 *G. edulis.* **(a)** Transverse section of a cystocarp showing pericarp with 9 to 14 rows of cells and gonimoblasts consisting of small cells; **(b)** Same section as in figure 4.11 (a) showing presence of nutritive filaments (arrows).

(a)



(b)

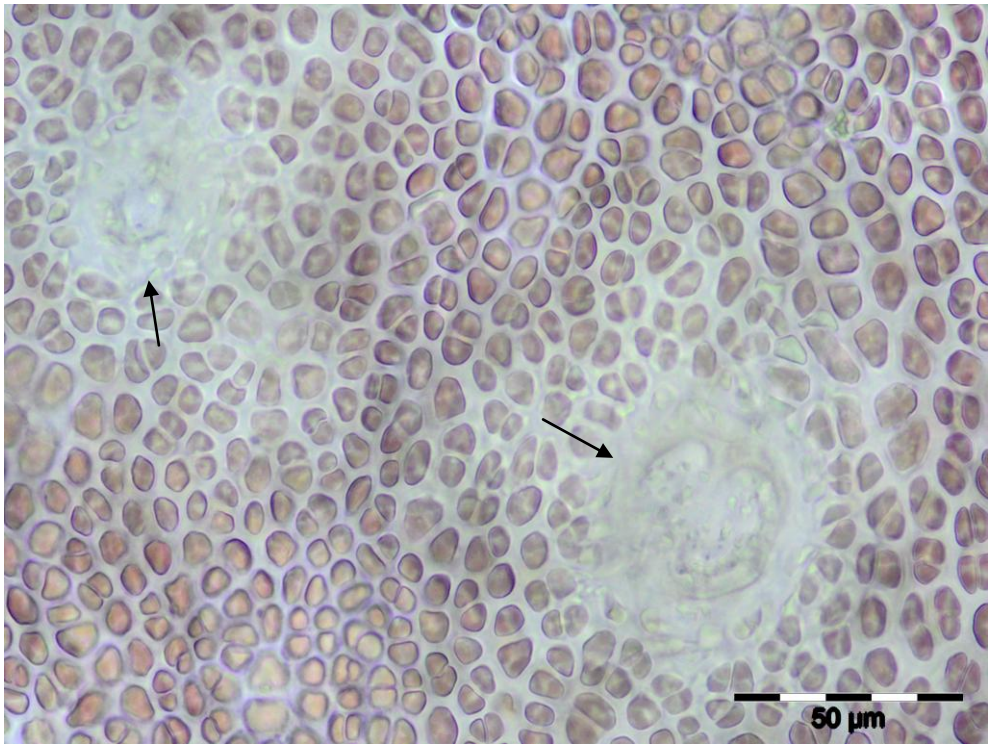


Figure 4.12 *G. edulis.* (a) Transverse section of spermatangial conceptacles-bearing branch showing *verrucosa*-type (arrowhead) to *polycavernosa*-type (arrow); (b) Surface view of conceptacles (arrows).

4.2 DEOXYRIBONUCLEIC ACID (DNA) EXTRACTION AND ISOLATION

For molecular studies, about 0.02 g dried tips were cut and ground in liquid nitrogen and total genomic DNA will be extracted using the commercially available DNeasy Plant Mini Kit (Qiagen, Germany) according to the instructions from manufacturer. A minor modification was included by incubating the disrupted samples with lysis buffer (AP1) and RNease for 20 minute at 65°C.

The purity and yield of isolated DNA can be measured by spectrophotometric at a ratio of the optical density (OD) readings at 260 nm and 280 nm (A_{260}/A_{280}). In this study, usually the OD readings of A_{260}/A_{280} were in range of 1.5 to 2.0. The concentration of isolated DNA was in range of 0.003-0.008 µg μL^{-1}.

4.3 GENE SEQUENCING

4.3.1 Polymerase Chain Reaction (PCR) of *rbc*L, *cox*2-3 Spacer and *cox*1

4.3.1.1 Large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (RUBISCO), *rbc*L

Primers used for amplification of the *rbc*L gene (primer F57, F753 and R*rbc*S Start R, Table 3.1) were successful in amplifying DNA of *G. changii*. The optimum annealing temperature for the *rbc*L gene was 48 ºC. The size of amplified product was estimated to be about 1400-1500 base pairs (bp).

4.3.1.2 Mitochondiral Cytochrome Oxidase Subunit 2 and 3 (*cox*2-3 Spacer)

The *cox*2-3 spacer was applied in the amplification reactions for the assessing of the use of the *cox*2-3 spacer and the *cox*1 gene as potential markers for inferring

intraspecific genetic variation of the *Gracilaria changii* from Peninsular Malaysia .The *cox*2-3 spacer region from *G. changii* was successfully amplified with the markers of *cox*2F and *cox*3R. The step-up PCR conditions for the marker were adapted from Zuccarello et al. (1999) and were found to be successful in amplifying the selected gene fragments. The optimal annealing temperatures for the step-up PCR to amplify the *cox*2-3 spacer in this study were 45ºC followed by 55ºC respectively. The sizes of amplified fragments of this region were in the range of 300-400 bp. The aligned sequences with 363 bp were used for the haplotype analysis.

4.3.1.3 Mitochondrial Cytochrome Oxidase Subunit I Gene (*cox*1)

Amplification of the *cox*1 gene was successfully conducted with primers of *cox*1 43F and *cox*1 1549R (Geraldino et al., 2006), and GazF1 and Gaz R1 (Saunders, 2005), respectively. Modification of PCR parameter was carried out with a lower temperature of annealing in the first five cycles where primers will be bound unspecifically to template DNA and followed by higher annealing temperature to enhance the specificity in amplification of the remaining 35 cycles. Optimal annealing conditions were: initial denaturation at 94ºC for 1 min, followed by five cycles of 94ºC for 1 min, 45ºC/ 72ºC for 1.5 min each, 35 cycles of 94ºC/ 50ºC for 30 second each, 72ºC for 2 min and final cycle at 72ºC for 10 min. The optimal annealing temperatures for the step-up PCR of both species to amplify the *cox*1 region in this study: 48ºC and 50ºC for *cox*1 43F and *cox*1 1549R; 45ºC and 48ºC for GazF1 and GazR1, respectively. Different markers worked best at different annealing temperatures and amplification conditions. The size of amplified fragment of *cox*1 43F and *cox*1 1549R primers was in the range of 1450-1500 bp, while about 700 bp for GazF1 and GazR1. Table 4.5 shows the optimized PCR condition for amplification of the *rbc*L region, *cox*2-3 spacer and *cox*1 region.

Table 4.5 A summary of optimised amplification conditions for the *rbc*L gene, *cox*2-3 spacer and *cox*1 region.

Optimised PCR Condition	**Primer**			
	***rbc*L region**	***Cox*2-3 spacer**	***Cox*1 region**	
	F57, F753 and *Rrbc*S Start R (Freshwater & Rueness, 1994)	*Cox*2F and *Cox*3R (Zuccarello et al. 1999)	*Cox*1 43F and *Cox*1 1549R (Geraldino et al., 2006)	GazF1 and GazR1 (Saunders, 2005)
dNTP	0.2 mM	0.2 mM	0.2 mM	0.2 mM
Primer	10 pmol	10 pmol	10 pmol	10 pmol
Taq polymerase	1.5 U	1.5 U	1.5 U	1.5 U
DNA	25 ng	25 ng	25 ng	25 ng
Annealing temperature	48ºC	45ºC & 55ºC	48ºC & 50ºC	45ºC & 48ºC

4.4 Phylogenetic Analysis of the *rbc*L Gene for identification of *Gracilaria changii*

The length of the *rbc*L gene for *G. changii* from selected localities in this study varied from 1452 bp to 1522 bp. The downloaded sequences of *rbc*L gene from GenBank varied from 1182 bp to 1492 bp. The lengths of all the sequences have to be standardized by complete alignment for the phylogenetic analysis. The aligned sequences with 1410 bp were used to generate ML (Maximum Likelihood) and BI (Bayesian Inference) trees.

The Maximum Likelihood and Bayesian Inference methods produced almost similar topology trees and formed two major assemblages (Fig. 4.13 & 4.14): a *Gracilaria sensu lato* clade (*Gracilaria sensu stricto* and *Hydropuntia*), and *Gracilariopsis* clade. *Gracilariopsis* was the basal genus for this tree and distant apart from *Gracilaria sento lato* clade and with 100% bootstrap support. Specimens collected from Selalang, Sarawak (Number of specimen: 322 Selalang, Sarikei, Sarawak) and Pulau Nunuyan Laut, Sabah (Number of specimen: 374 & 375 Pulau Nunuyan Laut, Sandakan, Sabah) with a high degree of morphological variability were grouped

together with *G. changii* from Malaysia, Thailand and Vietnam with full support values in *Hydropuntia* clade. Despite some differences in terms of their morphologies, the data of phylogenetic study showed that the specimens from Selalang and Pulau Nunuyan Laut are collectively known as *G. changii*, supported by full support values in ML and BI trees, respectively. *G. changii* has been transferred to *Hydropuntia* by Wynne in 1989 with new combination of *Hydropuntia changii* (Xia & Abbott) Wynne comb. nov. However, for consistency and ease of references, we retain it as *Gracilaria changii* in this study.

Figure 4.13 (a) The phylogenetic tree of *Gracilaria changii* and sequence data of the family Gracilariaceae based on *rbc*L gene sequences constructed using the maximum likelihood (ML) methods. The scale bar indicates number of the nucleotide substitutions and at the nodes denotes the bootstrap support values. *Curdiea crassa* and *Melanthalia abscissa* were selected as the outgroup taxa.

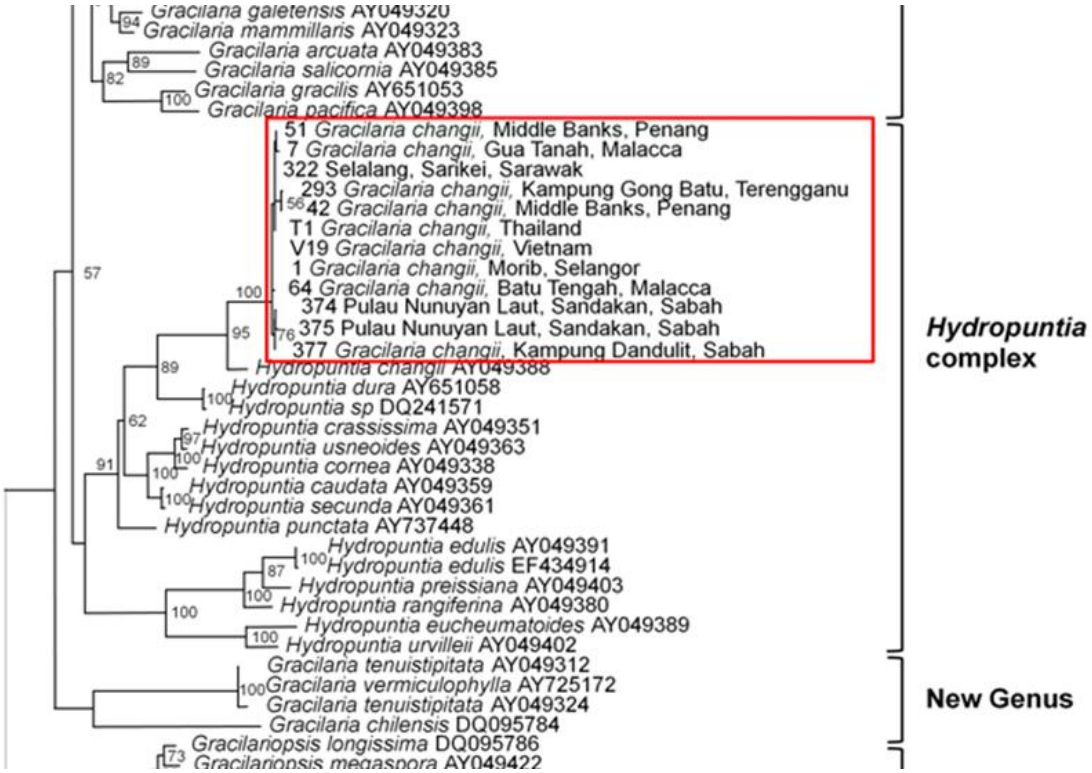


Figure 4.13 (b) Close-up view of figure 4.13 (a), showing *G. changii* from this study grouped in *Hydropuntia* clade in the maximum likelihood (ML) tree.

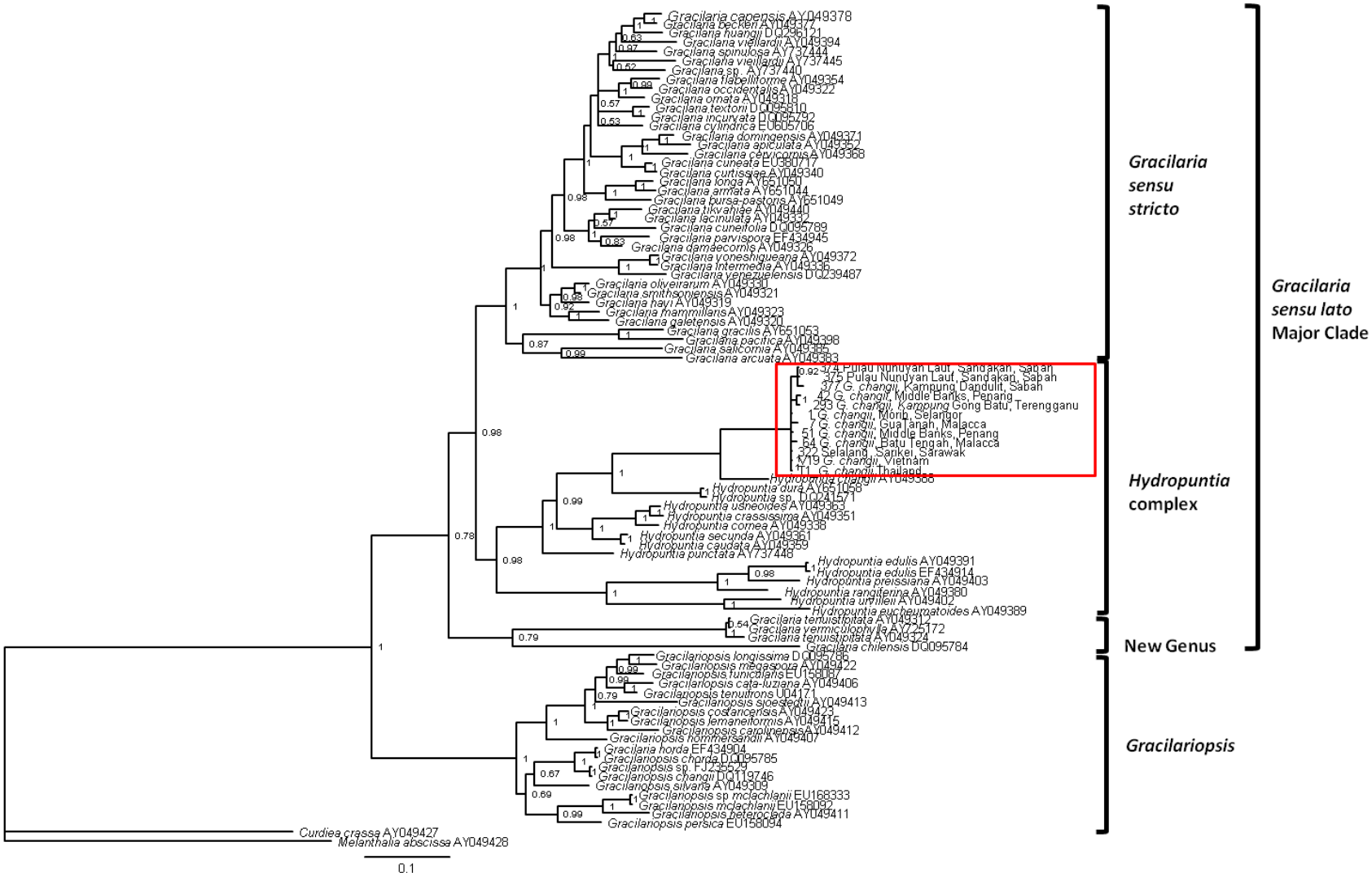


Figure 4.14 (a) The phylogenetic tree of *Gracilaria changii* and sequence data of the family Gracilariaceae based on *rbc*L gene sequences constructed using the BI (Bayesian Inference) methods. The scale bar indicates number of the nucleotide substitutions. Numbers at nodes indicate the posterior probabilities. *Curdiea crassa* and *Melanthalia abscissa* were selected as the outgroup taxa.

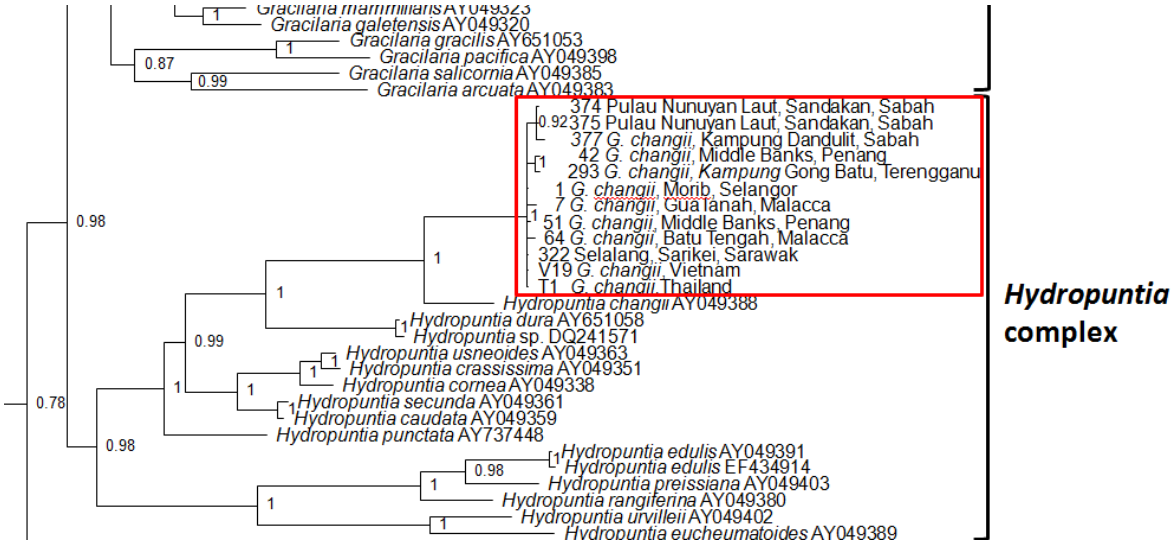


Figure 4.14 (b) Close-up view of figure 4.14 (a), showing *G. changii* from this study grouped in *Hydropuntia* clade in the Bayesian tree.

4.5 INTRASPECIFIC GENETIC DIVERSITY ANALYSIS

4.5.1 Intraspecific Genetic Diversity of *Gracilaria changii* from Peninsular Malaysia Based on the *cox*2-3 Spacer and the *cox*1 Gene

Assessing the usefulness of the *cox*2-3 spacer and the *cox*1 gene as potential markers for inferring intraspecific genetic variation of the *Gracilaria changii* from Peninsular Malaysia was undertaken. 40 specimens of *G. changii* were collected and identified from 11 locations: Kuala Sungai Merbok (Kedah), Middle Banks (Penang), Morib (Selangor), Teluk Pelanduk (Negeri Sembilan), Pantai Dickson (Negeri Sembilan), Gua Tanah (Malacca), Batu Besar (Malacca), Batu Tengah (Malacca), Sungai Pulai (Johore), Sungai Kong Kong (Johore) and Kampung Gong Batu (Terengganu).

(i) Distribution of haplotype using the cox2-3 spacer

A total of three *cox*2-3 spacer haplotypes (haplotype B1, B2 and B3) were observed with 363 characters which resulted in 0-4 bp nucleotide differences (Fig. 4.15a, Table 4.6). At each location, all specimens belonged to one haplotype with no significant genetic divergence. Haplotype B1 represented all individuals sequences from seven locations (Kuala Sungai Merbok, Middle Banks, Morib, Gua Tanah, Batu Tengah, Sungai Pulai and Sungai Kong Kong). Haplotype B1 was identified as the basal haplotype with 25 individuals. The specimens from Kampung Gong Batu were identical as haplotype B2 and differed from haplotype B1 by 3 bp: transitions occurred at position 218 (a guanidine to an adenine) and at position 242 (a cytosine to thymine), and deletion of an adenine at position 303. Haplotype B3 represented all individuals from Teluk Pelanduk, Pantai Dickson and Batu Besar with 1 bp difference from haplotype B1: a substitution base of thymine to guanine at position 153 bp. One

representative sequence of each haplotype for the *cox*2-3 spacer was deposited in GenBank. The GenBank accession numbers are as follow: B1=GU645707, B2=JX228064, B3=GU645715.

Table 4.6 Variation sites in DNA sequences of *G. changii* for haplotypes from the *cox*2-3 spacer.

Mitochondrial marker	Length of sequence (bp)	No. Of variable sites	Haplotype	Variation sites in DNA sequence ----------153-------- 218—242---- 303--------	GenBank accession No.
*cox*2-3 spacer	363	4	B1	----------T----------G------C-------A----------	GU645707
			B2	----------T----------A------T----------	JX228064
			B3	----------G----------G------C-------A----------	GU645715

*The position of the nucleotide sequences are in direction of 5' to 3'.
*Base abbreviation are A- Adenine, C-Cytosine, T-Thymine, G-Guanine.

(ii) Distribution of haplotype using the cox1 gene

A statistical parsimony network of the *cox*1 gene with 923 characters yielded a total of seven haplotypes (C1, C2, C3, C4, C5, C6 and C7) (Fig. 4.15b, Table 4.7) with 0-6 bp differences intraspecifically. Haplotype C1 represented all individuals sequenced from seven locations (Kuala Sungai Merbok, Middle Banks, Pantai Dickson, Gua Tanah, Batu Tengah, Sungai Pulai and Sungai Kong Kong). Three haplotypes (C1, C4, and C5) were identified from Morib, and two haplotypes (C1 and C2) from Batu Besar. Haplotype C1 may be the basal haplotype with 29 individuals. Haplotype C1 was the dominant haplotype in almost all the areas examined excluding specimens from Teluk Pelanduk with haplotype C3 and C6, and Kampung Gong Batu with haplotype C7, respectively. All individuals from Kampung Gong Batu were of haplotype C7 with 2 bp difference relative to haplotype C1 (transition mutation changes of a cytosine to thymine at position 359 bp and a guanidine to adenine at position 591 bp). One individual from Batu Besar was detected as haplotype C2 and differed 3 bp from haplotype C1 by three mutation changes: an adenine to cytosine at position 172 bp, a

thymine to cytosine at position 410 bp and an adenine to guanidine at position 728 bp. Two additional haplotypes of C4 and C5 were detected from Morib differing from haplotype C1 by 1 bp (a substitution of an adenine to guanidine at position 644 bp) and 3 bp (deletions of a thymine at position 16 bp and an adenine at position 43 bp with mutation change of adenine to guanidine at position 644 bp), respectively. Haplotype C3 and C6 were represented all the individuals from Teluk Pelanduk differing from haplotype C1 by 2 bp (an adenine to cytosine at position 25 bp and a cytosine to thymine at position 108 bp) and 1 bp (insertion of a cyanine at position 40 bp), respectively. One representative sequence of each haplotype for the *cox*1 gene was deposited in GenBank. The GenBank accession numbers are as follow: C1=GU645769, C2= GU645777, C3=GU645763, C4=GU645771, C5=GU645770, C6=GU645786, C7=JX228063.

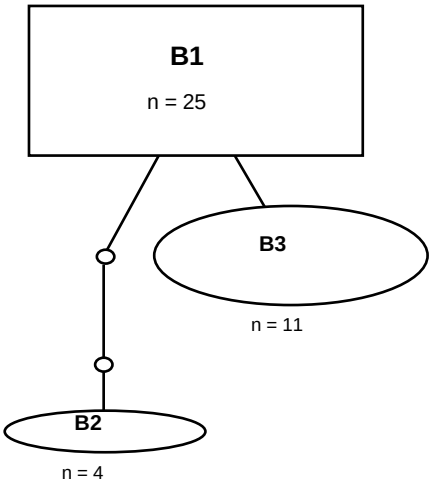
Figure 4.16 shows the haplotype distribution of the *cox*2-3 spacer and the *cox*1 for *G. changii* from Peninsular Malaysia.

Table 4.7 Variation sites in DNA sequences of *G. changii* for haplotypes from the *cox*1 gene.

Mitochondrial marker	Length of sequence (bp)	No. of variable sites	Haplotype	Variation sites in DNA sequence 16 25 40 43 108 172 359 410 591 644 728	GenBank accession No.
*cox*1 gene	923	6	C1	-T---A-------A---- C----A------C-----T----G-----A----A-	GU645769
			C2	-T---A-------A---- C----C------C-----C----G-----A----G-	GU645777
			C3	-T---C-------A---- T----A------C-----T----G-----A----A-	GU645763
			C4	-T---A-------A---- C----A------C-----T----G-----G----A-	GU645771
			C5	------A---------- C----A------C-----T----G-----G----A-	GU645770
			C6	-T---A---C--A---- C----A------C-----T----G-----A----A-	GU645786
			C7	-T---A-------A---- C----A------T-----T----A-----A----A-	JX228063

*The position of the nucleotide sequences are in direction of 5' to 3'.
*Base abbreviation are A- Adenine, C-Cytosine, T-Thymine, G-Guanine.

(a)



(b)

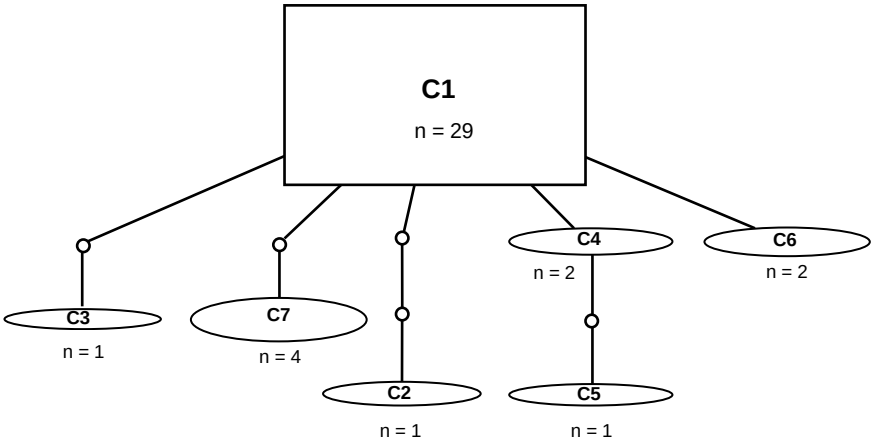


Figure 4.15 Statistical parsimony networks for **(a)** the *cox*2-3 spacer haplotypes and **(b)** the *cox*1 haplotypes of *G. changii.* A single line indicates one mutational step; small circles indicate missing haplotype; *n*, number of specimens with identical haplotypes. The size of square or oval is corresponds to the haplotype frequency. Haplotypes B1 and C1 were inferred as the hypothetical ancestral haplotype, respectively.

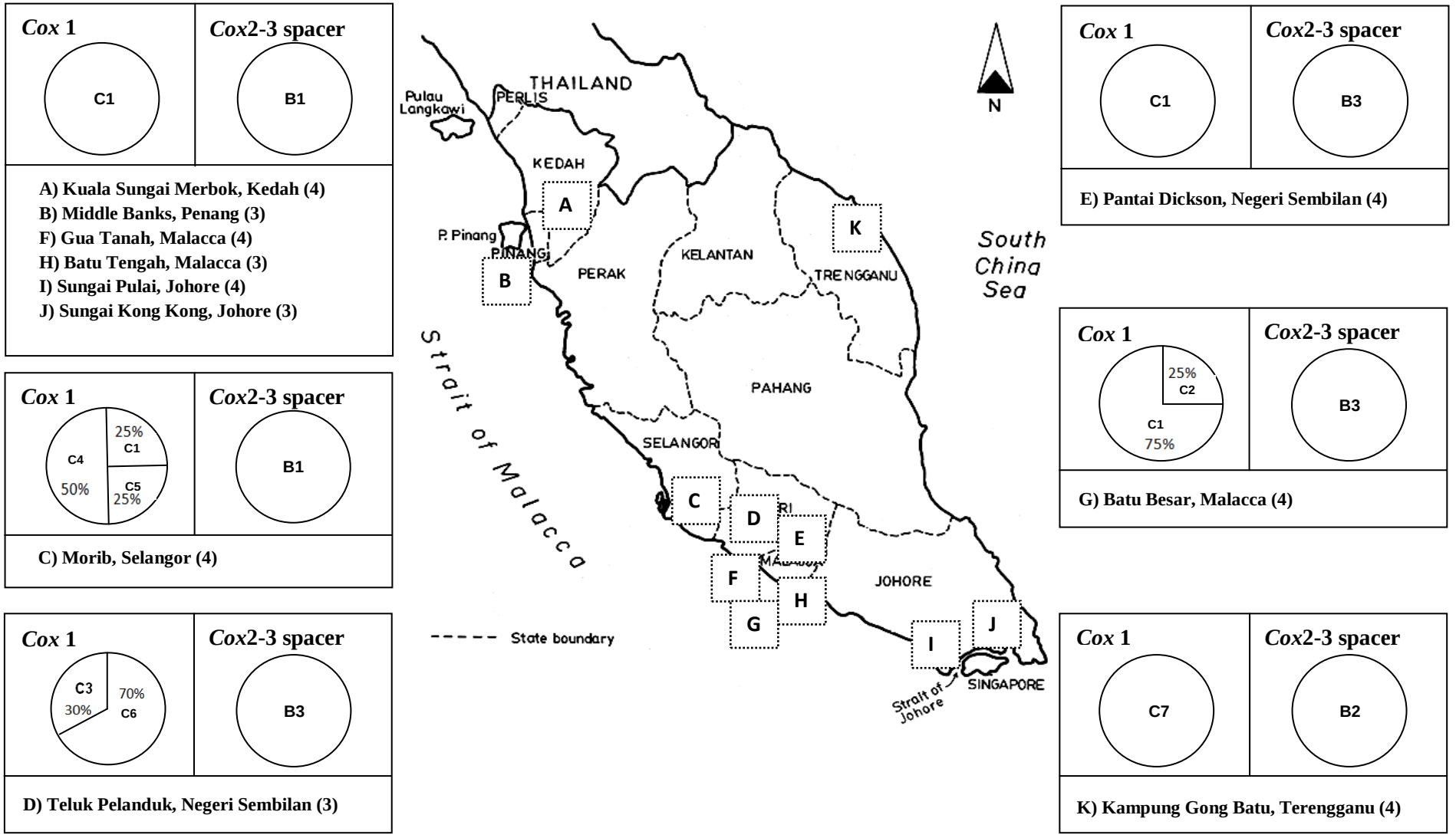


Figure 4.16 The haplotypes distribution of the *cox*1 gene and the *cox*2-3 spacer for *Gracilaria changii* from Peninsular Malaysia. The number of examined samples is shown in parentheses. Map from http://www.fao.org/docrep/field/009/ag160e/AG160E09.htm

The results showed that the mitochondrial marker of the *cox*1 gene is an ideal means of intraspecific marker for red algae and given higher resolution compared the *cox*2-3 spacer. This *cox*1 gene marker is selected and employed to infer the intraspecific genetic variation of *Gracilaria changii* and *Gracilaria edulis* from Malaysia, selected regions in Southeast Asian countries and Japan.

4.5.2 Intraspecific Genetic Diversity of *Gracilaria changii* from Malaysia and Southeast Asian Countries

4.5.2.1 Statistical Parsimony Network for *G. changii*

274 out of 278 specimens were obtained from 17 different biogeographic locations in Malaysia, and four specimens from neighbouring countries of Thailand (3) and Vietnam (1) were investigated to elucidate their genetic variation using the *cox*1 gene (Table 4.8).

A total of 923 bp were aligned in *G. changii*, 12 positions being variable (at position of 16, 22, 25, 40, 43, 108, 172, 359, 410, 591, 644 and 728; 1.3%) and 4 positions (at position of 22, 359, 591, 644; 0.4%) parsimoniously informative (Table 4.9). Haplotype and nucleotide diversity were 0.3755 and 0.00055, respectively (Table 4.14). The nucleotide composition of *G. changii* out of 923 bp: Adenine (A) = 27.5%; Thymine (T) = 40.7%; Guanine (G) = 18.2%; Cytosine (C) = 13.6%. Generally, the A+T (68.2%) composition was higher in comparison to G+C composition (31.8%). Nucleotide substitutions and indels were detected, where transition mutations rate are higher frequency than transversions. Table 4.10 shows the genetic distance among each haplotypes. The genetic distance between each haplotypes results in a 0.1-0.5% differences, with the largest difference between haplotype C2 with C7 and C2 with C3.

(i) Distribution of the mitochondrial cox1 haplotype in Malaysia

A statistical parsimony network of 274 taxa with 923 characters of the *cox*1 gene yielded a total of eight haplotypes, based on the specimens collected from the 17 localities in Malaysia, namely C1, C2, C3, C4, C5, C6, C7 and C8 (Fig. 4.17; Table 4.8 & 4.9). All of the haplotypes were closely related with 0-6 bp differences intraspecifically (0-0.7%). Two locations (Morib and Teluk Pelanduk) each had three haplotypes, one location (Batu Besar) had two haplotypes and all other 14 localities had a single haplotype. Among the examined populations, prevailing haplotype C1 was inferred as the basal haplotype with 217 individuals. It was found in the populations along the Malacca Straits and the coastline of Johore (Kuala Sungai Merbok, Middle Banks, Kuala Sepetang, Teluk Pelanduk, Pantai Dickson, Batu Besar, Batu Tengah, Gua Tanah, Sungai Pulai, Sungai Kong Kong, Teluk Sari), and populations in Sabah along the Sulu Sea (Kawasan Perumahan Green View, Kampung Dandulit and Pulau Nunuyan Laut). Haplotype C2 was detected in Batu Besar and differs from C1 by three mutation changes: an adenine to cytosine at position 172, a thymine to cytosine at position 410 and an adenine to guanidine at position 728. The haplotype C3 from Teluk Pelanduk was formed from the ancestral C1 with two base changes, where an adenine was substituted by cytosine at position 25 and followed by substitution of cytosine to thymine at position 108. There was a substitution of an adenine to guanidine at position 644 for C4, this haplotype was contributed by the Morib samples. The deletions of thymine at position 16 and an adenine at position 43 with a mutation change of adenine to guanidine at position 644 were found for haplotype C5 from Morib. An insertion of cytosine at position 40 was observed from Teluk Pelanduk and differentiated haplotype C6. All individuals from Kampung Gong Batu, Terengganu, were of haplotype C7 with 2 bp differences relative to haplotype C1: a cytosine was substituted by a thymine at

position 359 and a guanidine substituted by an adenine at position 591. In east Malaysia, basal haplotype C1 was detected to represent all the specimens collected from Sabah. Haplotype C8 was found for all the individuals from Selalang, Sarawak and differed from basal haplotype C1 by a substitution of a thymine to a cytosine at position 22. Figure 4.18 shows the haplotype distribution of the *cox*1 gene for *G. changii* from Malaysia.

*(ii) Distribution of the mitochondrial cox*1 *haplotype in Thailand and Vietnam*

Due to limited number of specimens at a location, three specimens of *G. changii* from Ao Cho, Thailand and one specimen from Phu Quoc Kien Giang, Vietnam were used in this study to compare their genetic variation with Malaysian *G. changii*. All individuals from Thailand and Vietnam were represented by the basal haplotype C1 of *G. changii* from Malaysia. Figure 4.19 shows the haplotype distribution of the *cox*1 gene for *G. changii* from Malaysia, Thailand and Vietnam.

Table 4.8 A summary of the *cox*1haplotype diversity of *G. changii* with number of individuals (*n*) from each location and number of specimens with identical haplotypes.

LOCATION	*n*	HAPLOTYPE							
		C1	C2	C3	C4	C5	C6	C7	C8
i)West Coast of Peninsular Malaysia									
Kuala Sungai Merbok, Kedah	9	9							
Middle Banks, Penang	18	18							
Kuala Sepetang, Perak	18	18							
Morib, Selangor	33	1			31	1			
Teluk Pelanduk, Negeri Sembilan	13	9		1			3		
Pantai Dickson, Negeri Sembilan	11	11							
Batu Besar, Malacca	16	15	1						
Batu Tengah, Malacca	19	19							
Gua Tanah, Malacca	10	10							
Sungai Pulai, Johore	29	29							
ii) East Coast of Peninuslar Malaysia									
Sungai Kong Kong, Johore	7	7							
Teluk Sari, Johore	5	5							
Kampung Gong Batu, Terengganu	15							15	
iii) East Malaysia									
Selalang, Sarawak	9								9
Kawasan Perumahan Green View, Sabah	14	14							
Kampung Dandulit, Sabah	24	24							
Pulau Nunuyan Laut, Sabah	24	24							
(iv) THAILAND									
Ao Cho	3	3							
(v) VIETNAM									
Phu Quoc Kien Giang	1	1							

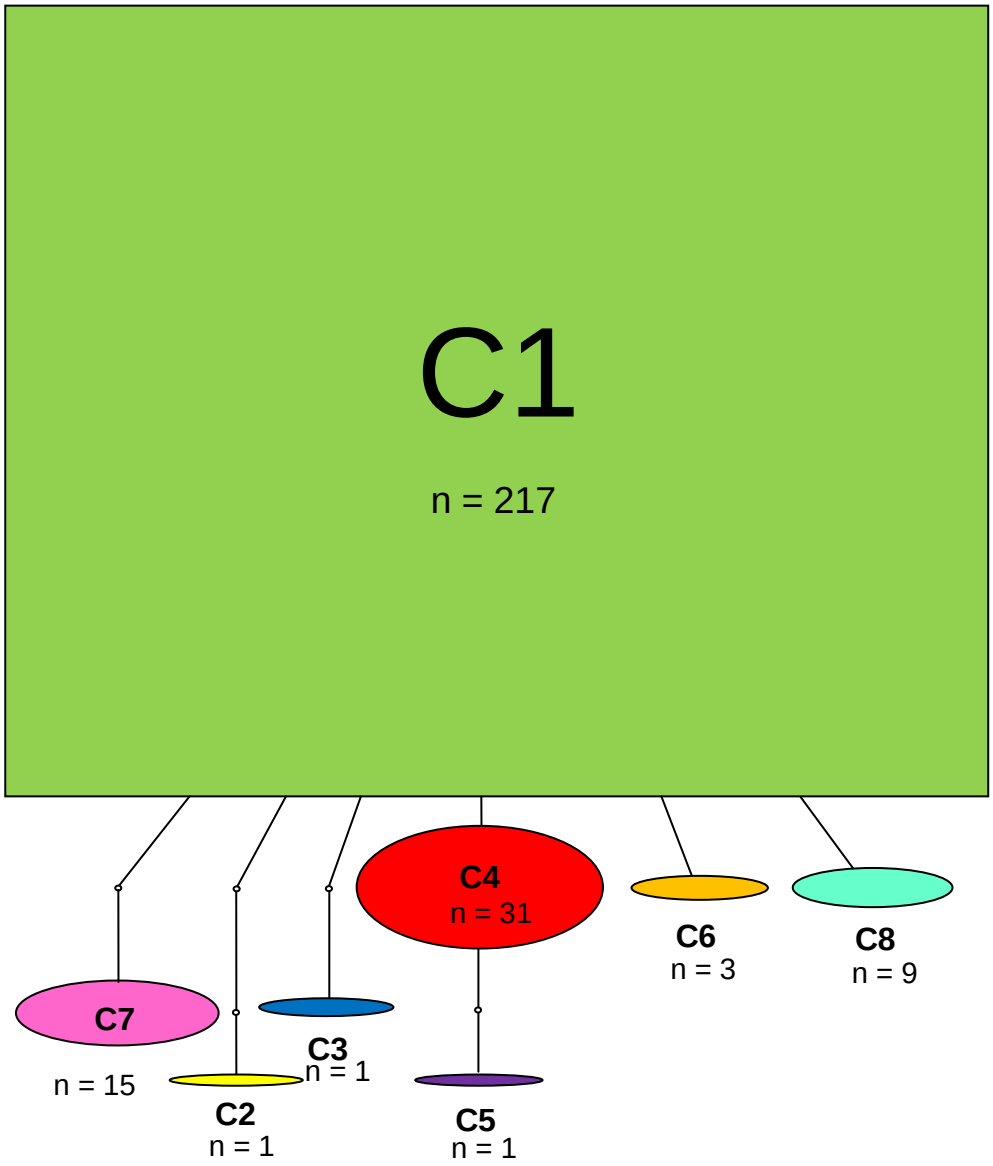


Figure 4.17 Statistical parsimony networks for the *cox*1 haplotypes of *G. changii*. A single line indicates one mutational step; small circles indicate missing haplotype; *n*, number of specimens with identical haplotypes. The size of rectangle or oval is corresponds to the haplotype frequency. Haplotype C1 in rectangle was inferred as the most probable ancestral haplotype as calculated by TCS.

Table 4.9 Variation sites in DNA sequences of *G. changii* for haplotypes from the *cox*1 gene.

Haplotype	Country/ State	Collection site	Variation sites in DNA sequence -16--22--25--40-43---108--172---359---410--591--644--728-
C1	Malaysia/ Kedah	Kuala Sungai Merbok (100%)	-T----T---A-------A-----C----A------C-----T----G-----A----A-
	Malaysia/ Penang	Middle Banks (100%)	
	Malaysia/ Perak	Kuala Sepetang (100%)	
	Malaysia/ Selangor	Morib (3%)	
	Malaysia/ Negeri Sembilan	Teluk Pelanduk (69%)	
	Malaysia/ Negeri Sembilan	Pantai Dickson (100%)	
	Malaysia/ Malacca	Batu Besar (94%)	
	Malaysia/ Malacca	Batu Tengah (100%)	
	Malaysia/ Malacca	Gua Tanah (100%)	
	Malaysia/ Johore	Sungai Pulai (100%)	
	Malaysia/ Johore	Sungai Kong Kong (100%)	
	Malaysia/ Johore	Teluk Sari (100%)	
	Malaysia/ Sabah	Kawasan Perumahan Green View (100%)	
	Malaysia/ Sabah	Kampung Dandulit (100%)	
	Malaysia/ Sabah	Pulau Laut Nunuyan (100%)	
	Thailand	Ao Cho (100%)	
	Vietnam	Phu Quoc Kien Giang (100%)	
C2	Malaysia/ Malacca	Batu Besar (6%)	-T----T---A-------A-----C----**C**------C-----**C**----G-----A----**G**-
C3	Malaysia/ Negeri Sembilan	Teluk Pelanduk (8%)	-T----T---**C**-------A-----**T**----A------C-----T----G-----A----A-
C4	Malaysia/ Selangor	Morib (94%)	-T----T---A-------A-----C----A------C-----T----G-----**G**----A-
C5	Malaysia/ Selangor	Morib (3%)	------T---A----------C----A------C-----T----G-----**G**----A-
C6	Malaysia/ Negeri Sembilan	Teluk Pelanduk (23%)	-T----T---A---**C**--A-----C----A------C-----T----G-----A----A-
C7	Malaysia/ Terengganu	Kampung Gong Batu (100%)	-T----T---A-------A-----C----A------**T**-----T----**A**-----A----A-
C8	Malaysia/ Sarawak	Selalang (100%)	-T----**C**---A-------A-----C----A------C-----T----G-----A----A-

*The position of the nucleotide sequences are in direction of 5' to 3'.
*Base abbreviation are A- Adenine, C-Cytosine, T-Thymine, G-Guanine.

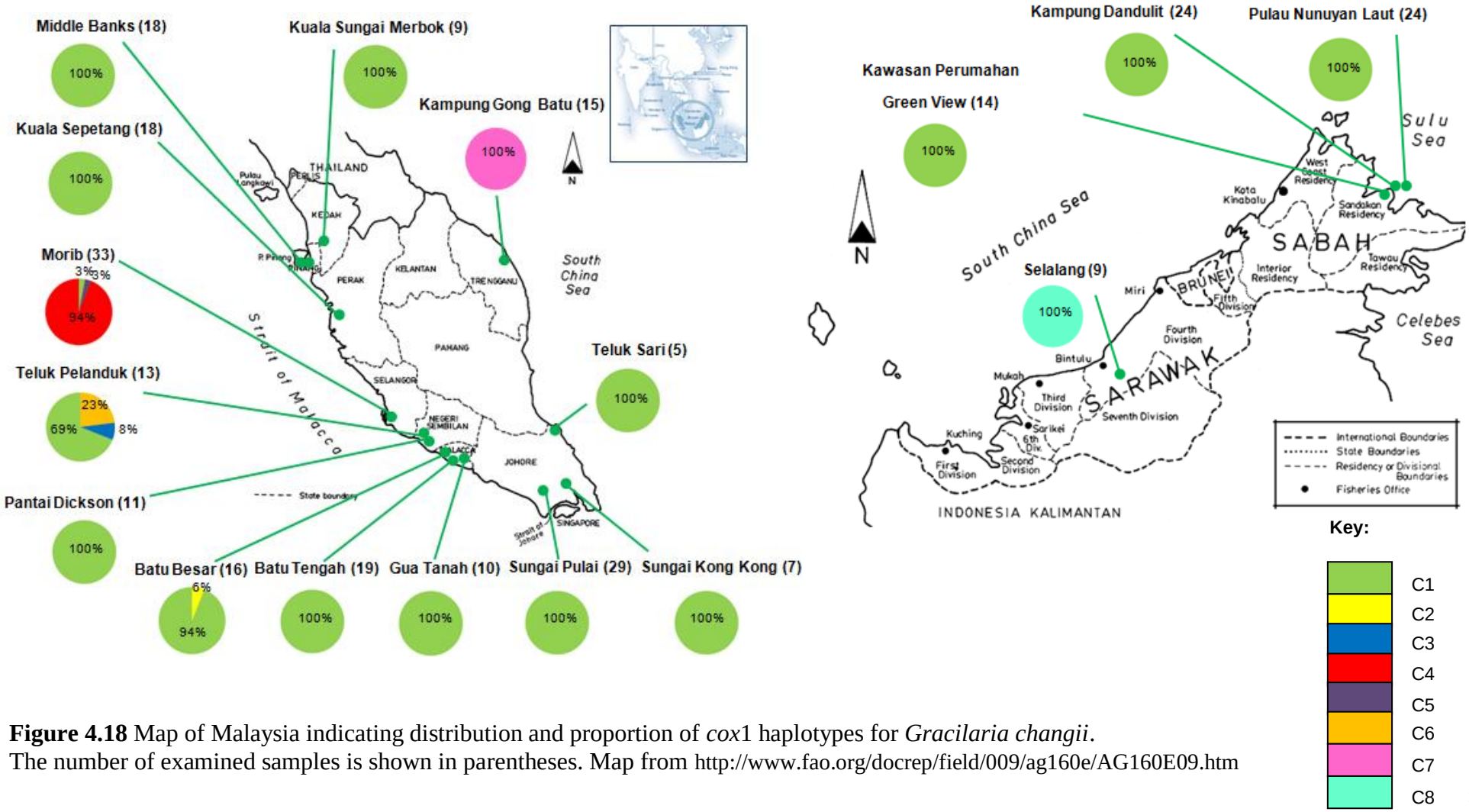


Figure 4.18 Map of Malaysia indicating distribution and proportion of *cox*1 haplotypes for *Gracilaria changii*. The number of examined samples is shown in parentheses. Map from http://www.fao.org/docrep/field/009/ag160e/AG160E09.htm

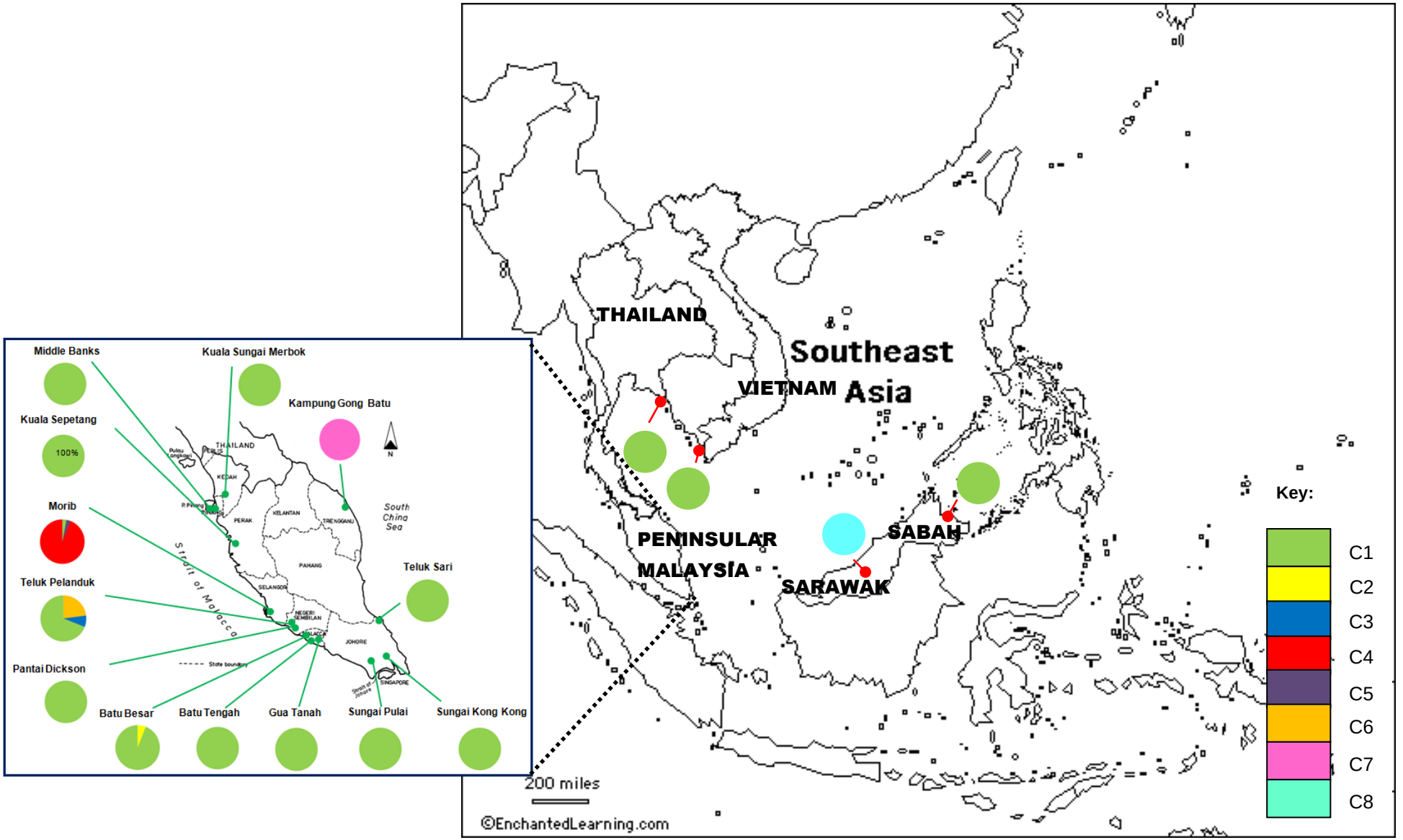


Figure 4.19 Map of Southeast Asian indicating distribution and proportion of *cox*1 haplotypes for *Gracilaria changii*. Inset map of Peninsular Malaysia indicates detailed collection sites and distribution of the *cox*1 haplotypes for each site. Map of Southeast Asian is modified from http://www.enchantedlearning.com/geography/asia/seoutlinemap/map.GIF.

Table 4.10 Genetic distance of the mitochondrial haplotypes for *G. changii*.

	C1	C2	C3	C4	C5	C6	C7	C8
C1	0	0.32538	0.21692	0.10846	0.108889	0	0.217558	0.109209
C2	0.32538	0	0.542299	0.433839	0.434272	0.325027	0.544291	0.435956
C3	0.21692	0.542299	0	0.325380	0.326099	0.217551	0.435998	0.327634
C4	0.10846	0.433839	0.325380	0	0	0.108342	0.325901	0.217551
C5	0.108889	0.434272	0.326099	0	0	0.108771	0.327043	0.21798
C6	0	0.325027	0.217551	0.108342	0.108771	0	0.217551	0.108342
C7	0.217558	0.544291	0.435998	0.325901	0.327043	0.217551	0	0.325380
C8	0.109209	0.435956	0.327634	0.217551	0.21798	0.108342	0.325380	0

4.5.2.2 Intraspecific Phylogenetic Analyses for *G. changii*

A total of eight sequences for haplotype C1 to C8 (923 bp) were aligned, with two sequences of *Gracilariopsis longis* and *Gracilariopsis chorda* as outgroups to root the phylogenetic trees in ML and BI analyses. The intraspecific phylogenetic trees of the ML and BI analyses were similar in term of tree topology but were not identical. Phylogram of ML and BI are shown in Figure 4.20 and Figure 4.21, respectively. Nodes without values in dendrogram of ML and BI have support values below 50 or 0.50, respectively.

BI analysis resulted in two trees with similar topology. Phylogenetic analysis using BI revealed a very similar topology to the ML. In ML and BI trees, two major clades (Clade A and Clade B) were produced and outgroups formed the fully-supported clade of Clade B. Both trees revealed a major clade (Clade A) of *G. changii* samples from Peninsular Malaysia (C1-C7), Sabah (C1), Thailand (C1) and Vietnam (C1) while *G. changii* from Sarawak with haplotype C8 was basal to the Clade A. The Clade A consisting of haplotype C1 to C7 was not supported in ML analysis but it was supported by posterior probabilities of 0.66. In BI analysis, the Clade A1 was subdivided into two clades: Clade A1 including haplotypes C4 and C5; and Clade A2 including haplotypes C1, C2, C3, C6, and C7 were divided by a polytomy. In ML tree, haplotype C8 was separated from all others, which were divided into Clade A1 (C2 & C3); Clade A2 (C4

& C5); and haplotype of C1, C6, and C7. The Clade A1 including C2 and C3, and that Clade A2 including C4 and C5 were grouped, but without significant bootstrap value was obtained in this tree, except a moderate bootstrap support (69%) for the Clade A2. Haplotype C4 was closely related to C5 and they shown to be distinct than other haplotypes with moderate bootstrap support (68.9%) in ML (Clade A2), but well supported in BI (0.95) (Clade A1). In ML and BI analysis, haplotype C1 to C7 were grouped and are clearly regarded as monophyletic. All *G. changii* samples from Peninsular Malaysia region, Sabah, Thailand and Vietnam formed a single cluster in ML and BI trees (Clade A, Fig. 4.20 & 4.21), while haplotype C8 from Sarawak was shown to be distinct from all other haplotypes, but no significant support value was obtained in this study.

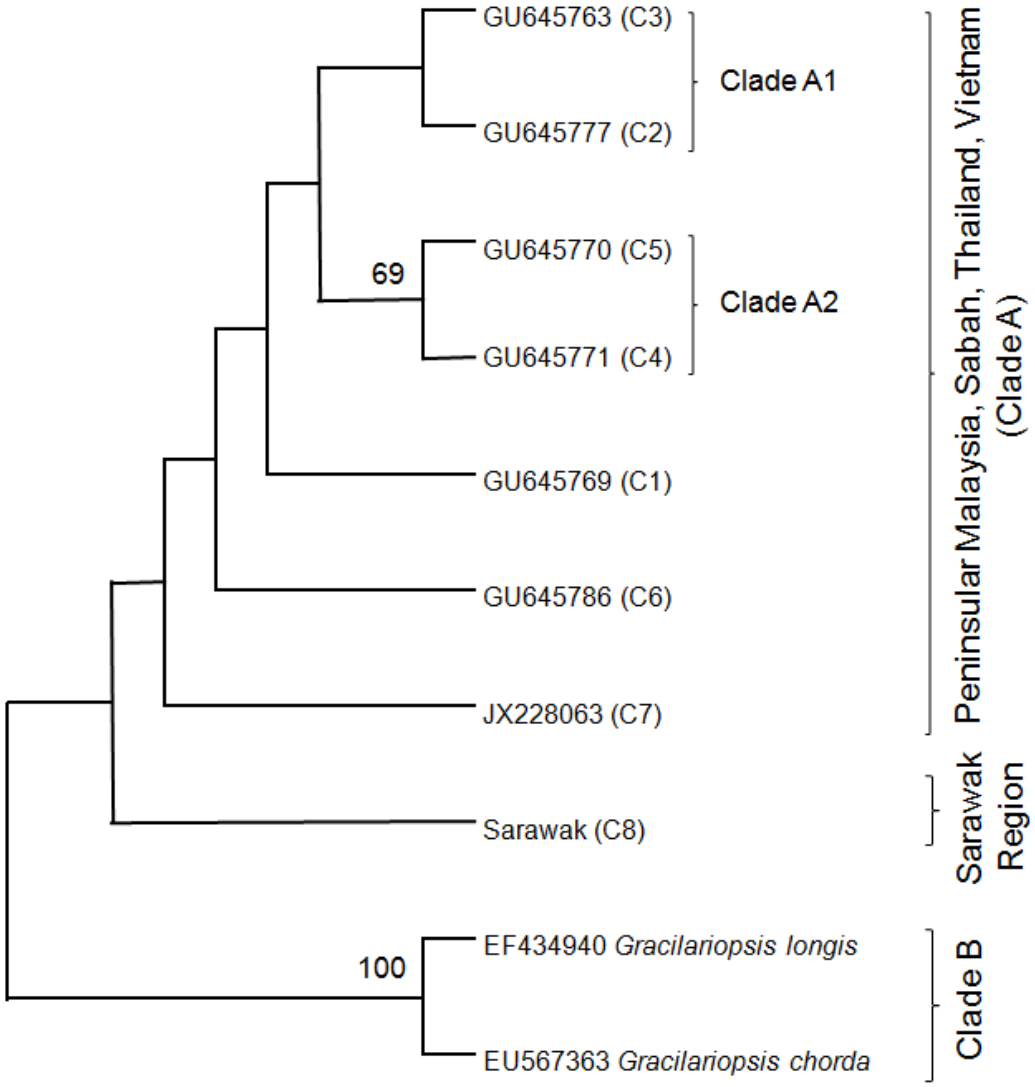


Figure 4.20 Phylogram shows ML analysis of *cox*1 haplotype sequences from *G. changii*. Numbers at nodes indicate bootstrap value in percentage. Abbreviations refer to representative of each haplotypes in populations listed in Table 3.5. *Gracilariopsis longis* and *Gracilariopsis chorda* were selected to root the tree. Value > 50% were shown above the branches.

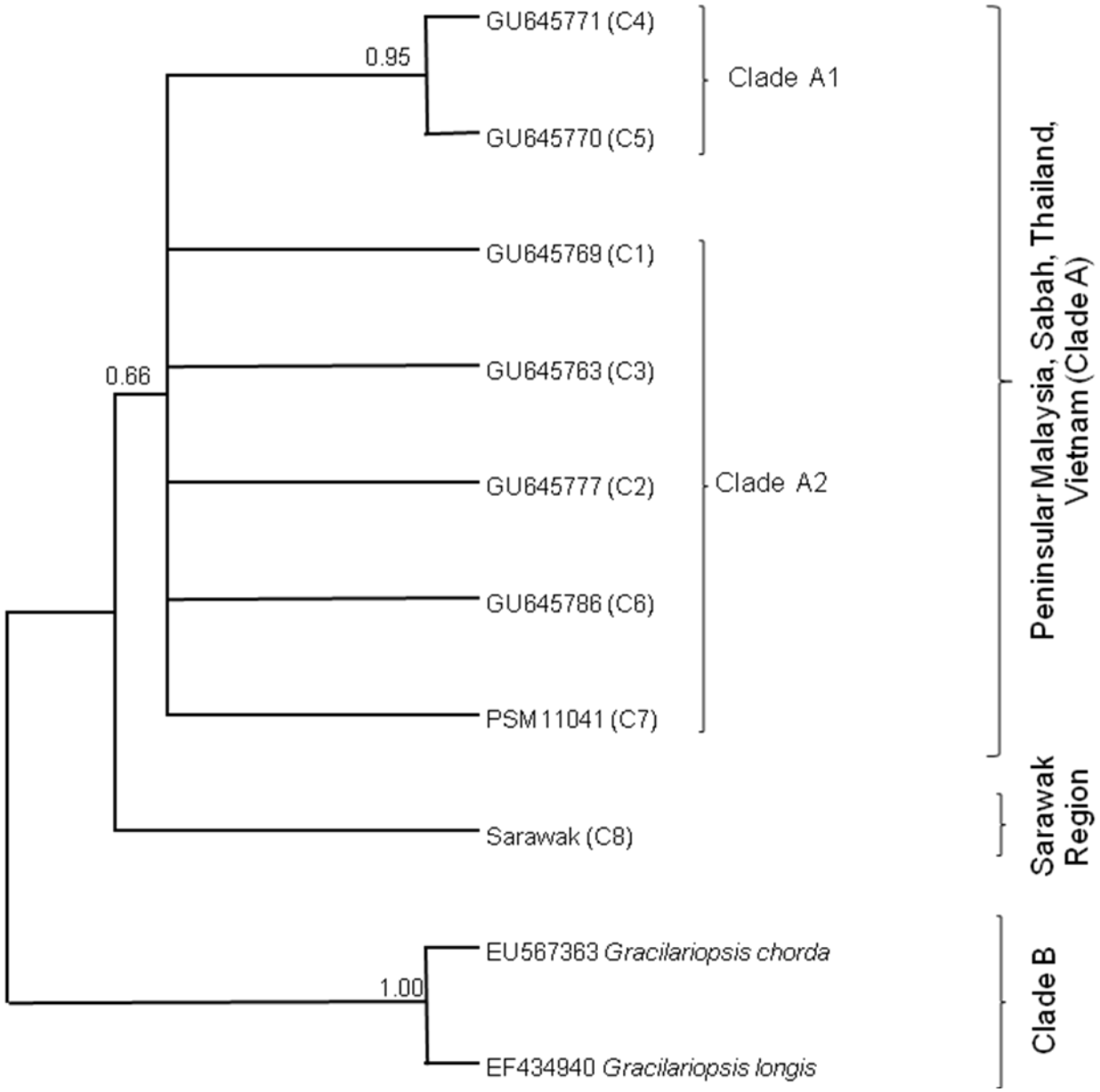


Figure 4.21 Phylogram shows BI analysis of *cox*1 haplotype sequences from *G. changii*. Numbers at nodes indicate the posterior probabilities. Abbreviations refer to representative of each haplotypes in populations listed in Table 3.5. *Gracilariopsis longis* and *Gracilariopsis chorda* were selected to root the tree. Only nodes with Bayesian posterior probabilities (PP) greater than 0.5 as a validity supported clade for the construction of a 50% majority rule consensus tree.

4.5.3 Intraspecific Genetic Diversity of *Gracilaria edulis* from Malaysia, Southeast Asian Countries and Japan

4.5.3.1 Statistical Parsimony Network for *G. edulis*

120 specimens of *G. edulis* from 16 different biogeographic locations in Malaysia and 20 specimens from other Asia countries of Thailand (2), Indonesia (9) and Japan (9) were investigated to elucidate their genetic variation using the *cox*1 gene (Table 4.11). Figure 4.22 shows the statistical parsimony network of 140 taxa with 846 bp characters of the *cox*1 gene for *G. edulis.*

A total of 846 bp were aligned in *G. edulis*, 31 positions (3.7%) being variable and parsimoniously informative, respectively (at position of 7, 14, 29, 46, 54, 60, 101, 127, 136, 148, 237, 242, 249, 261, 327, 348, 399, 402, 423, 483, 591, 585, 630, 642, 786, 796, 820, 836, 842, 845, and 846) (Table 4.12). Haplotype and nucleotide diversity were 0.5291 and 0.00318, respectively (Table 4.14). The nucleotide composition of *G. edulis* out of 846 bp: Adenine (A) = 27.4%; Thymine (T) = 41.3%; Guanine (G) = 18.5%; Cytosine (C) = 12.8%. The A+T composition (68.7%) was higher in comparison to G+C composition (31.3%). Nucleotide substitutions and indels were detected. Table 4.13 shows the genetic distance between each haplotypes. The genetic distance between each haplotypes results in a 0.1-2.2% differences, with the largest difference between haplotype C11 with C15.

(i) Distribution of mitochondrial cox1 haplotype in Malaysia

A statistical parsimony network of 120 taxa with 846 characters of the *cox*1 gene revealed a total of 12 haplotypes (E1-E12), based on the specimens collected from the 16 localities in Malaysian waters (Table 4.12, Fig. 4.22) with 0-10 bp differences intraspecifically (0-1.2%). Three locations in Peninsular Malaysia (Teluk Pelanduk,

Batu Tengah and Kukup) each had three haplotypes, one location (Batu Besar) had two haplotypes and all other 14 localities had a single haplotype. Haplotype E1 was positioned as basal haplotype for *G. edulis* as it was the dominant haplotype among the examined populations with 89 individuals. It was found in all the populations in Peninsular Malaysia, but undetectable in populations of East Malaysia. Haplotype E1 was found in Batu Feringgi, Middle Banks, Morib, Teluk Pelanduk, Batu Besar, Batu Tengah, Pulau Besar, Kukup, Merambung Shore, Pulau Che Kamat, Sungai Kong Kong, Teluk Ramunia and Pulau Sayak. Haplotype E1 represented all individuals sequenced from eight localities (Batu Feringgi, Morib, Batu Besar, Pulau Besar, Merambung Shore, Pulau Che Kamat, Teluk Ramunia and Pulau Sayak). Haplotype E2 was detected in Middle Banks and differs from C1 by an insertion of an adenine at position 101. The haplotype E3 from Teluk Pelanduk was formed from the ancestral C1 with two insertions of thymine at position 237 and 261. A deletion of an adenine at position 7 was detected as haplotype E4 in Batu Tengah. All individuals from Gua Tanah were belonged to haplotype E5 with 2 insertions relative to haplotype E1: a guanidine at position 46 and a cytosine at position 786. Haplotype E6 was found in Batu Tengah and differentiated from haplotype E5 with an insertion of a cytosine at position 836. There were four insertions of cytosine at position 786, 796, 820 and 836 for E7, this haplotype was contributed by individuals of Kukup. A mutation change of cytosine to thymine at position 483 was found for E8 from Sungai Kong Kong. Haplotype E9 from Kukup was formed from E1 with a deletion of thymine at position 127. Haplotype E10 was detected in Pulau Nunuyan Laut, Sabah, and differed from E1 with three substitution changes: a thymine substituted by cytosine at position 642; an adenine by guanine at position 845; cytosine by adenine at position 846. A substitution of an adenine to guanine at position 348 was found for haplotype E11 from Pulau Nunuyan

Laut and Kawasan Perumahan Green View compared to E10. Haplotype E12 from Teluk Pelanduk was differentiated from E1 with a substitution of a guanine at position 842 by adenine. Figure 4.23 shows the haplotype distribution of the *cox*1 gene for *G. edulis* from Malaysia.

(ii) Distribution of mitochondrial cox1 haplotype in Thailand, Indonesia and Japan

Two specimens of *G. edulis* from Thailand, nine from Indonesia and Japan, respectively, were used in this study to compare their genetic variation with Malaysian *G. edulis.*

Based on the network, 0-2.4% nucleotide differences were found among *G. edulis* from Malaysia, Thailand, Indonesia and Japan. All individuals from Thailand were represented by the basal haplotype E1 of *G. edulis* from Malaysia. Substitution of a cytosine by thymine at position 148 was detected in E13 and E14, respectively, for specimens from Indonesia. There was an addition substitution of adenine by cytosine found for E14 at position 519. All individuals from Japan were of haplotype E15 and displayed 14 bp differences from the dominant haplotype E1 in Malaysia: a cytosine was substituted by a thymine at position 14, 60 and 148; a cytosine to adenine at position 29; a thymine to cytosine at position 54, 242, 249, 327 and 399; an adenine to thymine at position 423; an adenine to guanidine at position of 136, 402, 585 and 630. Figure 4.24 shows the haplotype distribution of the *cox*1 gene for *G. edulis* from Malaysia, Indonesia, Thailand and Japan.

Table 4.11 A summary of the *cox*1haplotype diversity of *G. edulis* with number of individuals (*n*) from each location and number of specimens with identical haplotypes.

LOCATION	*n*	HAPLOTYPE														
		E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12	E13	E14	E15
i)West Coast of Peninsular Malaysia																
Pulau Sayak, Kedah	6	6														
Batu Feringgi, Penang	6	6														
Middle Banks, Penang	11	10	1													
Morib, Selangor	13	13														
Teluk Pelanduk, Negeri Sembilan	9	6		1									2			
Batu Besar, Malacca	2	2														
Batu Tengah, Malacca	6	4			1		1									
Gua Tanah, Malacca	1					1										
Pulau Besar, Malacca	5	5														
Kukup, Johore	3	1						1		1						
Merambung Shore, Johore	6	6														
ii) East Coast of Peninuslar Malaysia																
Pulau Che Kamat, Johore	16	16														
Sungai Kong Kong, Johore	13	8							5							
Teluk Ramunia	6	6														
iii)East Malaysia																
Pulau Nunuyan Laut	11										7	4				
Kawasan Perumahan Green View	6											6				
(iv) Thailand	2	2														
(v)Indonesia	9	4												3	2	
(vi)Japan	9															9

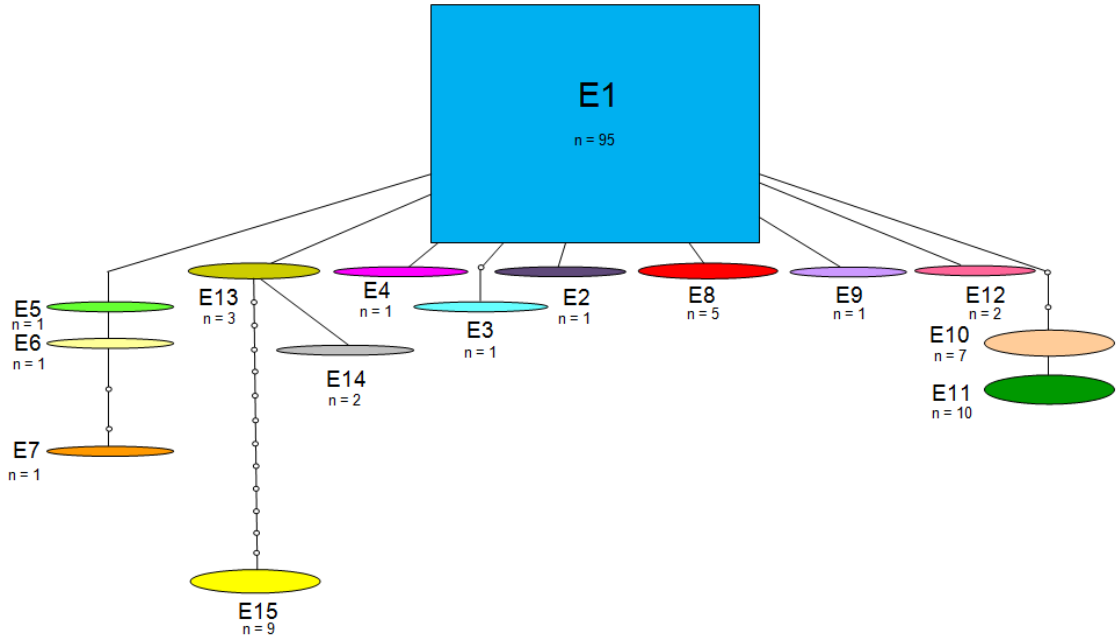


Figure 4.22 Statistical parsimony networks for the *cox*1 haplotypes of *G. edulis.* A single line indicates one mutational step; small circles indicate missing haplotype; *n*, number of specimens with identical haplotypes. The size of rectangle or oval is corresponds to the haplotype frequency. Haplotype E1 in rectangle was inferred as the most probable ancestral haplotype as calculated by TCS.

Table 4.12 Variation sites in DNA sequences of *G. edulis* for haplotypes from the *cox*1 gene.

Haplotype	Country/ State	Collection site	Variation sites in DNA sequence -7-14-29-46-54-60-101-127-136-148--237-242-249-261--327-348-399-402-423-483-519-585-630-642--786-796-820-836-842-845-846
E1	Malaysia/ Kedah Malaysia/ Penang Malaysia/ Penang Malaysia/ Selangor Malaysia/ Negeri Sembilan Malaysia/ Malacca Malaysia/ Malacca Malaysia/ Malacca Malaysia/ Johore Malaysia/ Johore Malaysia/ Johore Malaysia/ Johore Malaysia/ Johore Thailand Indonesia	Pulau Sayak (100%) Batu Feringgi (100%) Middle Banks (91%) Morib (100%) Teluk Pelanduk (67%) Batu Besar (100%) Batu Tengah (66%) Pulau Besar (100%) Kukup (34%) Merambung Shore (100%) Pulau Che Kamat (100%) Sungai Kong Kong (62%) Teluk Ramunia (100%) Prachuap Khiri Khan (100%) Gili Meno, Gili Genting & Landangluar (45%)	-A-C--C------T--C---------T----A---C---------T---T---------T----A---T----A---A---C----A---A----A---T----------G---A---C
E2	Malaysia/ Penang	Middle Banks (9%)	-A-C--C------T--C---**A**----T----A---C---------T---T---------T----A---T----A---A---C----A---A----A---T----------G---A---C
E3	Malaysia/ Negeri Sembilan	Teluk Pelanduk (11%)	-A-C--C------T--C---------T----A---C----**T**----T---T----**T**----T----A---T----A---A---C----A---A----A---T----------G---A---C
E4	Malaysia/ Malacca	Batu Tengah (17%)	----C--C------T--C---------T----A---C---------T---T---------T----A---T----A---A---C----A---A----A---T----------G---A---C
E5	Malaysia/ Malacca	Gua Tanah (100%)	-A-C--C-**G**--T--C---------T----A---C---------T---T---------T----A---T----A---A---C----A---A----A---T-----**C**----------G---A---C
E6	Malaysia/ Malacca	Batu Tengah (17%)	-A-C--C-**G**--T--C---------T----A---C---------T---T---------T----A---T----A---A---C----A---A----A---T-----**C**----------**C**---G---A---C
E7	Malaysia/ Johore	Kukup (33%)	-A-C--C------T--C---------T----A---C---------T---T---------T----A---T----A---A---C----A---A----A---T----**C**----**C**----**C**---**C**---G---A---C
E8	Malaysia/ Johore	Sungai Kong Kong (38%)	-A-C--C------T--C---------T----A---C---------T---T---------T----A---T----A---A---**T**----A---A----A---T----------G---A---C
E9	Malaysia/ Johore	Kukup (33%)	-A-C--C------T--C----------A---C---------T---T---------T----A---T----A---A---C----A---A----A---T----------G---A---C
E10	Malaysia/ Sabah	Pulau Nunuyan Laut (64%)	-A-C--C------T--C---------T----A---C---------T---T---------T----A---T----A---A---C----A---A----A---**C**----------G---**G**--**A**
E11	Malaysia/ Sabah	Pulau Nunuyan Laut (36%) Kawasan Perumahan Green View (100%)	-A-C--C------T--C---------T----A---C---------T---T---------T----**G**---T----A---A---C----A---A----A---**C**----------G---**G**--**A**
E12	Malaysia/ Negeri Sembilan	Teluk Pelanduk (22%)	-A-C--C------T--C---------T----A---C---------T---T---------T----A---T----A---A---C----A---A----A---T----------**A**---A---C

Table 4.12 (continued).

E13	Indonesia	Gili Genting (11%) Batu Kijok (22%)	-A-C--C------T--C---------T----A---**T**----------T---T----------T----A---T----A---A---C----A---A----A---T----------G---A---C
E14	Indonesia	Rambang (22%)	-A-C--C------T--C---------T----A---**T**----------T---T----------T----A---T----A---A---C----**C**---A----A---T----------G---A---C
E15	Japan	Okinawa (100%)	-A-**T**--**A**------**C**--**T**---------T---**G**---**T**----------**C**---**C**---------**C**----A---**C**---**G**---**T**---C----A---**G**----**G**---T----------G---A---C

*The position of the nucleotide sequences are in direction of 5’ to 3’.
*Base abbreviation are A- Adenine, C-Cytosine, T-Thymine, G-Guanine.

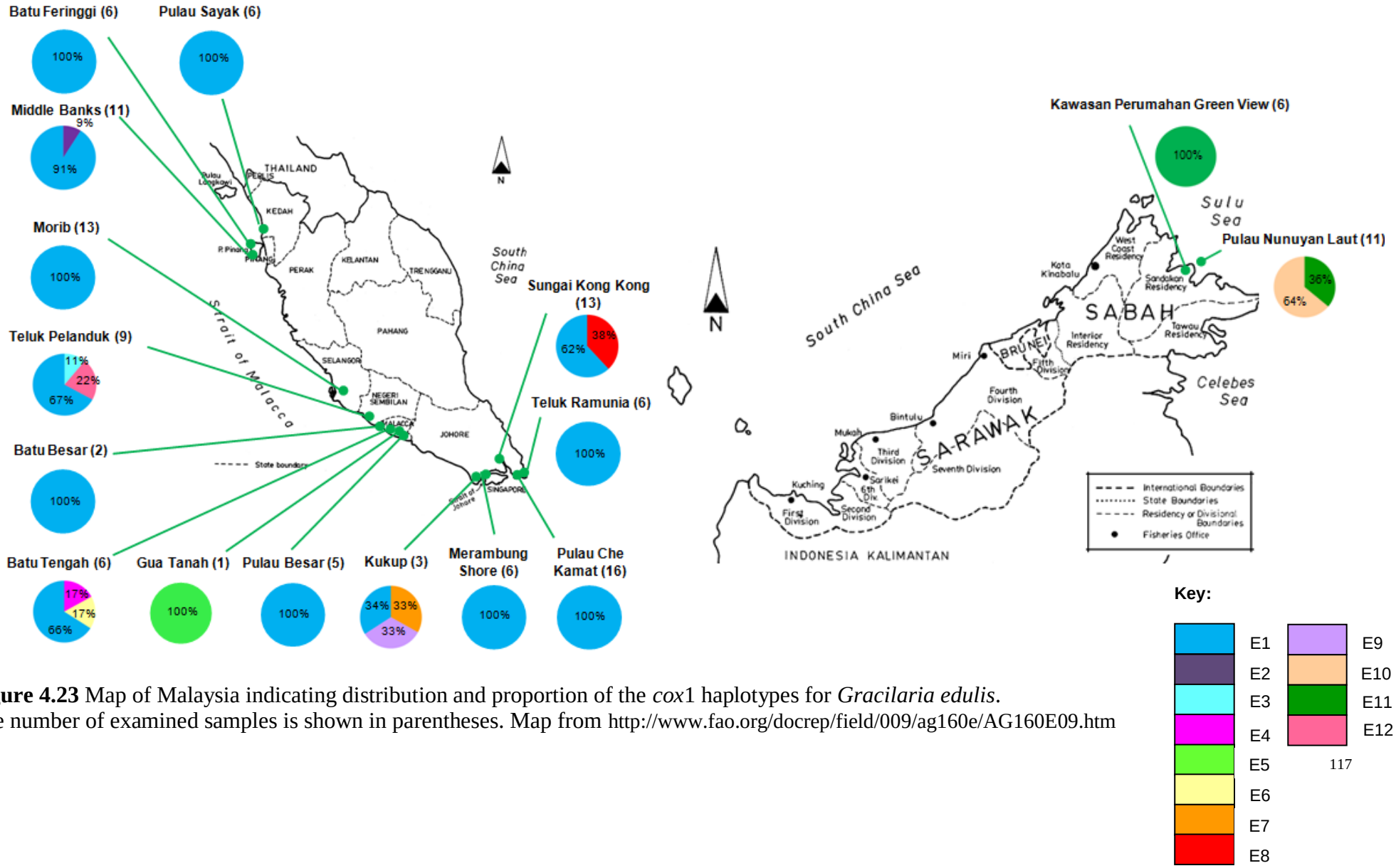


Figure 4.23 Map of Malaysia indicating distribution and proportion of the *cox*1 haplotypes for *Gracilaria edulis*. The number of examined samples is shown in parentheses. Map from http://www.fao.org/docrep/field/009/ag160e/AG160E09.htm

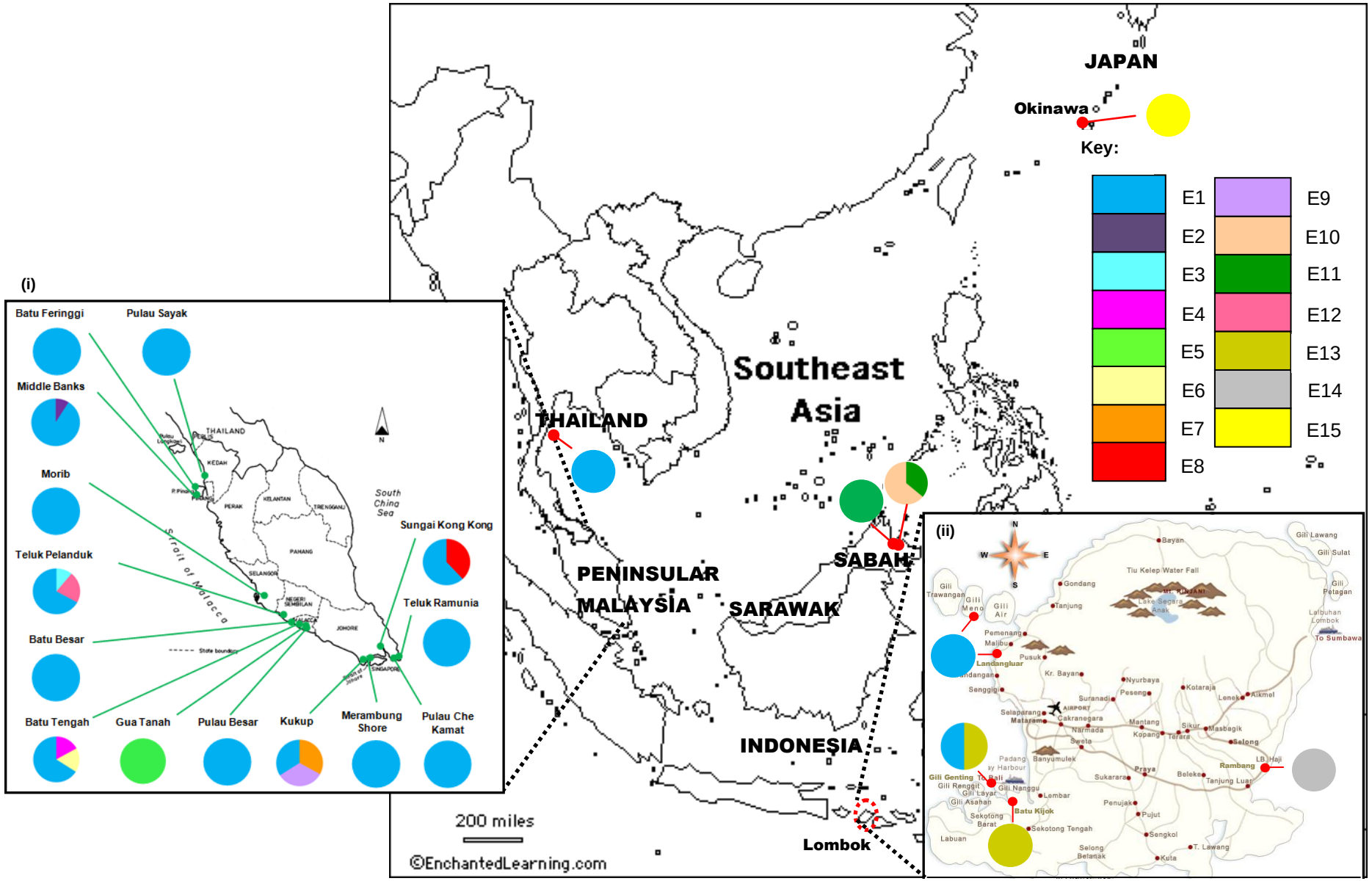


Figure 4.24 Map of Southeast Asian indicating distribution and proportion of *cox*1 haplotypes for *Gracilaria edulis*. Inset maps of (i) Peninsular Malaysia and (ii) Lombok indicate detailed collection sites and distribution of the *cox*1 haplotypes for each site. Map of Southeast Asian is modified from http://www.enchantedlearning.com/geography/asia/seoutlinemap/map.GIF. Map of Lombok, Indonesia is modified from http://www.lombok-travelnews.com/images/map-home.jpg

Table 4.13 Genetic distance of the mitochondrial haplotypes for *G.edulis*.

	E1	**E2**	**E3**	**E4**	**E5**	**E6**	**E7**	**E8**	**E9**	**E10**	**E11**	**E12**	**E13**	**E14**	**E15**
E1	0	0	0	0	0	0	0	0.119332	0	0.357995	0.477327	0.119332	0.119332	0.238663	1.670644
E2	0	0	0	0	0	0	0	0.11919	0	0.358087	0.477794	0.11919	0.11919	0.238897	1.671244
E3	0	0	0	0	0	0	0	0.119048	0	0.357831	0.476879	0.119048	0.119048	0.238095	1.670107
E4	0	0	0	0	0	0	0	0.119332	0	0.358516	0.47785	0.119851	0.119332	0.238663	1.671175
E5	0	0	0	0	0	0	0	0.12016	0	0.358255	0.477303	0.119816	0.12016	0.239208	1.671117
E6	0	0	0	0	0	0	0	0.121129	0	0.358941	0.477847	0.119673	0.121129	0.240035	1.673575
E7	0	0	0	0	0	0	0	0.123205	0	0.360734	0.479499	0.118765	0.123205	0.24197	1.680467
E8	0.119332	0.11919	0.119048	0.119332	0.12016	0.121129	0.123205	0	0.119677	0.477327	0.596659	0.238663	0.238663	0.357995	1.789976
E9	0	0	0	0	0	0	0	0.119677	0	0.357995	0.477327	0.119332	0.119677	0.239008	1.672032
E10	0.357995	0.358087	0.357831	0.358516	0.358255	0.358941	0.360734	0.477327	0.357995	0	0.119332	0.477327	0.477327	0.596659	2.02864
E11	0.477327	0.477794	0.476879	0.47785	0.477303	0.477847	0.479499	0.596659	0.477327	0.119332	0	0.596659	0.596659	0.71599	2.147971
E12	0.119332	0.11919	0.119048	0.119851	0.119816	0.119673	0.118765	0.238663	0.119332	0.477327	0.596659	0	0.238663	0.357995	1.789976
E13	0.119332	0.11919	0	0.119332	0.12016	0.121129	0.123205	0.238663	0.119677	0.477327	0.596659	0.238663	0	0.119332	1.551313
E14	0.238663	0.238897	0	0.238663	0.239208	0.240035	0.24197	0.357995	0.239008	0.596659	0.71599	0.357995	0.119332	0	1.670644
E15	1.670644	1.671244	0	1.671175	1.671117	1.673575	1.680467	1.789976	1.672032	2.02864	2.147971	1.789976	1.551313	1.670644	0

4.5.3.2 Intraspecific Phylogenetic Analyses for *G. edulis*

A total of 15 sequences for haplotype E1 to E15 (846 bp) were aligned, with two sequences of *Gracilariopsis longis* and *Gracilariopsis chorda* as outgroups to root the phylogenetic trees in ML and BI analyses. The intraspecific phylogenetic trees of the ML and BI analysis were similar in term of tree topology but were not identical. Dendrograms of ML and BI are shown in Figure 4.25 and Figure 4.26, respectively. Nodes without values in dendrogram of Ml and BI have support values below 50 or 0.50, respectively.

BI analysis resulted in two trees with similar topology. Phylogenetic analysis using BI revealed a very similar topology to the ML. In ML and BI trees, the outgroups formed the fully-supported clade of Clade C. Based on the ML and BI trees, all of the haplotypes (E1 to E15) were divided into three main groups according to the regions: (i) Peninsular Malaysia, East Malaysia and Thailand; (ii) Indonesia region; (iii) Japan region. Both trees revealed a major clade (Clade A) of *G. edulis* samples from Malaysia (E1- E12) and Thailand (E1), while *G. edulis* from Indonesia formed a clade (Clade B) including haplotypes E13 and E14. Samples from Japan with haplotype E15 was basal to the Clade A and Clade B. Within the Clade A, haplotypes E1-E10 and E12 were resulted in polytomy. Almost all clades, however, were supported by no or low support value (below 70%) except samples with haplotypes E10 and E11from Sabah, East Malaysia (in Clade A) formed a particular cluster with significant bootstrap support in ML (93%) and in BI (0.98). Haplotypes of E13 and E14 contributed from Indonesia were more closely related to haplotype of E1-E12 from Malaysian and Thailand than E15 from Japan. Haplotype E15 of *G. edulis* samples from Japan was distantly related to all others, but with no significant bootstrap value was obtained in these trees.

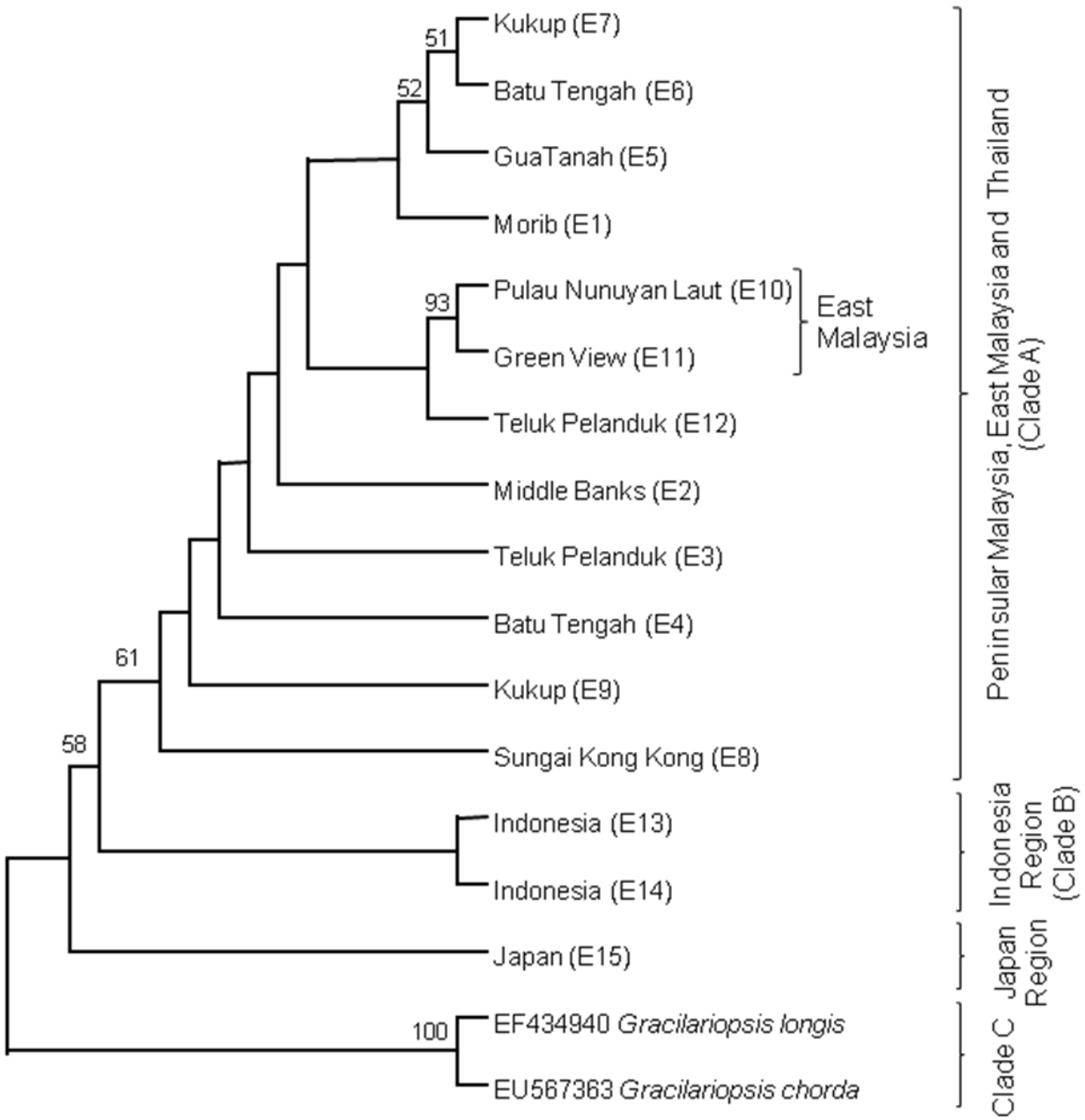


Figure 4.25 Phylogram shows ML analysis of *cox*1 haplotype sequences from *G. edulis*. Numbers at nodes indicate bootstrap value in percentage. Abbreviations refer to representative of each haplotypes in populations listed in Table 3.5. *Gracilariopsis longis* and *Gracilariopsis chorda* were selected to root the tree. Value > 50% were shown above the branches.

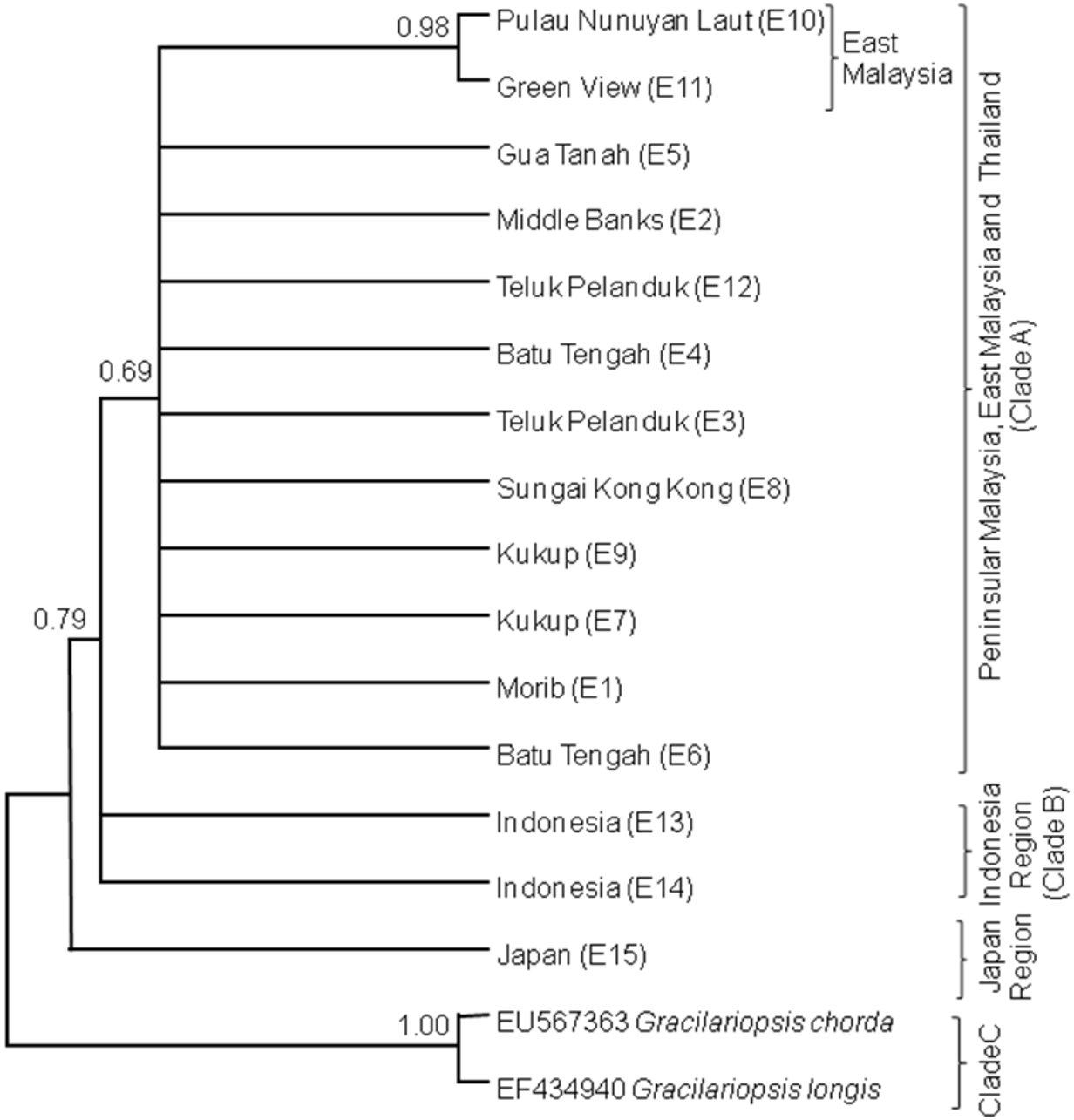


Figure 4.26 Phylogram shows BI analysis of *cox*1 haplotype sequences from *G. edulis*. Numbers at nodes indicate the posterior probabilities. Abbreviations refer to representative of each haplotypes in populations listed in Table 3.5. *Gracilariopsis longis* and *Gracilariopsis chorda* were selected to root the tree. Only nodes with Bayesian posterior probabilities (PP) greater than 0.5 as a validity supported clade for the construction of a 50% majority rule consensus tree.

Table 4.14 A summary of sequence variability for *G. changii* and *G. edulis* based on the *cox*1 gene.

	G. changii	***G. edulis***
No. of taxa	278	140
No. of haplotype	8	15
Length (bp)	923	846
No. of variable site (%)	12 (1.3%)	31 (3.7%)
No. of Parsimony informative sites (%)	4 (0.4%)	31 (3.7%)
Haplotype diversity (*Hd*)	0.3755	0.5291
Nucleotide diversity (*Pi*)	0.00055	0.00318

CHAPTER 5

DISCUSSION

5.1 MORPHOLOGICAL STUDIES

In this study, individuals of *G. changii* were sampled from 17 localities in Malaysian waters. A minimum sample size of five from each site was collected. Additionally, three individuals from Thailand and one individual from Vietnam were included. Individuals of *G. edulis* for this study were obtained from 16 locations in Malaysia. Two individuals were obtained from Thailand, nine from Indonesia and Japan, respectively. Healthy specimens were collected randomly from field sites. The number of individuals collected for *G. changii* was higher in comparison to *G. edulis.* All of the specimens were identified based on the morphological descriptions from Xia & Abbott (1987) and Lim & Phang (2004).

Throughout the sampling collections on *G. changii* and *G. edulis*, they were often found to share the same habitat (e.g. mangrove roots, fish cages, rocks, coral and intertidal mud flats). Species identification of *G. changii* and *G. edulis* was based on the criteria of the presence of main axis, the type of branching (alternate, di or trichotomous, irregular, or secund), obcuneate articulation, location and grade of the constrictions in the thallus, and shape of thallus (Zhang & Xia, 1988; Phang & Lewmanomont, 2001). The presence of distinct morphological characteristics between *G. changii and G. edulis* are essential to identify both samples accurately. They can be distinguished by their morphological features, whereby the former is reddish while the latter is bushier and dark red (Phang, 1994). However, the delineation of these criteria was difficult as they were very subjective to individual observation. Gross morphology of both species can be subjected to environmental factors as well.

Almost all of the *G. changii* collected for this study has no significant morphological variation regardless of the populations. However, specimens collected from Selalang, Sarawak and Pulau Nunuyan Laut, Sabah showed differences in their morphological features compared to specimens from other populations. Despite the specimens sharing some similarities on reproductive structures of *G. changii*, as described by Xia & Abbott (1987) and Lim & Phang (2004), however, the plants from Selalang, Sarawak showed absence of main axis (Fig. 4.2), while specimens from Pulau Nunuyan Laut showed absence of main axis with rounded to blunt branching apex (Fig. 4.3). The appearance of blunt branching apex could be due to the plants were eaten by fish or other marine organisms (e.g. aquatic insects, snails and mussels) and new branches were produced (Fig. 4.5). *Gracilaria* species are infamously difficult to identify based on morphological characters alone (Bellorin et al., 2008) likely due to their ordinary morphology and anatomy and rampant phenotypic plasticity (Saunders, 2005). Further identification of the plants from Selalang and Pulau Nunuyan Laut using DNA-based molecular marker of the *rbc*L gene were carried out. Based on the ML and BI trees analyses, the specimens from Selalang and Pulau Nunuyan Laut to be *G. changii*. A high degree of morphological variability of *G. changii* from Selalang and Pulau Nunuyan Laut is due to their environmentally-influenced morphological plasticity. The phenotypic characters are subjective and affected by biotic and abiotic factors of habitats and represent the adaptation capability of an organism (Kraufvelin et al., 2009). Environmental parameter of salinity is the main factor in influencing the morphological appearance of several *Fucus* sp. in Portugal (Cairrão et al., 2009). It is worth noting that a number of red algae have high morphological plasticity, e.g., *Halymenia pseudofloresii* Collins et M. Howe from Bermuda has broad range of morphological variation and has been reported as a phenotypically plastic species using a molecular analysis (Schneider et al., 2010); *Eucheuma* and *Kappachycus* in Hawaii

(Conklin et al., 2009); *Batrachospermum arcuatum* by Vis et al. (2010). Thus morphological characteristics alone are not sufficient to delineate morphologically similar species of *Gracilaria*. Hence, molecular analyses in combination with morphological studies are crucial for better identification of seaweed.

For sampling collections of *G. edulis*, all specimens had no significant morphologically differences regardless of the geographical locations.

5.2 DISTRIBUTION OF *GRACILARIA* SPP. IN THIS STUDY

Extensive sampling collections on *G. changii* and *G. edulis* have been conducted along the shorelines of Malaysia. The specimens of *G. changii* and *G. edulis* were naturally found in mangroves swamp, muddy areas and fish cages. Throughout the sampling collections for this study, more plants were found in fields during dry season (from May to September) than rainy season (from November to March). The reproductive plants are present throughout the year. Specimens were haphazardly collected from each sampling site to prevent collecting individuals from the same mother plant. The genus *Gracilaria* abundantly distributed in mangrove areas fringing along west coast of Peninsular Malaysia as reported by Phang et al. (1996). Approximately 91,779 ha of mangrove distributed along the west coast of Peninsular Malaysia (Shamsudin & Nasir, 2005) and encompassed highest generic richness of seaweeds (Ooi et al., 2006). It is notable that the density of *Gracilaria* species along the east coast of Peninsular Malaysia was low and only found in Terengganu and Johore as this coastline is characterizing by sandy and rocky shore (Phang et al., 1996) with 5738 ha of mangrove (Satyanarayana et al., 2010). The absence of seaweeds along the coastline of Pahang has also been reported by Du et al. (2009). Likewise, the east coast

of Peninsular Malaysia is undergoes heavy rain falls and strong winds of the northeast monsoon from November to March. The relatively low density of *Gracilaria* species along the east coast Malaysia could be due to seasonal variation and large tidal amplitude. *Gracilaria* specimens were subjected to numerous inherent contaminants, e.g. mud, epiphytic, endophytic organisms and salt from marine. The removal of existing contaminants is crucial to prevent any of them from interfering the quality and yield of extracted DNA. In order to extract a high purity of contamination-free DNA, precaution must be taken during selection and cleaning on specimens. After washing, it is important to dry the fresh samples completely and store in a dry condition to avoid growth of bacteria and fungi.

5.3 MOLECULAR STUDIES

5.3.1 Extraction of Deoxyribonucleic Acid (DNA)

High quality DNA is prerequisite ensuring successful PCR amplification with reproducible results. Isolation of DNA from *Gracilaria* species is difficult and it might due to the co-isolation of hydrocolloids, presence of polysaccharide and secondary metabolites (Kumari, 2013). In this study, genomic DNA of *Gracilaria* species were extracted from dried tips ground in liquid nitrogen with DNeasy Plant Mini Kit (Qiagen, Germany). Young thallus is preferable as it may contain less polyphenolic and terpenoid compounds (Jobes et al., 1995; Amani et al., 2011). According to Porebski et al. (1997), polyphenolic is one of the oxidizing agents to reduce the purity and yield of isolated genomic DNA. Hence, only tips were plucked from cleaned samples and used in DNA extraction as they might contain less polysaccharide (Sim et al., 2007) and others secondary metabolites. The amount of starting material for DNA isolation should not

excess 20 mg of dried specimens to reduce inhibitory agents interface with the PCR amplification.

Commercially available DNeasy Plant Mini Kit (Qiagen, Germany) found to be useful for genomic DNA extraction for *Gracilaria* species. Dried samples were ground into a very fine form to release all nucleic acid contained within the cells. Red algae are characterized by rigid phycocolloid production in the cell walls for structural enhancement purpose. Therefore, an additional step of sonification for 20 minute was carried out for further disruption of cells with lysis buffer and RNAase. This modification is to enhance the process of cell wall breakage. DNA isolation kits are efficient, user friendly, small amount of starting material needed, minimised handling to harmful chemicals such as phenol or chloroform as in conventional methods. This technology makes use of spin columns, which contain a silica-gel-based membrane that binds with DNA. The DNA bound to the membrane can be washed and cleaned to remove contaminants and then eluted from the column (membrane) using UHQ water or provided elution buffer. The extracted DNAs from commercially available kits gave significant results in terms of DNA quality and quantity. Hence, the use of commercially available kits in DNA extraction is reliable and efficient for this study as a large number of specimens need to be analysed rapidly. Determination of quantity of yielded DNA by electrophoresis is not recommended as the yield of DNA from isolation kits is lower compare to conventional methods.

5.3.2 Optimization of PCR Amplification

Amplification of the *cox*2-3 spacer for *G. changii* was successfully conducted with primers and PCR condition adapted from Zuccarello et al. (1999). No modification on PCR protocol is needed for PCR amplification of this species. There was not much problem encountered in gene amplification. The annealing temperature of

PCR amplification for *G. changii* is agreed with the finding of Zuccarello et al. (1999). The size of amplified fragments of this region was in the range of 300-400 bp.

For amplification of the *cox*1 gene for *G. changii* and *G. edulis*, optimizations of the annealing temperature from 45-55ºC were adopted. However, the success rate of the *cox*1gene amplification is low, especially for *G. edulis* from Kukup. Modification in the PCR protocol was made using Step-up PCR with two different annealing temperatures: 48°C and 50°C for *cox*1 43F and *cox*1 1549R (Geraldino et al., 2006); 45°C and 48°C for GazF1 and GazR1 (Saunders, 2005), respectively. Modification of PCR parameter was undertaken with a lower annealing temperature in the first five cycles where primers will be bounded unspecifically to template DNA to increase the number of putative gene, and followed by higher annealing temperature to enhance the specificity in amplification for 35 cycles. Step-up PCR is useful in amplification of both species due to the presence of chemical compounds in cells, which may inhibit the function of DNA polymerase (Jin et al., 1997). It greatly increased the success rate of the amplifications for both species. The size of amplified fragment of *cox*1 43F and cox1 1549R primers was in the range of 1450-1500 bp. The amplification product of GazF1 and GazR1 is about 700 bp. The final aligned *cox*1 gene sequences of *G. changii* were 923 bp and *G. edulis* were 846 bp, where the A+T content were higher in comparison to G+C content. This eliminates the possibility of jumping PCR and to reduce the probability of inefficiency of the PCR amplification caused by high G+C content (Baldwin et al., 1995). According to Wintzingeorde et al. (1997), additional of 0.25% BSA into the mixture of PCR greatly improved the reliability of success in amplification to prevent or reduce the inhibition effects of contaminants from field samples. 1-2 µl of DNA solution will work well for a standard PCR reaction for all the adopted markers.

5.4 HAPLOTYPE DIVERSITY OF *G. CHANGII* AND *G. EDULIS*

An overview of biodiversity in Malaysia has been reported in detail by Manokaran (1992) and Mazlan et al. (2005). Genetic diversity studies of marine organism in Malaysian waters have been undertaken using numerous molecular markers (e.g. Dinoflagellates using ITS markers by Pin et al., 2001; Fish bleeker using mtDNA marker by Kamarudin & Esa, 2009; Marine diatom using ITS marker by Lim et al., 2012; Horseshoe crab using mtDNA marker by Rozihan & Ismail, 2012), but have been scanty in marine seaweeds. Geographic distributions of seaweed and species richness in Malaysia have been well studied by Ooi et al. (2006), Phang et al. (2006) and Du et al. (2009). Rhodopyhta has the highest species richness and distributes widely in Peninsular Malaysia, Sabah and Sarawak. Phaeophyta and Chlorophyta are mostly distributing in Peninsular Malaysia and Sabah. Prior before this study, there were no determined *cox*1 sequences of *G. changii* and *G. edulis* available through GenBank. This is the first study of genetic diversity and phylogeographic structure for red algae in Malaysia using the *cox*1 gene.

Anthropogenic activities leading to pollution, costal degradation, climate change, transoceanic introduction of alien species and invasive species, including natural seaweed populations have impacted on the diversity of the seaweed genetic resources. In addition, ecological factors and natural evolutionary processes also influence a range of population, community and ecosystem responses that may generate changes in allele sequences or genotype frequency in seaweeds (Fussmann et al., 2007). Regular sampling at various coastal areas in Peninsular Malaysia has indicated that the wild populations of *G. changii* and *G. edulis* are diminishing. The high demand for both species for a wide array of applications has increased dramatically and resulted in the overharvesting of wild populations. According to Yow et al. (2013), there is no

commercial scale cultivation of *Gracilaria* species in Malaysia, except for experimental culture in a shrimp farm using the monofilament method. Information on intraspecific genetic diversity of this economically important genus is relatively unknown (Yow et al., 2011). To meet the surging global market demand of *G. changii* and *G. edulis* for industrial use, the necessity of selection of tolerant strains for successful mariculture is crucial to conserve natural resources and as an economic option.

Genetic variation could be introduced into a population by manners of immigration or mutation. The rate of evolutionary changes relies on several factors such as mutation rates, the effective size of local populations, and the amount of interchange of individuals between populations (Wright, 1920). The understanding of genetic diversity of *G. changii* and *G. edulis* is essential to provide insights into conservation, management and strain selection for cultivation for this economically important species. Characteristics like growth rate and agar yields may be species- or strain- specific and the study of genetic diversity can assist in strain selection for commercialisation.

In this study, a distance method of the statistical parsimony network (Clement, 2000) is adopted to analyse all the sequences data. TCS software is used to construct the haplotype network. This network can be applied to elucidate genetic diversity distribution and allows tracking of common ancestral haplotype. Ordinarily, old haplotypes with wide geographical distribution are located internally in a network tree (Keirstein et al., 2004; Uwai et al., 2006b). This software is working based on the coalescent assumptions of alleles geneology. The intraspecific phylogenetic relationship among the *cox*1 haplotypes for *G. changii* and *G. edulis* were inferred using Maximum Likelihood (ML) and Bayesian Inference (BI) analyses.

5.4.1 Genetic Diversity of *Gracilaria changii* from Peninsular Malaysia Based on the *cox*2-3 Spacer and the *cox*1 Gene

The usefulness of mitochondrial markers, *cox*1 (e.g. Robba et al., 2006; Yang et al., 2008; Yow et al., 2011; Yow et al., 2013) and *cox*2-3 spacer (e.g. Zuccarello & West 2002; Vidal, 2008) as molecular markers in the measurement of genetic diversity have been reported. The *cox*2-3 spacer was shown to be a useful marker for phylogeographical study of the Rhodophytes (Zuccarello & West, 2002; Zuccarello et al., 2006a; Andreakis et al., 2007; Vis et al., 2008, Halling et al., 2013). This region was reported variable within populations of the red algae *Caulacanthus ustulatus* (Zuccarello et al., 2002b). The *cox*2-3 spacer and the *cox*1 markers have also been employed to elucidate the phylogeographic pattern of freshwater red algae of the genus *Batrachospermum* (Chiasson et al., 2003; Sherwood et al., 2008). The latter marker serves well as a phylogeopraphic marker in taxa of Batrachospermales (House et al., 2010), compared to inter-simple sequence repeat (Hall & Vis, 2002) and nuclear internal transcribed spacer (Vis et al., 2001).

We compared the use of two markers, the *cox*1gene (primers of *cox*1 43F and *cox*1 1549R) and the *cox*2-3 spacer, as a potential marker to infer the intraspecific genetic variation of this species from Peninsular Malaysia. The results have been published in Yow et al. (2013), which was the first report on comparison of reliability of the *cox*1 gene with the *cox*2-3 spacer for intraspecific genetic variation of *G. changii*. Analysis of the haplotype networks indicated that higher genetic variation was obtained from the *cox*1 gene (7 haplotypes) compared to the *cox*2-3 spacer (3 haplotypes). Intraspecific nucleotide differences ranged from 0-6 bp over 923 bp for the *cox*1 and 0-4 bp over 363 bp for the *cox*2-3 spacer, respectively. Haplotype C1 was found in the majority of the populations studied and accounted for 73% of the 40 specimens examined. Kuala Sungai Merbok, Middle Banks, Gua Tanah, Pantai Dickson, Batu

Tengah, Sungai Pulai and Sungai Kong Kong exhibited the lowest genetic diversity or no phylogeographic pattern for *G. changii* as only the basal haplotype (C1) was observed. Haplotype C1 was shown to be the common ancestor and the most widespread haplotype, due to its prevalence in all the localities analysed. Specimens from Morib (Selangor) showed the greatest divergence with identification of three haplotypes (C1, C4 and C5), followed by samples from Batu Besar (C1 and C2) and Teluk Pelanduk (C3 and C6). The results from the *cox*1 analysis suggest that the ancestral population of *G. changii* may have originated in Morib and having colonized earlier, had more time to diverge from the dominant haplotype of C1 to C4 and C5. In contrast, there was only a single haplotype with no genetic differences that has been detected with the *cox*2-3 spacer in Morib (B1), Teluk Pelanduk (B3) and Batu Besar (B3), respectively, and is not as variable as the *cox*1 gene. 63% specimens possessed haplotype B1, indicating that B1 may be the common ancestor because it is the most widely distributed haplotype at Kuala Sungai Merbok, Middle Banks, Morib, Gua Tanah, Batu Tengah, Sungai Pulai and Sungai Kong Kong. The ancestral population of this species is unresolved with the *cox*2-3 spacer.

The non-coding regions are expected to manifest higher intraspecific and interspecific genetic variation than coding regions in the main. The use of non-coding region, *cox*2-3 spacer, to study intraspecific relationship in red algae has been noted elsewhere likely due to its higher mutation rate (Zuccarello et al., 1999; Zuccarello & West, 2002; Zuccarello et al., 2002b). The *cox*2-3 spacer has higher mutation rate which evolved four and 14 times faster in comparison to the plastid *rbc* spacer and the nuclear LSU region respectively in red algae *Asparagopsis* spp. (Andreakis et al. (2007). Based on the phylogeographic study of *Spongites* by Vidal (2008), this region was shown to be highly divergent marker and revealed 14 haplotypes in 37 individuals from Chile. However, our data do not support these statements because the *cox*2-3 spacer appears to

be highly conserved and showed no significant intraspecific genetic variation compared to the *cox*1 gene for *G. changii*. Limited intraspecific variation for red algae at this spacer has been reported elsewhere (Milstein et al., 2008; Sherwood, 2008; Teasdale & Klein, 2010; Lim et al., 2014).

Our analyzed results showed that the *cox*1 gene is a potential molecular marker to infer intraspecific genetic variation in *G. changii*. The *cox*1 marker is more variable compared to the *cox*2-3 spacer and revealed genetic variation and phylogeographic structure for this species. The size of analysed sequences of the *cox*1 gene in the present study was much larger than the *cox*2-3 spacer, where larger sized gene may contain more informative data on molecular evolution (Yang et al., 2008). Thus, the mitochondrial *cox*1 gene has been used to further study the intraspecific genetic variation of these ecologically and economically important species, *G. changii* and *G. edulis*.

5.4.2 Genetic Diversity of *G. changii* and *G. edulis* based on the *cox*1 Gene

The *cox*1 gene has also been suggested as a potential DNA barcode marker for red algae, brown algae and for some other diatom genera (Saunders, 2005; Geraldino et al., 2006; Robba et al., 2006; Evans et al., 2007; Kucera & Saunders, 2008; Le Gall & Saunders, 2010). In addition it is also useful for revealing population structure and the hidden diversity of red algae and for use in conservation purposes (Robba et al., 2006).

Malaysia has a coastline of 4,800 km: 2,100 km for Peninsular Malaysia and 2,700 km for East Malaysia. Biogeographically, there are two distinct regions to Malaysia: (i) Peninsular Malaysia is bounded by the Straits of Malacca on the west, the narrow Tebrau Straits on the south and the South China Sea on the east; (ii) East Malaysia (Malaysian Borneo) is made up of Sarawak and Sabah and it is bounded by the South of China Sea on the west, Sulu Sea on the North-West and Celebes Sea on the

south. The coastlines of Malaysia are endowed with a variety of plentiful marine ecosystem such as mangrove swamps, sandy beaches, rocky shores, mudflat, coral reefs and seagrass beds (Phang, 1998; Sidik et al., 2012). Geographic distributions of seaweed and species richness in Malaysia have been well studied by by Phang et al. (2006) and Du et al. (2009). A total of 386 specific and intraspecific taxa have been recorded in Malaysia. Rhodophyta comprise the highest number of taxa.

5.4.2.1 *Gracilaria changii* from Malaysia and Southeast Asian Countries

The eight haplotypes from 17 localities in Malaysia were distributed throughout Peninsular Malaysia and East Malaysia (Fig. 4.18). The haplotype C1 was clarified as the common ancestor and the most widespread haplotype for *G. changii*, due to its prevalence in almost all of localities examined, excluding specimens from Kampung Gong Batu with haplotype C7 and Selalang with haplotype C8, respectively. Along the west coast of Peninsular Malaysia, six haplotypes (C1, C2, C3, C4, C5 and C6) were discovered from Kuala Sungai Merbok, Middle Banks, Kuala Sepetang, Morib, Teluk Pelanduk, Pantai Dickson, Batu Besar, Batu Tengah, Gua Tanah and Sungai Pulai. All samples from Kuala Sungai Merbok, Middle Banks, Kuala Sepetang, Pantai Dickson, Batu Tengah, Gua Tanah, and Sungai Pulai shared a single and identical haplotype of C1. It is possibly exhibiting the lowest genetic diversity or no phylogeographic pattern for *G. changii* since only the basal haplotype (C1) was observed. Specimens from Morib (Selangor) and Teluk Pelanduk (Negeri Sembilan) showed the greatest divergence with identification of three haplotypes for each site (C1, C4 and C5 from Morib; C1, C3 and C6 from Teluk Pelanduk), followed by specimens from Batu Besar (Malacca) with two haplotypes (C1 and C2). Despite the type species of *G. changii* was described for the first time from the Middle Banks, Penang, by Xia & Abbott (1987) (Phang, 1994), the results suggest that the ancestral population of *G. changii* in

Malaysia may have originated in Morib (Selangor) or Teluk Pelanduk (Negeri Sembilan).

The Malacca Straits, being one of the world's busiest commercial shipping lane has been threatened by man's activities of shipping traffic, land reclamation and fishing since the 1980s. Most of the Malaysian rivers flow westward into the Malacca Straits and deposited sediments, silt, vegetation and pollutants onto the shorelines. Phang (1998) reported that the sheltered coastline of the west coast of Peninsular Malaysia was dominated by mangrove forests or muddy swamps with water temperature ranging from 23 - 31ºC, salinity from 28 - 34 ppt and semi-diurnal tides. Figure 5.1 showed the distribution of intertidal mangroves along the shoreline of South China Sea. Mangrove areas of Morib are dominated by *G. changii*, *G. edulis* and *G. salicornia*. These three *Gracilaria* species also have been found from Teluk Pelanduk beach. Teluk Pelanduk beach is lined with coarse pebbles and the waters are a little murky. This beach is a fishing village with an active fishing community and is a good location for water activities and scuba diving. The Morib beach and Teluk Pelanduk beach are well known as a tourist destination. This region is also surrounded by factories and poultry farms. Waste and discharge from these activities contributes to coastal pollution which washes ashore. *G. changii* and other species in this region may have had to adapt to these stressed environments by evolving and diverging from the basal haplotype of C1 to various haplotypes in Malaysia waters. Based on the phylogenetic analysis, haplotype C4 and C5 from Morib were distinct from all other haplotypes, but these haplotypes only differed from C1 by 1 and 3 bp, respectively. A potential explanation is that water pollution in this region profoundly caused an increase in the rate of evolution of *G. changii* with haplotype C1. Haplotype C4 and C5 were independently derived from haplotype C1 for adapting to the polluted areas.

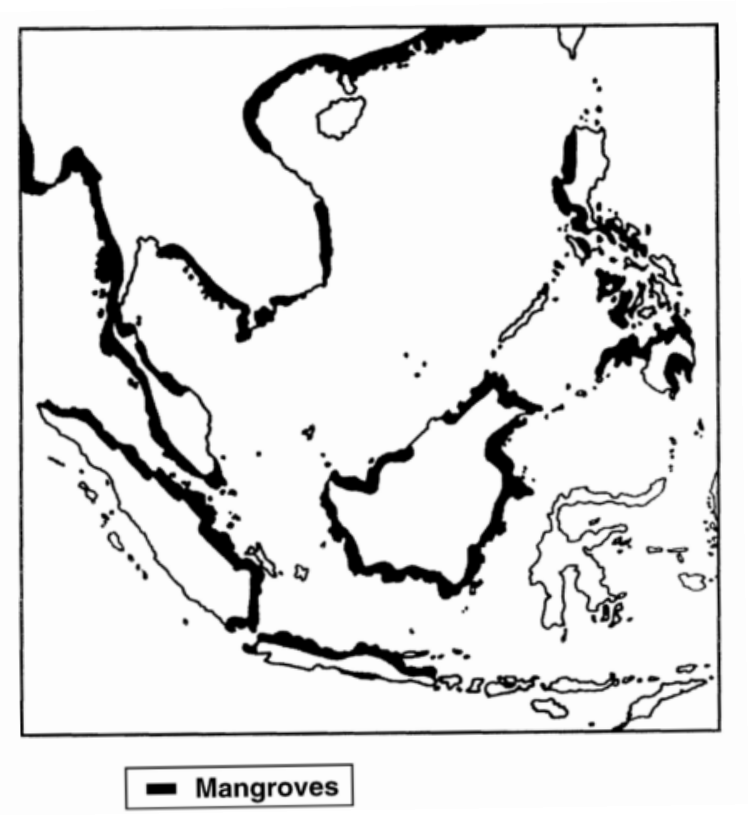


Figure 5.1 The distribution of intertidal mangroves along the shorelines of South China Sea. (Adapted from Morton & Blackmore, 2001).

The east coast Peninsular Malaysia is characterising by sandy and rocky shores, which is thoroughly exposed to the South China Sea and has a mixed tidal system. Activities of rice cultivation, fishing, timber harvesting, and oil and gas production has enriched the economy. The discovery of petroleum in the east coast of Peninsular Malaysia has contributed many environmental problems most notably in Terengganu. In this study, *G. changii* was collected from three sampling sites: Kampung Gong Batu (Terengganu, east coast Peninsular Malaysia), Teluk Sari (Johore, South Peninsular Malaysia) and Sungai Kong Kong (Johore, South Peninsular Malaysia). Extensive search for *G. changii* were conducted along the east coast of Peninsular Malaysia, however, number of locations for specimen collection were limited. Basal haplotype of C1 was represented in all the specimens from Teluk Sari and Sungai Kong Kong showing no genetic divergence. All specimens from Kampung Gong Batu revealed a unique haplotype C7 that differed from the basal haplotype by 2 bp (0.2%). The absence of the basal haplotype C1 in northern east coast Peninsular Malaysia is possibly owing to the separation of the Malacca Straits (west coast Peninsular Malaysia) and the South China Sea (east coast of Peninsular Malaysia), which are only connected by the narrow Tebrau Straits (in the southern Peninsular Malaysia). The pollution level in the Tebrau Straits is getting apprehensive and may adversely affect the seaweed populations along the Tebrau Straits. Elevation of water temperature in this straits has been reported (Sittamparam, 2007). There are also other plausible explanations that the plants with basal haplotype C1 were introduced to Terengganu but then evolved over time and derived into haplotype C7 for survival in the polluted areas.

The coastlines of Sabah and Sarawak are sandy with coral reefs and have a mixed tidal system. The specimens collected from east Malaysia (Sabah and Sarawak) were from habitats of muddy areas, sandy beaches with coarse pebbles and mangrove swamps. Interestingly, phenotypic variation was observed for *G. changii* collected from

Pulau Nunuyan Laut. The plants from Pulau Nunuyan Laut showed the absence of main axis, with rounded to blunt branching apex. This sampling site was show to possess the most abundant *G. changii* and carposporophytes in comparison to other sampling localities. This may suggest the oceanographic and environmental conditions are favorable and contributed to high abundance of carposporophytes. It may also be due to seasonal variation. The phylogenetic study using mitochondrial marker of the *cox*1 gene and chloroplast marker of the *rbc*L gene showed that the plasicity of specimens from Pulau Nunuyan Laut was phenotypic and not genetic-based. Perhaps *G. changii* with basal haplotype C1 from this site is highly morphologically plastic, yet genetically homogenous, likely owing to its dispersal capability and ability to survive and adapt to a wide range of conditions.

Based on the haplotype distribution in East Malaysia, the basal haplotype C1 was represented in all the *G.changii* from three localities (Kawasan Perumahan Green View, Kampung Dandulit and Pulau Nunuyan Laut) in Sabah; while only one haplotype, C8, was represented in all the specimens from Selalang, Sarawak. In comparison to the *cox*1 haplotype distribution of *G. changii*, haplotype C8 was demonstrated only in specimens from Selalang, Sarawak and it was closely related to haplotypes from Peninsular Malaysia (C1-C6) by differing by 1-4 bp. The low level of genetic divergence in *G. changii* sequences between haplotypes from Peninsular Malaysia and Sarawak could be due to the Pleistocene glaciation. Geographically, Peninsular Malaysia, East Malaysia, southern parts of Thailand and Vietnam, Sumatra and Java were once part of the Sunda shelf (Fig. 5.2) (Mohsin & Ambak, 1983). During the last Pleistocene, approximately 10,000 years ago, the formation of the Pleistocene land bridge traversed by the Sunda River, permitted the migration or dispersal of haplotypes from Peninsular Malaysia to East Malaysia by intermix of seawaters between rivers in Peninsular Malaysia and Sarawak. The great river system of Sunda

River was inundated because of the Pleistocene event and the sea level rose between 40-100 meters and caused the separation of the East Malaysia from mainland of Peninsular Malaysia by the South China Sea, about 1,200 km apart (Mohsin & Ambak, 1983). Geographic isolation of allopatric populations may lead to restriction of gene flow between two populations. It permits occurrence of genome evolution to adapt to new ecological conditions (Hall, 1993). The intraspecific phylogenetic trees showed that the haplotype C8 from Sarawak was distinct from all other haplotypes, but no significant support value was obtained. The samples were from Selalang river, Sarawak, located at the west coast of Sarawak, facing the South China Sea. As a major industrial area, Selalang coast is subjected to numerous threats of urbanization, industrialization and oil pollution. Oil pollution has relentlessly affected the marine environment of Malaysia since 1950s (Law & Hii, 2006). In congruence with the Pleistocene event, the *G. changii* with basal haplotype of C1 were dispersed from Peninsular Malaysia to East Malaysia. *G. changii* with haplotype C1 then gradually evolving into haplotype C8 in Selalang in order to cope with the polluted waters. Migration of fauna and flora by interconnection of Sunda River during the last Pleistocene have been reported by several researchers (Kamarudin & Esa, 2009; Rozihan & Ismail, 2011).

All samples of *G. changii* from Sabah were represented by an identical basal haplotype C1. The lack of genetic variation for specimens from three geographically close locations in Sabah, in comparison to haplotypes from Peninsular Malaysia is surprising due to the large geographical separation primarily over the ocean. During the last Pleistocene, no interconnection between Sunda River and Sabah rivers was found (Voris, 2000) (Fig. 5.3). No basal haplotye C1 was detected in Sarawak. The discontinuous distribution of the basal haplotype C1 that was discovered in Peninsular Malaysia presumably may show the recent introduction of *G. changii* into Sabah region. *G. changii* of haplotype C1 from Peninsular Malaysia may have dispersed to Sabah, east

Malaysia as floating debris, through the current circulation in South China Sea or through artificial introductions.

Three specimens of *G. changii* from Ao Cho, Trat, Thailand and one specimen from Phu Quoc Kien Giang, Vietnam were examined in this study to compare their genetic variation with the Malaysian *G. changii*. On the basis of current results from the haplotype network, all individuals from Thailand and Vietnam were represented by the basal haplotype C1 of *G. changii* from Malaysia. No significant genetic divergence is detected with large geographic distances. In other words, *G. changii* of Thailand and Vietnam were genetically identical with basal haplotype C1 from Malaysia. The absence of basal haplotype C1 along the northern part of east coast of Peninsular Malaysia suggested recent dispersal events of *G. changii* into Thailand and Vietnam through artificial translocation, but how is unknown. Likewise, current circulation has been proven to play a vital role in influencing the community structure of seaweeds (Lobban & Harrison, 1994; e.g. *Undaria pinnatifida* in Japan by Uwai et al., 2006b and *Sargassum hemiphyllum* in northwestern Pacific by Cheang et al., 2010). It could be speculated by the inflow of *G. changii* with haplotype C1 dispersed by coastal current from Malaysian waters and colonized into Thailand and Vietnam coastlines during the Southwest Monsoon, between May and September. In summer, the warm surface water enters from the Java Sea northwards to east coast of Peninsular Malaysia sweeping up into the Gulf of Thailand and Vietnam (Fig. 5.4). The detached thalli or fragments of the basal haplotype C1 *G. changii* from Malaysian waters could be dispersed to Thailand and Vietnam by drifting along with the currents.

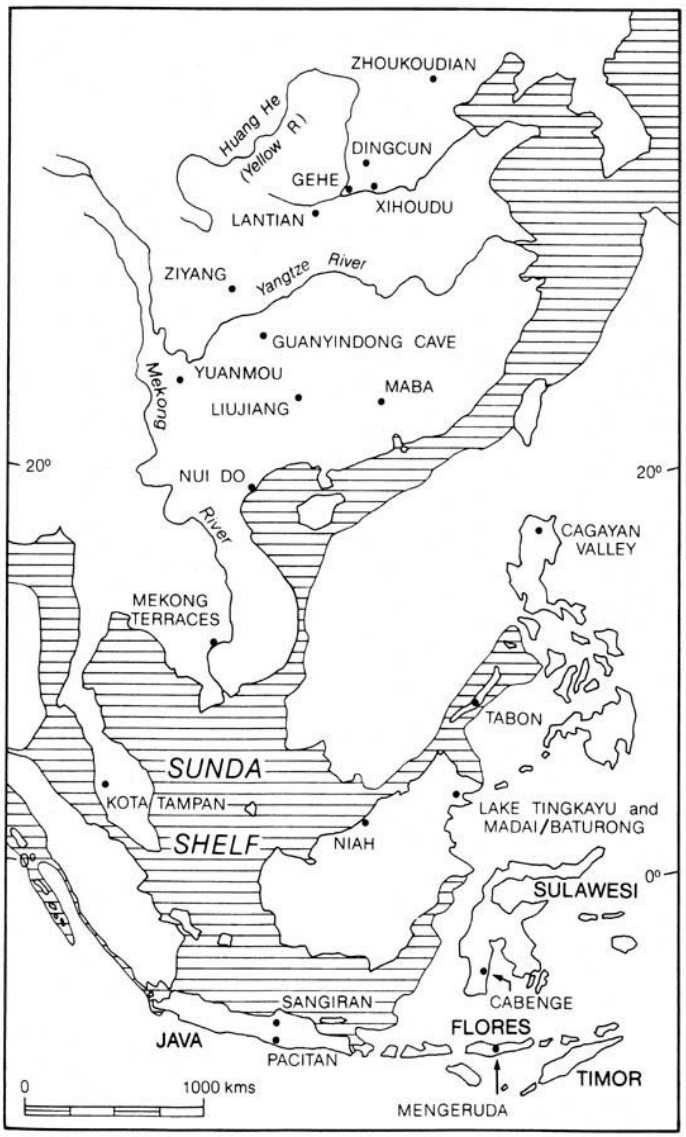


Figure 5.2 Island Southeast Asia and Last Glacial Maximum extent of the Sunda shelf (parallel lines).
(Adapted from http://www.ic.arizona.edu/~anth427/427_527a/maps/southchina.jpg)

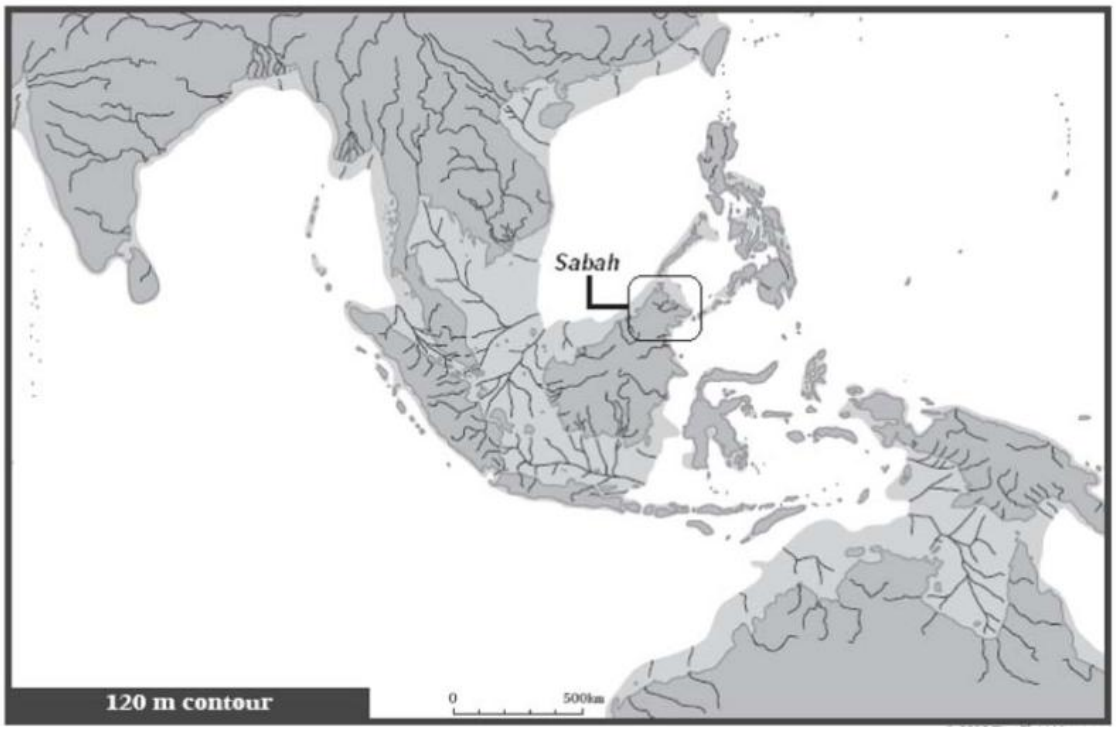


Figure 5.3 Map of Pleistocene sea level for Southeast Asia based on depth contour of 120 m below present level. No interconnection of Sunda River with Sabah rivers is shown. (Adapted from Kamarudin & Esa (2009).

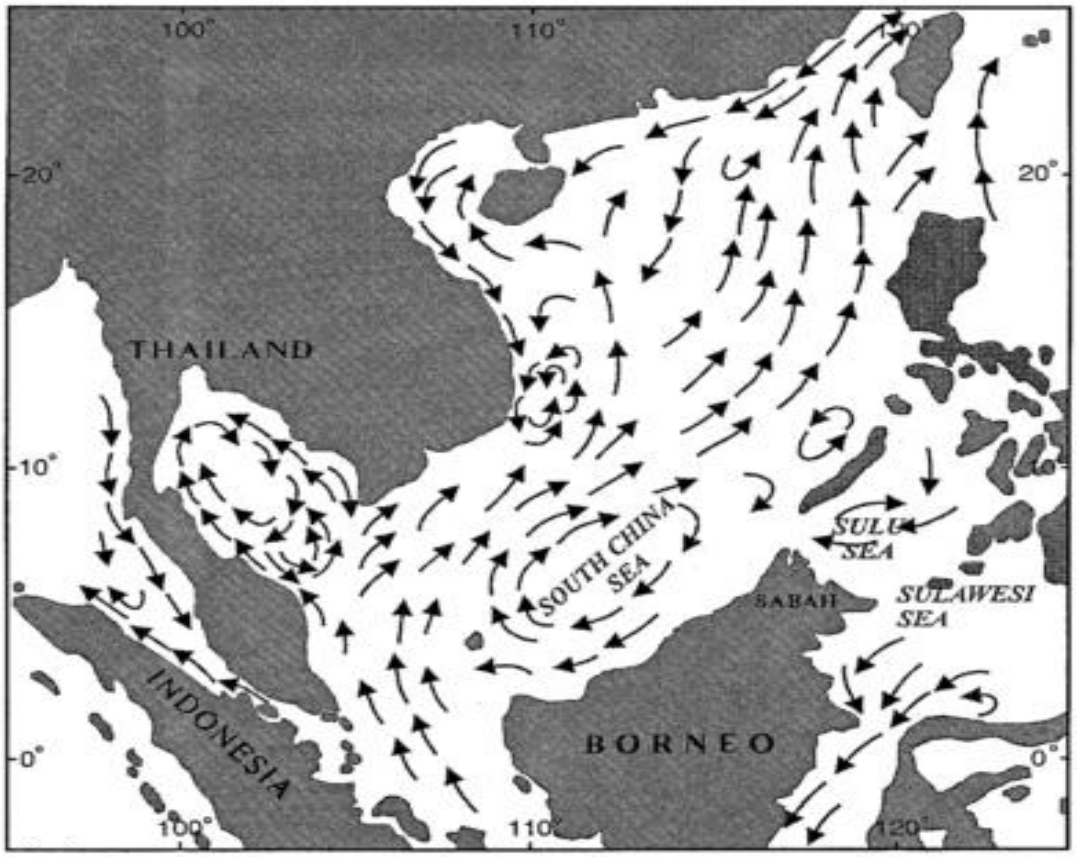


Figure 5.4 The sea surface currents of the South China Sea in summer. (Adapted from Morton & Blackmore, 2001).

5.4.2.2 *Gracilaria edulis* from Malaysia, Southeast Asian Countries and Japan

The present study revealed intraspecific genetic diversity in the *cox*1 gene of *G. edulis* from Malaysian waters, Thailand, Indonesia and Japan. A total of 12 *cox*1 haplotypes (10 from Peninsular Malaysia; two from East Malaysia) were identified in 120 individuals of *G. edulis* from 16 different geographically distinct populations in Malaysia. The intraspecific nucleotide differences range from 0-10 bp (0-1.2%) among *G. edulis* of Malaysia.

Based on the current findings, higher genetic diversity in Peninsular Malaysia than East Malaysia was found. The phylogenetic trees, haplotypes from Peninsular Malaysia (E1-E9 and E12) and Sabah (E10 and E11) were closely related. Haplotype E1 was detected in 89 out of 120 individuals and was dominant throughout majority of the populations in Peninsular Malaysia, but undetectable in populations of East Malaysia. Haplotype E1 was shown to be the common ancestor and the most widespread haplotype in Malaysia, excluding samples from Gua Tanah and two locations in Sabah. Along the coastlines of Peninsular Malaysia, ten haplotypes were observed (E1-E9 and E12). The hypothetical haplotype E1 represented all individuals sequenced from Pulau Sayak, Batu Feringgi, Morib, Batu Besar, Pulau Besar, Merambung shore, Pulau Che Kamat and Teluk Ramunia. It is possibly showing the lowest genetic diversity or no phylogeographic pattern for *G. edulis* in these populations since only the basal haplotype (E1) was found. A unique haplotype of E5 was found in a sample from Gua Tanah and differed from basal haplotype E1 by 2 bp. Sequence similarity between haplotype E1, E5 and E6 suggests that haplotype E5 could be evolved and derived from both E1 and E7. Specimens from Teluk Pelanduk (Negeri Sembilan), Batu Tengah (Malacca) and Kukup (Johore) showed the greatest divergence with identification of three haplotypes for each site (E1, E3 and E12 from Teluk Pelanduk; E1, E4 and E6 from Batu Tengah; E1, E7, and E9 from Kukup), followed by

individuals from Middle Banks (Penang) and Sungai Kong Kong with two haplotypes (E1 and E2; E1 and E8), respectively. The present results suggest that the ancestral population of *G. edulis* in Malaysia may have originated in Teluk Pelanduk (Negeri Sembilan), Batu Tengah (Malacca) or Kukup (Johore) as the highest levels of genetic variation were observed there. However, only three specimens from Kukup examined. Teluk Pelanduk and Batu Tengah are located in the Malacca Straits. The coastlines along the west coast of Peninsular Malaysia have suffering relentlessly from human-induced activities. The mangrove forests in west coast of Selangor, Negeri Sembilan, Malacca and South Johore were threatening by activities of land reclamation and conversion into other uses (e.g. aquaculture pond, extension of commercial port and tourism) (Mazlan et al., 2005). The coastline along Tebrau Straits has heavy traffic and has suffered heavily from human activities such as aquaculture, fisheries, coastal tourism and factories. Of these threats, oil spill caused by shipping accidents in this narrow and shallow channel is the greatest. The mangrove forests in Johore are being depleting at the rate of 5,000 trees yearly (The Star, 2nd December, 2011). According to Kasmin (2010), there were 144 cases of oil spills into the Malacca Straits from 2000-2005. The study for detrimental effects of oil pollution in Kukup has been conducted and it showed the devastation of mangrove area. Marine pollution enhances the rate of evolution of genomes of *G. edulis* with haplotype E1 and derived into newly haplotypes for adapting to local populations in order to cope with environmental changes. On the east coast of Peninsular Malaysia, *G. edulis* only could be found in Johore. The possibly owing to the deficiency accessibility of two water bodies between the Malacca Straits and the South China Sea, which are only connected by the narrow Tebrau Straits.

Two haplotypes E10 and E11 were revealed in Sabah (Kawasan Perumahan Green View and Pulau Nunuyan Laut). Both of these haplotypes were not detected elsewhere in Peninsular Malaysia. A unique haplotype E11, found in only two locations

in Sabah, was linked to basal haplotype E1 and haplotype E10 through 4 bp and 1 bp, respectively. According to Voris (2000), there was no interconnection between Sunda River and Sabah rivers during the last Pleistocene. For this reason, we postulate that the distribution of *G. edulis* from Peninsular Malaysia to East Malaysia could be considered by anthropogenic introduction with potential vectors including aquaculture, ship hulls and ballast waters. We have no data on genetic diversity of *G. edulis* from Sarawak. The level of genetic variation of *G. edulis* from Sarawak was not possible to clarify due to the sampling failure.

Two specimens of *G. edulis* from Thailand (Prachuap Khiri Khan), nine specimens from Lombok, Indonesia (collected from five locations: Landangluar, Gili Meno, Gili Genting, Batu Kijok and Rambang) and nine specimens from Japan (Bise Motobu, Okinawa) were examined to compare their genetic variation with Malaysian *G. edulis*.

In Thailand, about 333 species belonging to 132 genera were reported and the red algae have the most abundant diversity (Lewmanomont & Ogawa, 1995). The distribution of lineage *Gracilaria sensu lato* from Thailand is well documented by Chirapart (2008). The vernacular name of *G. edulis* in Thailand called *sarai woon*. According to the study of the distribution of mangrove forests in Thailand by Vibulsresth et al. (1975), the mangrove forest in Prachuap Khiri Khan considered as the smallest, approximately 4 km^2. It is located in the Gulf of Thailand. On the basis of current results from the haplotype network, both two individuals from Prachuap Khiri Khan provided identical *cox*1 gene mitochondrial DNA sequences. The single haplotype was also identical to the basal haplotype E1 of *G. edulis* from Malaysia. No significant genetic variation was show from the large geographic distances. However, the absence of basal haplotype E1 along the east coast of Peninsular Malaysia (excluding Johore) suggested recent colonization of *G. edulis* into Thailand.

The vernacular names of *G. edulis* in Indonesia are sayur karang, bulung embulung or janggut monyet. The low level of sequence variation of this species (three haplotypes namely E1, E13 and E14, differing by 1-2 bp) was revealed in nine specimens from five locations throughout Lombok, Indonesia. The basal haplotype E1 and haplotype E13 were detected from the western coast of Lombok, while a unique haplotype, E14, was found in eastern coast of Lombok. A small genetic variation between the basal haplotype E1 from Malaysia and Indonesia with haplotype E13 and E14 from Indonesia were obtained with larger geographic distances. Sequence similarity suggests that haplotype E13 could be derived from haplotype E1 in western Lombok, while haplotype E14 found in eastern Lombok may be independently derived from E13 through single substitution. The phylogenetic analyses (Fig. 4.22 & 4.23) showed that all haplotypes were divided into three main groups according to the regions: (i) Peninsular Malaysia, East Malaysia and Thailand; (ii) Indonesia region; (iii) Japan region. The close phylogenetic position of Malaysian and Thailand haplotypes (E1-E12) to Indonesian haplotypes (E1, E13 and E14) compared to Japanese haplotype (E15) in phylogenetic trees suggests that this species with haplotype E1 moved from Indonesia northwards to Malaysian waters in summer, as the type locality of *G. edulis* is Indonesia (Millar & Xia, 1997). Through the influences of the Southwest Monsoon in summer, the warm surface water flows in from the Java Sea via the Karimata Straits to Malaysian waters (Fig. 5.4).

High genetic divergence was observed for *G. edulis* from Japan in comparison to specimens from Southeast Asian countries. A unique haplotype E15 was found and identical for nine specimens from Japan (Bise, Motobu Okinawa). E15 differed from the basal haplotype E1 by 18 bp, and showed the greatest intraspecific variation of all the specimens examined. The relatively high level of the *cox*1 sequence variation revealed between haplotypes from Malaysia and Indonesia (differing by 1-6 bp) compared to

Japan in the haplotype network raises the possibility of hidden diversity here. It seems likely that the present Japanese populations originated from Indonesia, because haplotype E15 is relatively close to haplotypes from Indonesia (E1, E13 and E15) considering the TCS network (Fig. 4.22). Such high genetic divergence in Japan could be also due to the large geographical separation and influenced by both ecophysiological and biological factors (e.g. ocean current pattern, temperature and salinity of seawaters etc). Ordinarily, the larger the geographical separation of population is, the greater the level of genetic divergence would be (e.g. *Sargassum muticum* from Shandong Peninsular by Zhao et al. (2008)). We postulate that the great distance (more than 3500 km) would form a geographical barrier to gene flow among populations in Southeast Asian and Japan. A high *cox*1 intraspecific variation (11 bp nucleotide differences) of *Gracilaria gracilis* between two regions situated in South West England, about 120 km apart, was also reported by Robba et al. (2006). Additional sampling from the coastlines of Sarawak, Taiwan, Korea and China may enhance the accuracy of resolution for intraspecific genetic diversity study and phylogeographic distribution study in *G. edulis*.

5.4.3 Genetic Diversity of *G. changii* and *G. edulis*

The level of genetic homogeneity and heterogeneity within a species population may reflect the diversity of gene pool (Kidgell & Winzeler, 2005). Little intraspecific genetic variation was revealed for both species in Southeast Asian countries. It is likely that these species have a shallow genetic history (sharing a recent common ancestor) and rapid dispersal (high gene flow). The wide geographic distribution of different haplotypes may possibly be due to anthropogenic activities as suggested by Zuccarello et al. (2002). Haplotype distributions could correlate with exotypes that differ in their genotypic and/or phenotypic adaptations. *Gracilaria* has remarkable ecological traits

that are capable of adapting to the harsh silted mangrove and polluted areas, as well as with an ability to adapt to a wide range of ecological conditions (Phang et al., 1996). It is a euryhaline (grow well in salinity range of 20-28 ppt) and eurythermal algae (optimal development obtains between 20 and 28ºC) (Causey et al., 1946). Each species might respond differently to the environment change by shown unique strategy to adapt themselves to a range of variable environmental conditions for surviving. Ongoing natural processes of mutation, migration, genetic drift and natural selection determine the current level of genetic diversity within a species. Overall, the low distance values between haplotype diversity, *Hd* (0.00 to 0.3755), low nucleotide diversity, *Pi* (0.00055) and genetic distance differences (0.1-0.5%) showed a very close genetic relationship between *G. changii* specimens from Malaysia and Southeast Asian. This suggests that all *G. changii* examined were derived from a single ancestral strain that may have dispersed relatively rapidly and evolved over time in a range of populations by anthropogenic activities or natural distribution pathways. The specimens of *G. edulis* revealed a higher value of haplotype diversity, *Hd* (0.00 to 0.5291), nucleotide diversity, *Pi* (0.00318) and genetic distance differences (0.1-2.1%) in comparison to *G. changii*. In some localities examined, only a single haplotype was found in *G. edulis*. It is probably due to the influence of both ecophysiological and biological factors which prevented the recruitment of strains of other haplotypes. Despite the different in level of genetic differentiation in *G. changii* and *G. edulis*, both species do appear to share a similar broad geographic pattern. The lower haplotype diversity in *G. changii* relative to *G. edulis* in Malaysia indicates either a more recent introduction or a higher rate of gene flow of *G. changii* relative to *G. edulis*. These species do not share similar histories. Their genetic variation and phylogeographic distributions may have been affected differently by the Malaysian continental drift, and recent colonization events through artificial introductions or natural distributions. No or low intraspecific genetic diversity

variation with large geographic distances have been reported in studies of other red algae (*Murrayella periclados* by Zuccarello et al., 2002a; *Gracilariopsis lemaneiformis* by Pang et al., 2010); *Batrachospermum gelatinosum* by House et al., 2010; *Batrachospermum arcuatum* by Vis et al., 2010)

Haplotype C1 and haplotype E1 have been suggested as the common ancestor for *G. changii* and *G. edulis*, respectively. Individuals with haplotype C1 or E1 may possess high allele diversity and have greater capacity for evolutionary adaptation. These individuals have greater prospects in mariculture as they may possess a diversity of desirable properties like high growth rate, etc.

5.4.4 Factors Affecting Genetic Diversity of Seaweed

The Pleistocene glaciations events and currents circulation have vastly influenced the distribution of fauna and flora in Malaysia (e.g. Kamarudin & Esa, 2009; Pin et al., 2001; Lim et al., 2012; Rozihan & Ismail, 2012). Current threats to seaweed resources in Malaysian coastal waters have been reported by several researchers (e.g. Mazlan et al., 2005; Phang et al., 2006), which include land reclamation, pollution (e.g. land-based industrial, oil pollution), devastation of mangrove forest, overexploitation of commercially important species, introduction of invasive species and shipping.

Seaweeds are distributed around the world by various processes. Natural or anthropogenic environmental derived changes have been reported in many species by several researchers (Thompson, 1998, Mousseau et al., 2000, Umina et al., 2005; Schaffelke et al., 2006; Provan et al., 2012), which influences the level of genetic diversity of a species. The rapid loss of habitat leading to decline in seaweed populations worldwide have been reported elsewhere (e.g. Mazlan et al., 2005). The translocation of species causes confusion in seaweed biogeography (Mineur et al., 2010). More than one hundred seaweed species have been documented that are widely

dispersed across their native ranges due to anthropogenic activities (Farnham et al., 1973; Rueness, 1989; Curiel et al., 1998; Fletcher & Farell, 1999; Rueness & Rueness, 2000; Boudouresque & Verlaque, 2002; Smith et al., 2002; Kim et al., 2004; Uwai et al., 2006a; Cho et al., 2007; Sherwood, 2008). There are a number of vectors for seaweed introductions such as aquaculture activities, hull-fouling, ballast and fishing gear. Aquaculture and fouling of vessel hulls are the major means for seaweed introductions (Williams & Smith, 2007). One of the human-assisted relocation of marine species is the transplantation of commercially important species from one population to another. Oysters were one of the vectors that introduced many groups of seaweed from Japan into Europe in the past two centuries (Farnham, 1994; Uwai et al., 2006a; Saunders, 2009; Cheang et al., 2010; Mineur et al., 2010). Approximately 12 species of algae have been introduced to California likely attributed to oyster aquaculture (Miller, 2004). The introduction of an alien *Gracilaria vermiculophylla* into United States from plants attached to transplanted oyster from the West Pacific was reported by Thomsen et al. (2005). According to Tseng (2001), *Saccharina japonica* in Japan was accidentally introduced to the coastline of China around 1927. Trans-oceanic introductions of marine organisms as a consequence of the globalization of shipping systems. Fisheries have also been documented by a number of researchers in such introductions (Carlton & Hodder, 1995; Ribera & Boudouresque, 1995; Nelson & Maggs, 1996; McIvor et al., 2001; Shaffelke et al., 2006; Uwai et al., 2006a). In Singaporean waters, vectors such as aquaculture and shipping have been reported (Lee et al., 2009).

The anthropogenic activities through the deposition of pollutants, habitat disturbance, over-harvesting, costal degradation, global climate change and transoceanic introduction of alien species and invasive species have caused substantial irreversible changes to the aquatic and terrestrial ecosystems. Elevation of global temperature is one

of the major issues at the global level. Temperature of seawater is the foremost abiotic factors accountable for the morphology (Chung et al., 2007), survival, reproduction and geographic distribution of seaweeds (Agrawal, 2009; Gao et al., 2013). Climate change would influence the distribution patterns of seaweed and cause the replacement of cold-water species by warm-water species (Jump & Peñuelas, 2005; Kantachumpoo et al., 2014). According to Schiel et al. (2004) and Harley et al. (2006), global warming may greatly decrease the abundance of seaweed by affecting the biology of the species, e.g. *G. verrucosa* with few other algae have completely extinct along the North Sea coast (Ducrotoy, 1999).

According to Loveless & Hamrick (1984), intrinsic factor of life history characteristics of a species can be applied to understand their genetic structure and genetic diversity. It is also influenced by the dispersal abilities of spores between populations. Genetic structure of populations for a given species is significantly affected by geographical distance through restricting the movement of spores, gametes, or drifting reproductive fragments. *Gracilaria* has a complex haploid-diploid isomorphic life cycle and is known to have a remarkable regeneration capacity from breaks or damaged areas on attached and detached plants (e.g. *G. salicornia* by Smit et al., 2004; *G. vermiculophylla* by Thomsen et al., 2005; *G. chilensis* by Guillemin et al., 2008). *Gracilaria* attaches to biogenic or other substrates (e.g. plastic bags and trawling nets) by its holdfast and might break into small fragments and drift to other populations via hydrodynamic events. *G. changii* and *G. edulis* are typically characterized by complex life cycles which can reproduce either sexually or asexually. According to Kumar & Singh (1979), there are two types of life cycles in red algae in general, namely the life cycle with two alternating haploid gametophytic and carposporophytic generations; and the life cycle with three generations (triphasic) consisting of a dioecious haploid sexual phase (the gametophyte), a diploid phase that develops directly on the female thallus

(the carposporophyte) and a free-living diploid phase bearing meiosporangia (the tetrasporophyte). The dioecious haploid gametophytes restrict the passive dispersal mechanism. Members of this genus can be propagated through the release of spores, either asexual tetraspores or sexual carpospores. In the state of suspension, the spore dispersal mainly relies on their longevity and viability. However, seaweed spores and sperm are not adapted to long-distance dispersal resulting in a low rate of fertilization between populations (van den Hoek, 1987) and short viability (Noormohammadi et al., 2011). The viability and longevity of spematia has been considered as the major reason of fertilization limitation in *Gracilaria* species. Destombe et al. (1990) showed that the non-motile spermatia of *G. verrucosa* survived for less than 5 hours with dispersion distance of 80 meters.

5.4.5 Molecular Approaches for Genetic Diversity and Phylogeographic Studies

Advances in DNA-based genetic markers provide the essential tools in measuring genetic diversity relating to the evolution, biogeography and systematics of red algae by exploiting genetic variation in the entire genome of organisms. The *cox*1 intraspecific nucleotide differences in many red algae range from 0-2 bp and interspecific nucleotide differences are more than 30 bp (Saunders, 2005). Generally, the intraspecific variation shown in the present study (0-6 bp in *G. changii*; 0-20 bp in *G. edulis*) principally agreed with the results reported by Robba et al. (2006) in red algae and by Yang et al. (2008) in *G. vermiculophylla*. The *cox*1 gene gave higher variability for *G. vermiculophylla* (variable up to 11 bp) compared to the *rbc*L gene. Genetic diversity studies of seaweeds using the mitochondrial gene have been carried out by several researches with intraspecific nucleotide differences ranging from 0-35 bp. Current researches on freshwater red algae proved that the *cox*1 is a potential candidate marker for phylogeographic studies (House et al., 2008; Sherwood, 2008; Sherwood et

al., 2008). The *cox*1 gene has also been suggested as a potential DNA barcode marker for red algae, brown algae and for some other diatom genera (Evans et al., 2007; Geraldino et al., 2006; Hu et al., 2009; Kim et al., 2010; Kucera and Saunders, 2008; Le Gall and Saunders, 2010; Yang et al., 2013).

A number of genetic diversity and phylogeographic studies on seaweeds have been published using nuclear markers and plastid markers. Ribosomal internal transcribed spacer (ITS) regions have been employed in studies of biogeographical, intraspecific, species identification and phylogenetic studies to Rhodophyta owing to its rapid evolution rate (Broom et al., 2002; Marston & Villard-Bohnsack, 2002; Hu et al., 2009). However, the overriding drawback of the high level of intra-individual ITS variation have been reported (Famà et al., 2000; Lange et al., 2002; Uwai et al., 2006b). In contrast, mitochondrial markers are uniparental inherited and intra-individual polymorphism is less known. Plastid markers of the gene for the large subunit of rubisco, *rbc*L, are used to investigate the phylogeographic pattern of red algae *Polysiphonia harveyi* in Japan (McIvor et al., 2001). Meanwhile, AFLP and SSR have been employed in genetic diversity analysis of *Gracilariopsis lemaneiformis* from China. Recent work in Phaeophyta has shown that ISSR is applicable in genetic structure analysis of *Sargassum muticum* (Zhao et al., 2008) compared to RAPD. Microsatellite DNA markers are codominant and readily applied in measurement of genetic diversity studies. This technique is being currently favoured owing to the high resolution observed. Waittier et al. (1997) reported the use of single-locus microsatellite to study the population genetics of *G. gracilis* due to the high variability of microsatellite loci. In addition, recent research on development of microsatellite DNA markers for genetic diversity of *G. chilensis* (Guillemin et al., 2005) has shown the potential of this molecular tool. However, genetic homogeneity and low rate of SSR polymorphism was reported for *Porphyra* species (Pang et al., 2010, unpublished data)

and *Gracilariopsis lemaneiformis* (Zhang et al., 2009a) between different geographical areas.

However, the direction and means of dispersal for both *Gracilaria* species remains uncertain in Malaysian waters given the present data. Haplotypes within a contiguous body of water may intermix continuously and disperse naturally by water current or by anthropogenic activities. For *G. changii*, an original colonization of Morib or Teluk Pelanduk with subsequent dispersal to the other locations is suggested; while Teluk Pelanduk, Batu Tengah or Kukup for *G.* edulis. The exact phylogeographic pattern of both species was difficult to ascertain. Phylogeographic distribution analysis for marine algae with taxonomically doubtful fossil evidence is crucial to reveal their biogeographic and population histories (Vidal, 2008) and requires an integration of morphological, molecular and life-history approaches (Zuccarello & West 2002). Increased sample sizes and more systematic sampling strategies should be employed for wider biogeographical areas.

CHAPTER 6

CONCLUSION

274 specimens of *G. changii* were collected from waters in Peninsular Malaysia and East Malaysia (Sabah and Sarawak) and 20 specimens from other Asia countries of Thailand (2), Indonesia (9) and Japan (9). For *G. edulis*, 120 specimens from Malaysia and 20 specimens from other Asia countries of Thailand (2), Indonesia (9) and Japan (9) were obtained for this study.

Two genetic markers, *cox*2-3 spacer and *cox*1 gene were investigated for genetic diversity analysis in Malaysian *G. changii*. The *cox*1 gene was found to be more suitable because it produced highest genetic diversity and was used for further studies for *G. changii* and *G. edulis* from other countries.

6.1 SUMMARY OF SIGNIFICANT RESULTS OF THIS STUDY

The significant results from this study can be summarized as follow:

1. Based on collection data, the diversity of *Gracilaria* species from west coast of Peninsular Malaysia is richer than in the east coast of Peninsular Malaysia and East Malaysia.
2. *G. changii* and *G. edulis* collected had no significant morphological variation from the morphological descriptions of Xia & Abbott (1987) and Lim & Phang (2004). However, *G. changii* from Selalang, Sarawak and Pulau Nunuyan Laut, Sabah were phenotypically dissimilar.
3. The use of the molecular analyses in combination with morphological studies is crucial for better species identification.

4. Commercial DNeasy extraction kit is suitable for genomic DNA extraction from *Gracilaria changii* and *G. edulis* with little modification. Step-up PCR was shown to be useful in enhancing the success rate of DNA amplification.
5. The *cox*2-3 spacer appears to be highly conserved and showed less significant intraspecific genetic variation for *G. changii* compared to the *cox*1 gene.
6. The present study showed that *G. changii* and *G. edulis* examined belong to different haplotypes. Alternative hypotheses supported.
7. A statistical parsimony network of *G. changii* yielded eight haplotypes (C1-C8) which were closely related. Populations at Morib (Selangor) or Teluk Pelanduk (Negeri Sembilan) were suggested as probable ancestral population of *G. changii*. Haplotype C1 is suggested as the common ancestor of *G. changii* and it colonized earlier in Morib or Teluk Pelanduk and having sufficient time to evolve into the various haplotypes.
8. *G. edulis* showed higher genetic divergence compared to *G. changii*. A total of 12 *cox*1 haplotypes (E1-E12) were found for *G. edulis* in Malaysian waters, three haplotypes (E1, E13, and E14) in Indonesia and only E15 in Japan. E15 showed the greatest intraspecific variation of all the specimens examined. Haplotype E1 was shown to be the common ancestor. The ancestral population of *G. edulis* in Malaysia may have originated in Teluk Pelanduk (Negeri Sembilan), Batu Tengah (Malacca) or Kukup (Johore) as contributing the highest intra-population diversity.
9. The lower haplotype diversity in *G. changii* relative to *G. edulis* in Malaysia shows either a more recent introduction or a higher rate of gene flow of *G. changii* relative to *G. edulis*. These species do not share similar histories. Their genetic variation and phylogeographic distributions may have been affected

differently by the Malaysian continental drift, and recent colonization events through artificial introductions or natural distributions.

10. The Pleistocene events, anthropogenic activities and natural distributions have been postulated to affect the dispersal, in turn the genetic populations structure of *G. changii* and *G. edulis.*
11. In contrast with molecular data, despite phenotypic variation were observed for *G. changii* from Selalang, Sarawak and Pulau Nunuyan Laut, Sabah, no correlation between morphological features of *G. changii* and the *cox*1 haplotypes were found. For *G. edulis*, no populations showed a unique range of morphological variation.
12. The *cox*1 gene was shown to be suitable for resolving intraspecific genetic variation and is a reliable marker for the study of genetic diversity and phylogeographic distribution in *Gracilaria* species and even of Rhodophytes. The *cox*1 gene has also been suggested as a potential DNA barcode marker for red algae.

However, the intraspecific genetic diversity of *G. changii* and *G. edulis* cannot be clarified accurately due to the sampling failures and limited size of samples collected in some regions. The exact phylogeographic pattern of both species was difficult to ascertain given the present data.

6.2 APPRAISAL OF THE STUDY AND RECOMMENDATIONS FOR FUTURE RESEARCH

This represents the first extensive genetic diversity study of two important tropical agarophytes. The study allowed the identification of a suitable molecular marker which may be useful for DNA barcoding for *Gracilaria.*

For the regional study, only few specimens were available for this study. Extensive sampling from a wider biogeographical areas and increased sample sizes from different geographical areas are essential to provide a better understanding of the genetic diversity this potentially economically important agarophyte. The findings in the present study augmented our understanding of the genetic diversity of *G. changii* and *G. edulis* in this region and highlighted several interesting sampling locations for further investigation and will contribute significantly to ongoing studies. Some of the interesting aspects that are necessary to be exploited including determining whether the various of haplotypes correlate with ecotypes that differ in their response to the environment, agar content and rheological analysis of gel strength on the common ancestor haplotype. This may provide insights into origin and evolutionary relationships of *G. changii* and *G. edulis* in Malaysian waters as well as the consequence of natural and anthropogenic impacts which have affected the geographic distribution of *G. changii* and *G. edulis.* This essential information contributes to plant breeding programs for the most tolerant strain or haplotype for cultivation in the future.

In conclusion, the alternative hypotheses (H_1) for *G. changii* and *G, edulis* were accepted.

H_1: *G. changii* collected from various localities belong to different haplotypes.

H_1: *G. edulis* collected from various localities belong to different haplotypes.

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PRESENTATIONS

ORAL PRESENTATIONS:

1. Application of Mitochondrial *cox*1 Gene for Genetic Diversity Studies on Malaysian *Gracilaria changii* and *Gracilaria edulis* (Gracilariaceae, Rhodophyta). 8th Asia Pacific Conference on Algal Biotechnology and 1st International Conference on Coastal Biotechnology, 09 July to 12 July 2012 at Adelaide, Australia, (International).

2. Application of mitochondrial *cox*1 gene in genetic diversity study of Malaysian *Gracilaria changii* (Gracilariaceae, Rhodophyta), 16th Biological Sciences Graduate Congress, 12 December to 14 December 2011 at Singapore, (International).

3. Genetic Diversity of Malaysian *Gracilaria changii* and *Gracilaria edulis* (Gracilariaceae, Rhodophyta), based on the Mitochondrial *cox*1 gene. 7th Asia-Pacific Conference on Algal Biotechnology, 01 December to 04 December 2009 at New Delhi, India, (International).

POSTER PRESENTATION:

1. Genetic Diversity of *Gracilaria changii* and *Gracilaria edulis* (Gracilariaceae, Rhodophyta) in Malaysia Waters. Sharing Knowledge, Resources and Technologies for a Sustainable South China Sea 2012, 21 October to 24 October 2012 at University of Malaya, Malaysia, (International).

LIST OF PUBLICATIONS ARISING FROM THIS RESEARCH

1. Yow, Y. Y., Lim, P. E., & Phang, S. M. (2011). Genetic diversity of *Gracilaria changii* (Gracilariacea, Rhodopyta) from west coast, Peninsular Malaysia based on mitochondrial *cox*1 gene analysis. *Journal of Applied Phycology*, *23*, 219-226.

2. Yow, Y. Y., Lim, P. E., & Phang, S. M. (2013). Assessing the use of mitochondrial *cox*1 gene and *cox*2-3 spacer for genetic diversity study of Malaysian *Gracilaria changii* (Gracilariaceae, Rhodophyta) from Peninsular Malaysia. *Journal of Applied Phycology*, *25*, 831-838.

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Genetic diversity of *Gracilaria changii* (Gracilariaceae, Rhodophyta) from west coast, Peninsular Malaysia based on mitochondrial *cox*1 gene analysis

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Abstract Mitochondrial cytochrome *c* oxidase subunit I (*cox*1) was employed to investigate the intraspecific genetic diversity of *Gracilaria changii* collected from various localities distributed along the west coast of Peninsular Malaysia. *Gracilaria changii* is an agarophyte with potential for commercialization in Malaysia as it has high yields of good quality agar with high gel strength for the production of food grade agar and agarose. The phylogeographic aspect of *G. changii* has not been studied despite its abundance and potential commercialization. In this study, six mitochondrial haplotypes (C1–C6) were revealed from 62 specimens varying by 0–3 bp over 923 bp. Results indicate that haplotype C1 is the common ancestor and the most widespread haplotype due to its prevalence in Morib, Gua Tanah, Middle Banks, Batu Besar, Batu Tengah, Sungai Pulai, and Kuala Sungai Merbok. In this study, Morib was suggested as contributing the highest intrapopulation diversity with the identification of three haplotypes. The mitochondrial marker *cox*1 is a highly divergent mitochondrial marker and is applicable for studies on species identification and assessment of genetic diversity of *G. changii*.

Keywords *cox*1 gene · *Gracilaria changii* · Phylogeography · Genetic diversity

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Introduction

Gracilaria is the second largest genus of the red algae (Brodie and Zuccarello 2007) comprising more than 150 species distributed worldwide (Byrne et al. 2002), many of which are of economic (Oliveira et al. 2000) and ecological importance (McLachlan and Bird 1986). A total of 20 species of *Gracilaria* have been recorded for Malaysia (Lim and Phang 2004).

Gracilaria changii from Malaysia was originally described by Xia and Abbott (1987) and is widely distributed in the mangrove areas fringing the west coast of Peninsular Malaysia (Phang et al. 1996; Lim and Phang 2004). The wide use of high-quality agar and agarose with good gel strength extracted from *G. changii* (Phang et al. 1996) has generated an increased interest among scientists and entrepreneurs, e.g., substitution for gelatin in food, cosmetic, and pharmaceutical applications. *G. changii* contains a notable amount of protein, fatty acids (Chu et al. 2003), and bioactive compounds (Wong et al. 2006). However, no information about the intraspecific genetic diversity of this species is available. The high demand for *G. changii* in agar production has increased dramatically and resulted in the overharvesting of wild populations. Losses in seaweeds are also attributed to human activities through habitat destruction, overharvesting, pollution, development of coastal areas for tourism, global climate change, and introduction of alien species. Buschmann et al. (2001) reported that there was a major shift in the algal industry in the 1980s to obtaining raw material from harvesting natural beds to predominantly mariculture-based production as a result of decline in wild populations due to overexploitation. Expansion of mariculture also led to introductions of marine algae from one geographic region to another, which has been documented by a

number of researchers (McIvor et al. 2001; Nelson et al. 1996; Uwai et al. 2006a).

Genetic diversity is the fundamental component of biodiversity that quantifies the magnitude of genetic variability within a population. Measurement of genetic diversity contributes important clues to an understanding of the nature of forces acting on genetic variation, pattern, level of genetic variation, and evolutionary history. Genetic diversity within a population also has ecological effects on productivity, growth, and sustainability, as well as interspecific interactions within communities and ecosystem-level processes (Hughes et al. 2008).

DNA-based molecular markers provide a pivotal role in the assessment of genetic diversity for a highly plastic genus such as *Gracilaria* (Bird and McLachlan 1982). Application of mitochondrial DNA in animal population studies and phylogenetic reconstruction has increased owing to the rapid evolution, lack of recombination, and uniparental inheritance of mtDNA (Avise 1994). The success of mitochondrial marker cytochrome oxidase subunit I (*cox*1) in animals led to the assessment of this marker for applications in DNA bar coding in red algae (Saunders 2005).

A number of intraspecific markers have been employed on phylogenetic and genetic diversity studies such as the *cox*1 gene, (Robba et al. 2006; Yang et al. 2007; Sherwood 2008), mitochondrial cytochrome oxidase subunit 3, *cox*3 (Steel et al. 2000; Coyer et al. 2004; Uwai et al. 2006b), the mitochondrial-encoded *cox*2-3 spacer (Zuccarello et al. 1999; Zuccarello and West 2002; Rueness 2005; Zuccarello et al. 2006b; Vidal 2008), nuclear-encoded internal transcribed spacers of the ribosomal cistrons, ITS1 and ITS2 (Bellorin et al. 2002; Marston and Villalard-Bohnsack 2002; Cho et al. 2007), the plastid-encoded RuBisCo spacer (Byrne et al. 2002; Zuccarello et al. 2006a), and *rbc*L gene (Nam et al. 2000; McIvor et al. 2001; Gurgel and Fredericq 2004). The first study on the haplotype analysis of the *cox*1 coding gene of *Gracilaria vermiculophylla* by Yang et al. (2007) showed it to be a reliable molecular marker for intraspecific study and useful for revealing species relationships, population structure, and the hidden diversity of red algae.

The present study aims to infer the geographic distribution of *G. changii* along the west coast of Peninsular Malaysia. Our work on comparing the suitability of the *cox*1 and the *cox* 2-3 spacer for genetic diversity of *G. changii* showed that the *cox*1 gene was better than the *cox* 2-3 spacer, giving higher resolution (unpublished data). Hence, in this study, the mitochondrial cytochrome *c* oxidase subunit I (*cox*1) gene was used, although the uniparental inheritance and limited variation of markers have been a handicap for certain circumstances. We have focused primarily on samples collected from the west coast of Peninsular Malaysia. However, from this study, we could not estimate the vectors, factors, and stresses that may have contributed to the existence of the various haplotypes shown in *G. changii* as details on the shipping, environmental changes, and introduction of alien marine algae into this region are not available.

Materials and methods

Healthy samples of *Gracilaria changii* (Xia et Abbott) Abbott, Zhang et Xia were collected randomly in the field from various localities distributed along the west coast of Peninsular Malaysia: Penang, Kedah, Selangor, Negeri Sembilan, Malacca, and Johore. Specimens examined in the present study are listed in Table 1. The specimens were cleaned with seawater and distilled water. Mud or dirt, epiphytes, epizoites, and fungi were removed by successive washing in seawater with a final rinse in distilled water. Specimens were dried in silica gel prior to isolation of their DNA.

Genomic DNA was extracted from approximately 10 mg of tips from dried specimens of *G. changii* (ground into powder using liquid nitrogen) with DNeasy Plant Mini Kit (Qiagen, Germany). All the isolation steps were carried out according to the instructions of the manufacturer with minor modification by incubating the disrupted samples with buffer AP1 for 20 min at 65°C.

The amplifications of extracted genomic DNA and DNA sequencing were carried out using mitochondrial primers of *cox*1 43F and *cox*1 1549R from Geraldino et al. (2006). The amplification of DNA was performed in a final volume of 25 μL containing 2.5 μL 10× buffer, 0.2 mM of each dNTP (dATP, dTTP, dCTP, and dGTP), 10 pmol of each forward and reverse primer, 1.5 U *Taq* polymerase, and 20–50 ng of genomic DNA. Ultrahigh-quality water was added to make up the final total volume of 25 μL. polymerase chain reaction (PCR) was carried out by GeneAmp® PCR system 2700 (Applied Biosystem) thermal cycle with an initial denaturation at 94°C for 10 min to denature the double-stranded DNA, followed by 35 cycles of amplification (denaturing at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 2 min) with a final extension for 10 min at 72°C. The amplified products were electrophoresed on 1% (*w*/*v*) TAE agarose gel pre-stained with ethidium bromide. PCR products were purified with the QIaquick PCR Purification Kit (Qiagen).

Sequencing data were analyzed and edited using Chromas 2.01 (Technelysium Pty Ltd., Australia) and BioEdit 7.0.9.0 (Hall 1999) software. Edited sequences were aligned by CLUSTAL X program (Thompson et al. 1997) followed by PAUP 4.0b10 (Swofford 2002). Haplotype networks (gene genealogies) were created using TCS 1.13 (Clement et al. 2000) to calculate the minimum

Table 1 Sampling location and haplotype identified for *G. changii* by *cox*1 gene

Collection site	Number of specimens	Collection number of specimen	Gene bank accession number	Haplotype
Kuala Sungai Merbok, Kedah	7	PSM11101_UMSS 0001	GU645726	C1
		PSM11102_UMSS 0002	GU645727	C1
		PSM11103_UMSS 0003	GU645728	C1
		PSM11104_UMSS 0004	GU645764	C1
		PSM11105_UMSS 0005	GU645765	C1
		PSM11106_UMSS 0006	GU645729	C1
		PSM11107_UMSS 0007	GU645730	C1
Middle Banks, Penang	11	PSM11108_UMSS 0008	GU645731	C1
		PSM11109_UMSS 0009	GU645732	C1
		PSM11110_UMSS 0010	GU645766	C1
		PSM11111_UMSS 0011	GU645733	C1
		PSM11112_UMSS 0012	GU645734	C1
		PSM11113_UMSS 0013	GU645735	C1
		PSM11114_UMSS 0014	GU645736	C1
		PSM11115_UMSS 0015	GU645737	C1
		PSM11116_UMSS 0016	GU645767	C1
		PSM11117_UMSS 0017	GU645768	C1
		PSM11118_UMSS 0018	GU645738	C1
Morib, Selangor	10	PSM11119_UMSS 0019	GU645769	C1
		PSM11120_UMSS 0020	GU645739	C5
		PSM11121_UMSS 0021	GU645740	C5
		PSM11122_UMSS 0022	GU645770	C4
		PSM11123_UMSS 0023	GU645741	C5
		PSM11124_UMSS 0024	GU645742	C5
		PSM11125_UMSS 0025	GU645771	C5
		PSM11126_UMSS 0026	GU645743	C5
		PSM11127_UMSS 0027	GU645772	C5
		PSM11128_UMSS 0028	GU645744	C5
Gua Tanah, Malacca	9	PSM11129_UMSS 0029	GU645773	C1
		PSM11130_UMSS 0030	GU645745	C1
		PSM11131_UMSS 0031	GU645774	C1
		PSM11132_UMSS 0032	GU645746	C1
		PSM11133_UMSS 0033	GU645747	C1
		PSM11134_UMSS 0034	GU645748	C1
		PSM11135_UMSS 0035	GU645749	C1
		PSM11136_UMSS 0036	GU645750	C1
		PSM11137_UMSS 0037	GU645751	C1
Batu Besar, Malacca	8	PSM11138_UMSS 0038	GU645775	C1
		PSM11139_UMSS 0039	GU645776	C1
		PSM11140_UMSS 0040	GU645777	C2
		PSM11141_UMSS 0041	GU645778	C1
		PSM11142_UMSS 0042	GU645752	C1
		PSM11143_UMSS 0043	GU645753	C1
		PSM11144_UMSS 0044	GU645754	C1
		PSM11145_UMSS 0045	GU645755	C1
Batu Tengah, Malacca	7	PSM11146_UMSS 0046	GU645756	C1
		PSM11147_UMSS 0047	GU645779	C1
		PSM11148_UMSS 0048	GU645780	C1
		PSM11149_UMSS 0049	GU645781	C1

Table 1 (continued)

Collection site	Number of specimens	Collection number of specimen	Gene bank accession number	Haplotype
		PSM11150_UMSS 0050	GU645757	C1
		PSM11151_UMSS 0051	GU645758	C1
		PSM11152_UMSS 0052	GU645759	C1
Sungai Pulai, Johore	6	PSM11153_UMSS 0053	GU645782	C1
		PSM11154_UMSS 0054	GU645783	C1
		PSM11155_UMSS 0055	GU645784	C1
		PSM11156_UMSS 0056	GU645785	C1
		PSM11157_UMSS 0057	GU645760	C1
		PSM11158_UMSS 0058	GU645761	C1
Teluk Pelanduk, Negeri Sembilan	4	PSM11159_UMSS 0059	GU645786	C6
		PSM11160_UMSS 0060	GU645762	C6
		PSM11161_UMSS 0061	GU645787	C6
		PSM11162_UMSS 0062	GU645763	C3

number of mutational steps by which the sequences can be joined with >95% confidence.

Results

Sixty-two specimens of *G. changii* from eight different biogeographic locations (i.e., Morib, Gua Tanah, Middle Banks, Batu Besar, Batu Tengah, Sungai Pulai, Kuala Sungai Merbok, and Teluk Pelanduk) were used for the study of genetic diversity using the *cox*1 gene.

A statistical parsimony network of 62 taxa aligned as 923 characters of the *cox*1 gene revealed six haplotypes based on the specimens collected from the eight localities along the west coast of Peninsular Malaysia, namely, C1, C2, C3, C4, C5, and C6 (Fig. 1 and Table 2). Among the examined populations, prevailing haplotype C1 was inferred as the basal haplotype. Haplotype C1 was found in Kuala Sungai Merbok, Middle Banks, Morib, Gua Tanah, Batu Besar, Batu Tengah, and Sungai Pulai. Haplotype C2 was detected in Batu Besar and differs from C1 by three mutation changes: an adenine to cytosine at position 172, a thymine to cytosine at position 410, and an adenine to guanidine at position 728. Haplotype C3 from Teluk Pelanduk was formed from the ancestral C1 with two base changes where an adenine was substituted by cytosine at position 25 and followed by substitution of cytosine to thymine at position 108. There was a substitution of an adenine to guanidine at position 644 for C4; this haplotype was contributed by the Morib samples. The deletions of thymine at position 16 and an adenine at position 43 with a mutation change of adenine to guanidine at site 644 were found for haplotype C5 from Morib. An insertion of cytosine at site 40 was observed from Teluk Pelanduk and differentiated haplotype C6.

Discussion

The *cox*1 gene has been reported as an ideal marker for DNA bar coding of red algae by several researchers (e.g., Saunders 2005; Geraldino et al. 2006; Robba et al. 2006). In addition, it is also useful for revealing the population structure and the hidden diversity of red algae (Robba et al. 2006). The specimens of *G. changii* collected from different biogeographical locations along the west coast of Peninsular Malaysia were used to elucidate the distribution of genetic diversity using the *cox*1 gene; their genetic distribution is shown in Fig. 2.

Based on the results of TCS software, haplotype C1 was clarified as the common ancestor and the most widespread

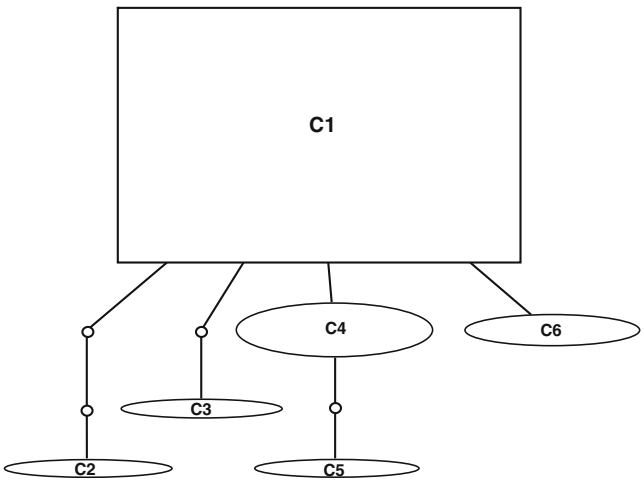

Fig. 1 Statistical parsimony networks for *cox*1 haplotypes of *G. changii*. *Lines* represent parsimonious connections between haplotypes with a probability higher than 95%, with each representing one mutational step, and the *small circles* indicate missing haplotype. The size of square or oval corresponds to the haplotype frequency. Haplotype C1 was inferred as the hypothetical ancestral haplotype

Table 2 Variation site in DNA sequences of *G. changii* for mitochondrial haplotype from various localities

Haplotype	Collection site[a]	Variation sites in DNA sequence								
		16	25	40	43	108	172	410	644	728
C1	Kuala Sungai Merbok (100%) Middle Banks (100%) Morib (10%) Gua Tanah (100%) Batu Besar (87%) Batu Tengah (100%) Sungai Pulai (100%)	T	A		A	C	A	T	A	A
C2	Batu Besar (13%)	T	A		A	C	C	C	A	G
C3	Teluk Pelanduk (25%)	T	C		A	T	A	T	A	A
C4	Morib (80%)	T	A		A	C	A	T	G	A
C5	Morib (10%)		A			C	A	T	G	A
C6	Teluk Pelanduk (75%)	T	A	C	A	C	A	T	A	A

[a] The percentage of haplotype in each collection site is shown in parentheses

haplotype for *G. changii* due to its prevalence in Kuala Sungai Merbok, Middle Banks, Morib, Gua Tanah, Batu Besar, Batu Tengah, and Sungai Pulai. *G. changii* in Morib, Selangor, provided the largest divergence with the identification of three haplotypes (i.e., C1, C4, and C5). Based on the distribution of the haplotype along the coastline of Malacca, two haplotypes were found from Batu Besar. Haplotype C1 and C2 differed from each other by three

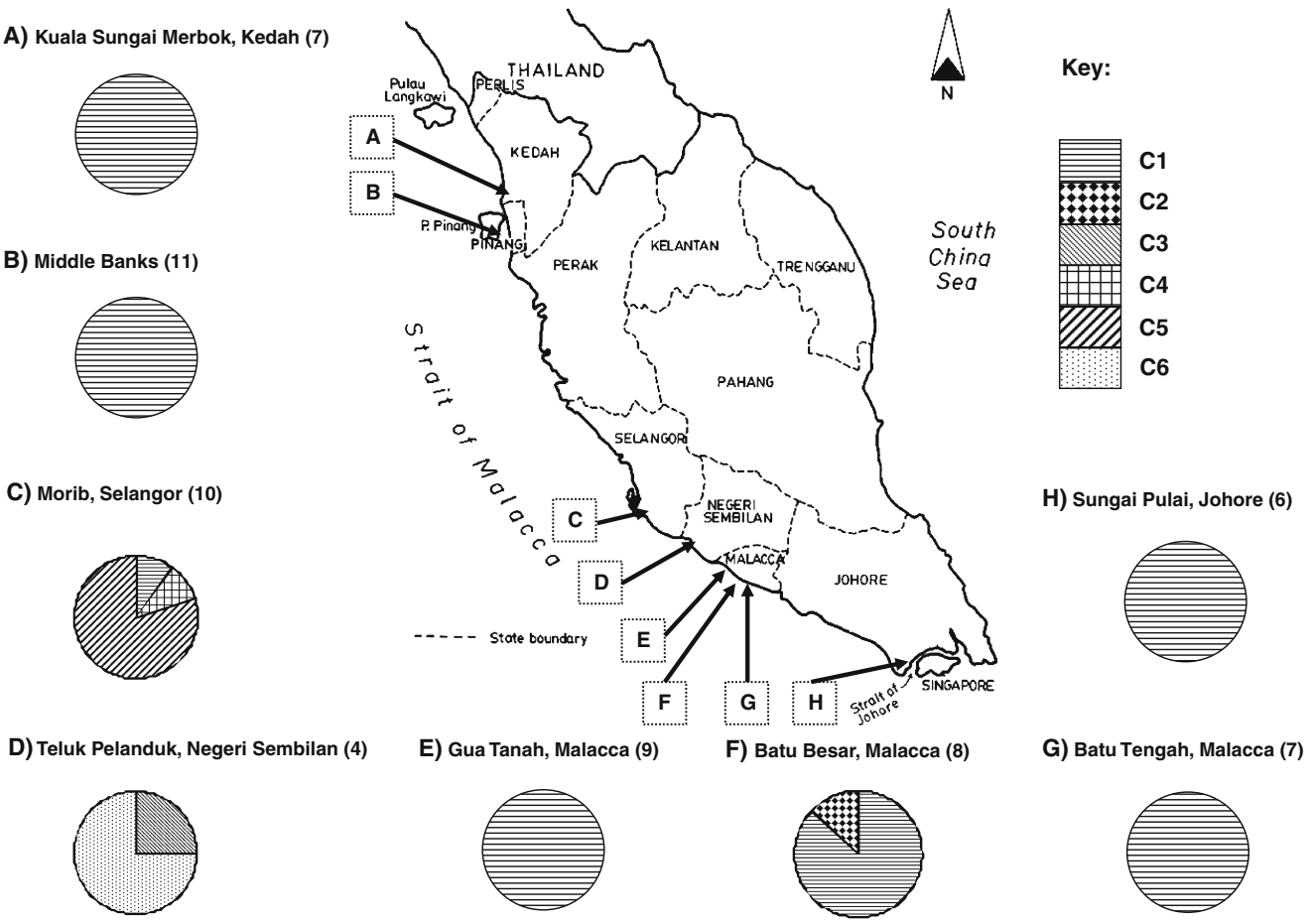

Fig. 2 Haplotype distribution (C1–C6) of *cox*1 for *G. changii* along the west coast of Peninsular Malaysia. The number of examined samples is shown in *parentheses* (map of west coast Peninsular Malaysia adapted from http://www.fao.org/docrep/field/009/ag160e/AG160E09.htm)

substitutions. Two haplotypes (C3 and C6) were discovered in Teluk Pelanduk with the absence of the common ancestor (C1). The samples from Kuala Sungai Merbok, Middle Banks, Gua Tanah, Batu Tengah, and Sungai Pulai exhibited the lowest genetic diversity of *G. changii* since only the basal haplotype (C1) was observed. Intraspecific nucleotide differences ranged from 0–3 bp (0.33%) over 923 bp. According to Saunders (2005), intraspecific nucleotide differences for red algae range from 0 to 2 bp, and interspecific nucleotide differences were more than 30 bp; our study showed similar results. The type species of *G. changii* was described for the first time from Middle Banks, Penang (Phang 1994). The study indicated that Morib may be the original locality of *G. changii* as the highest level of genetic variation was observed there; however, the origin of *G. changii* can only be verified with additional sampling from wider biogeographical areas and an increased number of individuals (a minimum of ten) for each locality examined.

The Malacca Straits is the world's second busiest commercial shipping lane and has been threatened by man's activities of shipping traffic, land reclamation, and fishing since the 1980s. Phang (1998) reported that the sheltered coastline of the west coast of Peninsular Malaysia was dominated by mangrove swamps with water temperature ranging from 23°C to 31°C, salinity from 28 to 34 ppt, and semi-diurnal tides. Mangrove areas of Morib are dominated by *G. changii*, *G. edulis*, and *G. salicornia*. Morib beach is well known as a tourist destination and is also surrounded by factories and poultry farms. Waste and discharge from these activities contributes to coastal pollution. *G. changii* and other species in this region may have had to adapt to these stressed environments. *G. changii* is capable of adapting to the harsh silted mangrove and polluted areas (Phang et al. 1996). We suggest that haplotype C1 is the common ancestor of *G. changii* and evolved over time into the various haplotypes, namely, C2, C3, C4, C5, and C6, in order to cope with environmental changes and as a consequence of geographic distribution along the coastal regions of the west coast of Peninsular Malaysia. Further study will be required to determine whether the various *cox* 1 haplotypes correlate with ecotypes that differ in their response to the environment.

Seaweeds are distributed around the world by various processes. More than 100 seaweed species have been documented that are widely dispersed across their native ranges due to anthropogenic activities (Farnham et al. 1973; Rueness 1989; Curiel et al. 1998; Fletcher and Farrell 1999; Rueness and Rueness 2000; Boudouresque and Verlaque 2002; Smith et al. 2002; Kim et al. 2004). The relocation of species causes confusion in seaweed biogeography. Natural or anthropogenic environmental derived changes have been reported in many species by several researchers (Thompson 1998; Mousseau et al. 2000; Umina et al. 2005). Oysters were one of the vectors that introduced many seaweeds from Japan into Europe in the past two centuries (Farnham 1994; Uwai et al. 2006a). Transoceanic introductions of marine organisms are an impact of the globalization of shipping systems. Fisheries have also been documented by a number of researchers in such introductions (Carlton and Hodder 1995; Ribera and Boudouresque 1995; Nelson et al. 1996; McIvor et al. 2001; Shaffelke et al. 2006; Uwai et al. 2006a). In Singaporean waters, introductions have been attributed to such vectors as aquaculture and shipping (Lee et al. 2009).

In conclusion, Morib was found to have the most divergent haplotypes of *G. changii* in this study. The C1 haplotype has been suggested as the common ancestor, with five haplotypes for *G. changii* along the west coast of Peninsular Malaysia. However, the genetic diversity *G. changii* cannot be clarified accurately due to the limited size of samples collected. Extensive sampling from a wider geographical area in Malaysia is essential to provide a better understanding of the genetic diversity of this potentially economically important agarophyte. The findings in the present study augmented our understanding of the genetic diversity of *G. changii* in this region and highlighted several interesting sampling locations for further investigation and will contribute significantly to ongoing studies. However, further conclusions can only be made with additional sampling of at least ten individuals from each location. The analysis on the genetic diversity of *G. changii* is ongoing with increased number of individuals from each location as well as from a wider geographical area.

Phylogeographic distribution analysis for marine algae with taxonomically doubtful fossil evidence is crucial to reveal their biogeographic and population histories (Vidal 2008) and requires an integration of morphological, molecular, and life history approaches (Zuccarello and West 2002). The mitochondrial marker *cox*1 gene was shown to be suitable for resolving intraspecies relationships and is a reliable marker for the study of genetic variation in *Gracilaria*. However, it is essential to involve two or more sets of DNA-based molecular markers to resolve different depths of evolutionary relationships at the species level (Bellorin et al. 2002; Hayden et al. 2003) and to reveal cryptic diversity within *G. changii* in order to enhance the accuracy of resolution for the study of phylogeographic distribution.

Research on various aspects of *G. changii* including genetic transformation (Gan et al. 2003), tissue culture, proteomics (Wong et al. 2006), protoplast generation (Yeong et al. 2008), and functional genomes (Teo et al. 2007; Wong et al. 2007; Ho et al. 2009; Teo et al. 2009) have been carried out in Malaysia. There is no commercial cultivation of *G. changii* in Malaysia; however, the

Department of Fisheries Malaysia carried out pond cultivation at Kuala Sungai Merbuk, Kedah (Phang 1998). Hence, large-scale cultivation of *G. changii* is essential for this economically important species. This study may provide insights into the origin and evolutionary relationships of *G. changii* in Malaysia and contribute to plant breeding programs for the most suitable strain or haplotype for cultivation. This study may also provide insights into the consequence of natural and anthropogenic impacts as well as evolutionary changes which have affected the distribution of *G. changii*. This information is important for the development of strategies for ex situ conservation of the ecologically important genetic resource of *G. changii*.

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Assessing the use of mitochondrial *cox*1 gene and *cox*2-3 spacer for genetic diversity study of Malaysian *Gracilaria changii* (Gracilariaceae, Rhodophyta) from Peninsular Malaysia

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Abstract Advances in DNA-based genetic markers provide the essential tools in measurement of genetic diversity relating to the evolution, biogeography, and systematics of red algae by exploiting genetic variation in the entire genome of organisms. The understanding of genetic diversity in *Gracilaria changii* (Gracilariaceae, Rhodophyta) will provide valuable information for conservation, plant breeding management, and strain selection for cultivation. However, information of intraspecific genetic variation is still rudimentary. In this study, two mitochondrial encoded markers, cytochrome oxidase subunit 1 (*cox*1) and intergenic spacer between the cytochrome oxidase subunits 2 and 3 (*cox*2-3 spacer) were used to investigate genetic diversity in 40 individuals of *G. changii* collected from 11 different geographically distinct populations from Peninsular Malaysia. Seven distinct mitochondrial haplotypes were identified with the *cox*1 gene and three mitochondrial haplotypes with the *cox*2-3 spacer. Intraspecific nucleotide differences ranged from 0 to 6 bp for the *cox*1 and 0–4 bp for the *cox*2-3 spacer, respectively. This is the first report comparing the suitability of mitochondrial markers of the *cox*1 gene and the *cox*2-3 spacer for genetic diversity studies on *G. changii*. The present study showed that the *cox*1 gene is a potential molecular marker to infer intraspecific genetic variation in red macroalgae. The *cox*1 marker is more variable compared to the *cox*2-3 spacer and revealed genetic variation and phylogeographic structure for this ecologically and economically important species.

Keywords *Gracilaria changii* · Rhodophyta · *cox*1 gene · *cox*2-3 spacer · Genetic diversity · Phylogeography

Introduction

The red algae of *Gracilaria changii* (Xia and Abbott) Abbott, Zhang and Xia is a potential agarophytic species for commercialization in Malaysia as it has high yields of good quality agar with high gel strength for production of food grade agar and agarose (Phang 1994; Phang et al. 1996). Due to potential commercial value in Malaysia, *G. changii* has been the subject of interest among researchers in various aspects: random amplified polymorphism DNA (RAPD) molecular markers for differentiation of different life stages (Sim et al. 2007), tissue and protoplast culture (Yeong et al. 2008), functional genomics (Teo et al. 2007; Ho et al. 2009; Teo et al. 2009; Siow et al. 2012), and genetic transformation (Gan et al. 2003).

Anthropogenic activities leading to pollution, costal degradation, climate change, transoceanic introduction of alien species and invasive species, including natural seaweed populations, have impacted diversity of the seaweed genetic resources. In addition, ecological factors and natural evolutionary processes also influence a range of population, community, and ecosystem responses that may generate changes in allele sequences or genotype frequency in seaweeds (Fussmann et al. 2007). Regular sampling at various coastal areas in Peninsular Malaysia has indicated that the wild populations of *G. changii* are decreasing. To date, there is no commercial-scale cultivation of *G. changii* in Malaysia, except for experimental culture in a shrimp farm using the monofilament method (Phang 1998). Information on intraspecific genetic diversity of this economically important species is scarce (e.g. Yow et al.

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2011). There is, therefore, an urgent need to study the genetic diversity of *G. changii* to provide information necessary for selecting suitable strains for cultivation.

Measurement of genetic diversity of seaweed with molecular tools has been accelerated with advances in molecular markers technologies. In recent years, application of molecular techniques has become important in species identification (Conklin et al. 2009). Adoption of genetic markers for genetic variation studies provide valuable informative data for gene flow, population structure, phylogenetic relationships, biogeographic studies, and parentage and relatedness analysis (Féral 2002). A number of molecular tools are available and are being widely used to elucidate genetic variability of marine macroalgae, such as DNA markers used in allozyme differentiation, restriction fragment length polymorphism (RFLP; Kamikawa et al. 2007), intersimple sequence repeat (ISSR; Zhao et al. 2008), microsatellites (Zhang et al. 2009), RAPD (Zhao et al. 2008), and amplified fragment length polymorphism (AFLP; Donaldson et al. 2000; Pang et al. 2010). A large set of intraspecific markers that have been used for studying genetics and genetic diversity of seaweeds include mitochondrial cytochrome oxidase subunit I gene (Sherwood 2008; Kim et al. 2010; Yow et al. 2011); mitochondrial-encoded *cox*2-3 spacer (Rueness 2005; Chiasson et al. 2007; Vidal 2008; Vis et al. 2010); mitochondrial cytochrome oxidase subunit 3, *cox*3 (Steel et al. 2000; Coyer et al. 2004; Uwai et al. 2006); nuclear-encoded internal transcribed spacers of the ribosomal cistrons, ITS1 and ITS2 (Cho et al. 2007; Lindstrom 2008; Moniz and Kaczmarska 2010), and the plastid-encoded RuBisCO spacer (Byrne et al. 2002; Zuccarello et al. 2006b); and *rbc*L gene (Nam et al. 2000; McIvor et al. 2001; Gurgel and Fredericq 2004).

The usefulness of mitochondrial markers, the *cox*1 (e.g., Robba et al. 2006; Yang et al. 2007; Yow et al. 2011) and the *cox*2-3 spacer (e.g., Zuccarello and West 2002) as molecular markers in the measurement of genetic diversity, has been reported. The mitochondrial DNA region has been reported to provide plausible resolution results for species identification of red macroalgae (Saunders 2005; Lane et al. 2007). The gene *cox*1 was reported to be an ideal marker for DNA barcoding of red algae by several researchers (Geraldino et al. 2006; Kim et al. 2010). It is also useful for revealing population structure and the cryptic diversity of red algae (Robba et al. 2006). Likewise, the *cox*2-3 spacer is an intergenic spacer separating the conserved mitochondrial gene of *cox*2 and *cox*3 and was shown to be a useful marker for phylogeographical study of the Rhodophytes (Zuccarello et al. 2006a; Andreakis et al. 2007; Vis et al. 2008). This region was reported variable within populations of the red algae *Caulacanthus ustulatus* (Zuccarello et al. 2002).

The genetic diversity of *G. changii* from the west coast of Peninsular Malaysia based on the *cox*1gene was described by Yow et al. (2011) and showed that a combination of two or more molecular markers would reveal a better genetic structure of the species. In the present study, we compared the use of two markers, the *cox*1gene and the *cox*2-3 spacer, to infer the intraspecific genetic variation of this species from Peninsular Malaysia. Part of the six *cox*1 haplotype data were obtained from Yow et al. (2011) and additional samples from new locations were used for this study.

Materials and methods

Individuals of *Gracilaria changii* were collected and identified from 11 locations in Malaysian waters: Kuala Sungai Merbok (Kedah), Middle Banks (Penang), Morib (Selangor), Teluk Pelanduk (Negeri Sembilan), Pantai Dickson (Negeri Sembilan), Gua Tanah (Malacca), Batu Besar (Malacca), Batu Tengah (Malacca), Sungai Pulai (Johore), Sungai Kong Kong (Johore), and Kampung Gong Batu (Terengganu). All specimens were identified based on the morphological descriptions from Xia and Abbott (1987) and Lim and Phang (2004). The collected specimens were sorted, washed, and stored as in Sim et al. (2007) prior to DNA extraction. Extractions of DNA were performed using commercially available DNeasy Plant Mini Kit (Qiagen, Germany) according to the instructions of manufacturer with minor modification by incubating the disrupted samples with buffer AP1 for 20 min at 65 °C.

Polymerase chain reaction (PCR) amplification for extracted DNA was carried out in either the GeneAmp® PCR system 2700 Thermal cycler (Applied Biosystem, USA) or Eppendorf Thermal Cycler (Eppendorf, Germany). The PCR amplification and sequencing of the mitochondrial *cox*1 region was carried out using the published primers, *cox*1 43 F and *cox*1 1549R (Geraldino et al. 2006). The PCR amplification and sequencing on the mitochondrial *cox*2-3 spacer region was carried out as reported in Zuccarello et al. (1999). PCR amplification was performed in a final volume of 25 μL containing 2.5 μL 10× buffer, 0.2 mM of each dNTP (dATP, dTTP, dCTP, and dGTP), 10 pmol of each forward and reverse primer, 1.5 U *Taq* polymerase, and 20–50 ng of genomic DNA. UHQ water was added to make up the final total volume of 25 μL. Thermacycler protocol was employed following Yow et al. (2011) for the *cox*1 gene and the *cox*2-3 spacer region. The amplified products were electrophoresed on 1 % (w/v) Tris-acetate-EDTA (TAE) agarose gel prestained with ethidium bromide. PCR products were purified with the QIaquick PCR Purification Kit (Qiagen, Germany) according to the manufacturer's instructions and subsequently, were sent to a commercial company for gene sequencing.

Sequences were analyzed and edited using Chromas 2.01 (Technelysium Pty. Ltd., Australia) and BioEdit 7.0.9.0 (Hall 1999) software, and aligned using CLUSTAL X program

(Thompson et al. 1997). Part of the *cox*1 data from Yow et al. (2011) (GenBank accession numbers: GU645726, GU645727, GU645747, GU645748, and GU645763–GU645787) were incorporated for analysis together with new sequences generated from this study. Haplotype networks were created for the *cox*1 gene and the *cox*2-3 spacer data using TCS 1.13 (Clement et al. 2000) to calculate the minimum number of mutational steps by which the sequences could be joined with >95 % confidence.

Results

Forty specimens of *G. changii* from 11 different biogeographic locations in Malaysia were investigated to elucidate their genetic variation using the *cox*1 gene and the *cox*2-3 spacer. Figure 1 shows the haplotype distribution of the *cox*1 and the *cox*2-3 spacer for *G. changii* from Peninsular Malaysia.

Distribution of haplotype using the *cox*1 gene

A statistical parsimony network of 40 taxa with 923 characters of the *cox*1 gene yielded a total of seven haplotypes (C1, C2, C3, C4, C5, C6, and C7) (Fig. 2a, Table 1) with 0–6 bp differences intraspecifically. Haplotype C1 represented all individuals sequenced from seven locations (Kuala Sungai Merbok, Middle Banks, Pantai Dickson, Gua Tanah, Batu Tengah, Sungai Pulai, and Sungai Kong Kong). Three haplotypes (C1, C4, and C5) were identified from Morib and two haplotypes (C1 and C2) from Batu Besar. Haplotype C1 may be the basal haplotype with 29 individuals. Haplotype C1 was the dominant haplotype in almost all the areas examined excluding specimens from Teluk Pelanduk with haplotypes C3 and C6, and Kampung Gong Batu with haplotype C7, respectively. All individuals from Kampung Gong Batu were of haplotype C7 with 2 bp difference relative to haplotype C1. One individual from Batu Besar was detected as haplotype C2 and differed by 3 bp from haplotype C1. Two additional haplotypes of C4 and C5 were detected from Morib differing from haplotype C1 by 1 and 3 bp, respectively. Haplotypes C3 and C6 were represented in all the individuals from Teluk Pelanduk differing from haplotype C1 by 2 bp and 1 bp, respectively. One representative sequence of each haplotype for the *cox*1 gene was deposited in GenBank. The GenBank accession numbers are: C1=GU645769, C2=GU645777, C3=GU645763, C4=GU645771, C5=GU645770, C6=GU645786, and C7=JX228063.

Distribution of haplotype using the *cox*2-3 spacer

A total of three *cox*2-3 spacer haplotypes (haplotypes B1, B2, and B3) were observed among 40 taxa with 363

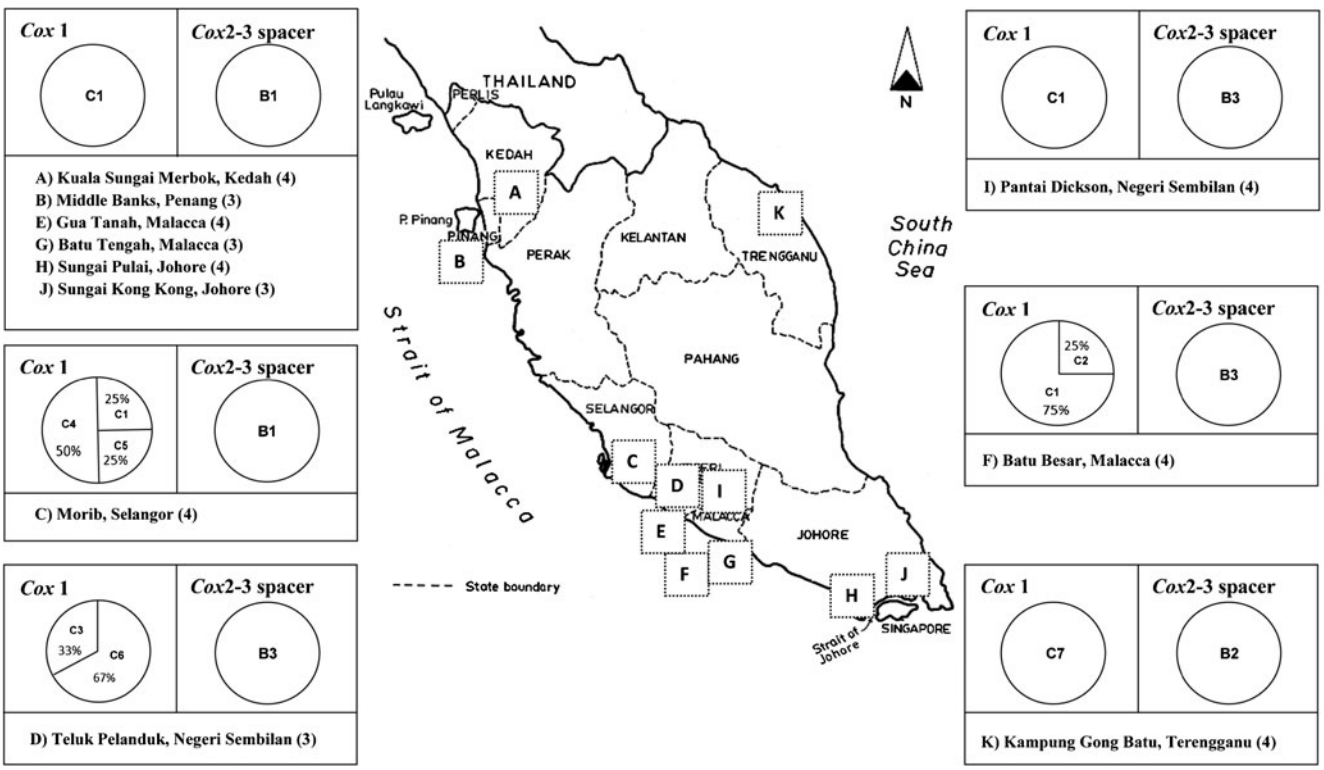

Fig. 1 The haplotype distribution of the *cox*1 gene and the *cox*2-3 spacer for *Gracilaria changii* from Peninsular Malaysia. The number of examined samples is shown in *parentheses*. Map of west coast Peninsular Malaysia is adapted from http://www.fao.org/docrep/field/009/ag160e/AG160E09.htm

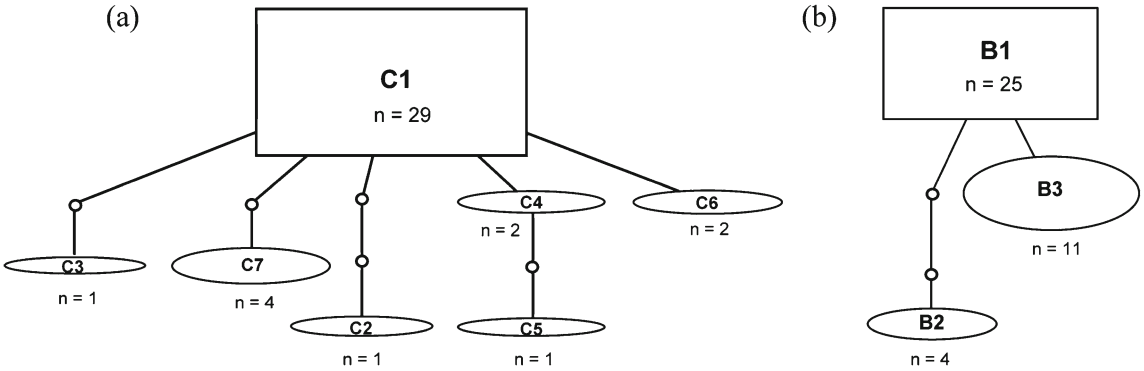

Fig. 2 Statistical parsimony networks for **a** the *cox*1 haplotypes and **b** the *cox*2-3 spacer haplotypes of *G. changii*. A *single line* indicates one mutational step; *small circles* indicate missing haplotype; *n* number of specimens with identical haplotypes. The *size of square or oval* is corresponds to the haplotype frequency. Haplotypes C1 and B1 were inferred as the hypothetical ancestral haplotype, respectively

characters which resulted in 0–4 bp nucleotide differences (Fig. 2b, Table 2). At each location, all specimens belonged to one haplotype. Haplotype B1 represented all individuals sequenced from seven locations (Kuala Sungai Merbok, Middle Banks, Morib, Gua Tanah, Batu Tengah, Sungai Pulai, and Sungai Kong Kong). Haplotype B1 was positioned as the basal haplotype with 25 individuals. The individuals from Kampung Gong Batu were identical as haplotype B2 and differed from haplotype B1 by 3 bp. Haplotype B3 represented all individuals from Teluk Pelanduk, Pantai Dickson, and Batu Besar with 1 bp difference from haplotype B1. One representative sequence of each haplotype for the *cox*2-3 spacer was deposited in GenBank. The GenBank accession numbers are: B1=GU645707, B2=JX228064, and B3=GU645715.

Discussion

The understanding of genetic diversity of *G. changii* is essential to provide insights into conservation, management, and strain selection for cultivation for this economically important species. Characteristics like growth rate and agar yields may be species- or strain-specific and the study of genetic diversity can assist in strain selection for commercialization.

In the present study, the genetic diversity of *G. changii* from Peninsular Malaysia was assessed using mitochondrial markers of the *cox*1 gene and the *cox*2-3 spacer. The *cox*2-3 spacer and the *cox*1 markers have also been employed to elucidate the phylogeographic pattern of freshwater red algae of the genus *Batrachospermum* (Chiasson et al. 2003; Sherwood et al. 2008). The latter marker serves well as a phylogeopraphic marker in taxa of Batrachospermales (House et al. 2010) compared to ISSR (Hall and Vis 2002) and nuclear internal transcribed spacer (Vis et al. 2001).

Analysis of the haplotype networks indicated that higher genetic variation was obtained from the *cox*1 gene (seven haplotypes) compared to the *cox*2-3 spacer (three haplotypes). Haplotype C1was found in the majority of the populations studied and accounted for 73 % of the 40 specimens examined. Kuala Sungai Merbok, Middle Banks, Gua Tanah, Pantai Dickson, Batu Tengah, Sungai Pulai, and Sungai Kong Kong exhibited the lowest genetic diversity or no phylogeographic pattern for *G. changii* as only the basal haplotype (C1)

Table 1 Variation sites in DNA sequences of *G. changii* for haplotypes from the *cox*1 gene

Mitochondrial marker	Length of sequence (bp)	No. Of variable sites	Haplotype	Variation sites in DNA sequence -16-25--40-43---108--172---359---410--591--644--728-	GenBank accession No.
*cox*1 gene	923	6	C1	-T---A-------A-----C----A-----C-----T-----G-----A----A-	GU645769
			C2	-T---A-------A-----C----C-----C-----C-----G-----A----G-	GU645777
			C3	-T---C-------A-----T----A-----C-----T-----G-----A----A-	GU645763
			C4	-T---A-------A-----C----A-----C-----T-----G-----G----A-	GU645771
			C5	------A----------C----A-----C-----T-----G-----G----A-	GU645770
			C6	-T---A---C--A-----C----A-----C-----T-----G-----A----A-	GU645786
			C7	-T---A-------A-----C----A-----T-----T-----A-----A----A-	JX228063

Table 2 Variation sites in DNA sequences of *G. changii* for haplotypes from the *cox*2-3 spacer

Mitochondrial marker	Length of sequence (bp)	No. of variable sites	Haplotype	Variation sites in DNA sequence ——153———218—242——303————	GenBank accession no.
*cox*2-3 spacer	363	4	B1	——T————G——C———A————	GU645707
			B2	——T————A——T————————	JX228064
			B3	——G————G——C———A————	GU645715

was observed. Haplotype C1 was shown to be the common ancestor and the most widespread haplotype due to its prevalence in all the localities analyzed. Specimens from Morib (Selangor) showed the greatest divergence with identification of three haplotypes (C1, C4, and C5), followed by samples from Batu Besar (C1 and C2) and Teluk Pelanduk (C3 and C6). The results from the *cox*1 analysis suggest that the ancestral population of *G. changii* may have originated in Morib and having colonized earlier, had more time to diverge from the dominant haplotype of C1 to C4 and C5. In contrast, there was only a single haplotype with no genetic differences being detected with the *cox*2-3 spacer in Morib (B1), Teluk Pelanduk (B3), and Batu Besar (B3), respectively and is not as variable as the *cox*1 gene. Sixty-three percent of specimens possessed haplotype B1, indicating that B1 may be the common ancestor because it is the most widely distributed haplotype at Kuala Sungai Merbok, Middle Banks, Morib, Gua Tanah, Batu Tengah, Sungai Pulai, and Sungai Kong Kong. The ancestral population of this species is unresolved with the *cox*2-3 spacer. The *cox*2-3 spacer appears to be highly conserved and showed no significant intraspecific genetic variation for this species. Limited intraspecific variation for red algae at this spacer has been reported elsewhere (Milstein et al. 2008; Teasdale and Klein 2010). The morphology of the collected *G. changii* was identical regardless of the location and habitat. *G. changii* can be naturally found in mangrove areas (e.g., attached to *Avicennia* trees), sandy beaches, rocks, muddy areas, and attached to fish cages. Despite high level of genetic variation obtained with the *cox*1 gene, no significant morphological variation was observed in collections from all locations.

Intraspecific nucleotide differences ranged from 0 to 6 bp over 923 bp for the *cox*1 and 0–4 bp over 363 bp for the *cox*2-3 spacer, respectively. The size of analyzed sequences of the *cox*1 gene was much larger than the *cox*2-3 space, where larger sized gene may contain more informative data on molecular evolution (Yang et al. 2007). The *cox*1 intraspecific nucleotide differences in many red algae ranged from 0 to 2 bp (0.3 %) and interspecific nucleotide differences was more than 30 bp (Saunders 2005). Generally, the intraspecific variation shown in the present study principally agreed with the results reported by Robba et al. (2006) in red algae and by Yang et al. (2007) in *G. vermiculophylla*. According to the first report of haplotype analysis of the *cox*1-coding region in red algae by Yang et al. (2007), the *cox*1 gene is a reliable molecular marker for intraspecific study and useful for revealing species relationships, population structure, and the hidden diversity of red algae. It gave higher variability for *G. vermiculophylla* (variable up to 11 bp) compared to *rbc*L gene. Current research on freshwater red algae proved that the *cox*1 is a potential candidate marker for phylogeographic studies (House et al. 2008; Sherwood et al. 2008). The *cox*1 gene has also been suggested as a potential DNA barcode marker for red algae, brown algae, and some other diatom genera (Evans et al. 2007; Kucera and Saunders 2008; Le Gall and Saunders 2010).

A number of phylogeographic studies on seaweeds have been published using nuclear markers and plastid markers. Ribosomal internal transcribed spacer (ITS) regions have been employed in studies of biogeographical, intraspecific, species identification, and phylogenetic studies to Rhodophyta owing to its rapid evolution rate (Broom et al. 2002; Marston and Villalard-Bohnsack 2002; Hu et al. 2009). However, the overriding drawback of the high level of intraindividual ITS variation has been reported (Famà et al. 2000; Lange et al. 2002; Uwai et al. 2006). In contrast, mitochondrial markers are uniparentally inherited and intraindividual polymorphism is less known. Plastid markers of the gene for the large subunit of RuBisCO, *rbc*L, are used to investigate the phylogeographic pattern of red algae *Polysiphonia harveyi* (McIvor et al. 2001). Meanwhile, AFLP and SSR have been employed in genetic diversity analysis of *Gracilariopsis lemaneiformis* from China. Recent work in Phaeophyta has shown that ISSR is applicable in genetic structure analysis of *Sargassum muticum* (Zhao et al. 2008) compared to RAPD. Microsatellite DNA markers are codominant and readily applied in measurement of genetic diversity studies. This technique is being currently favored owing to the high resolution observed. Waittier et al. (1997) reported the use of single-locus microsatellite to study the population genetics of *G. gracilis* due to the high variability of microsatellite loci. In addition, recent research on development of microsatellite DNA markers for genetic diversity of *G. chilensis* (Guillemin et al. 2005) has shown the potential of this molecular tool. However, genetic homogeneity and low rate of SSR polymorphism was reported for *Porphyra* species (Pang et al. 2010, unpublished data) and *G. lemaneiformis* (Zhang et al. 2009) between different geographical areas.

In conclusion, consistent with previous studies, our analysis shows that the mitochondrial marker *cox*1 gene is a potential highly divergent mitochondrial marker for resolving intraspecies relationships and revealing the level of genetic variation of *G. changii*. This is the first report on comparison of reliability of the *cox*1 gene with the *cox*2-3 spacer for intraspecific genetic variation of *G. changii*. The *cox*1 is potentially usable in a number of studies, namely, DNA barcoding, species identification, resolution of population structure, phylogeography, and conservation management. However, no single genetic marker is the best for multiapplications or studies as each might provide different insights at different levels of investigation. Multigene approaches will be crucial to resolve different depths of evolutionary relationships at the species level (Bellorin et al. 2002; Newmaster et al. 2006) and to enhance the statistical significance for intraspecific genetic variation study in *G. changii*. In contrast with the *cox*1 gene analysis, no correlation between morphological features of *G. changii* and the *cox*1 haplotypes was found.

The incorporation of the *cox*2-3 spacer did not enhance the intraspecific genetic variation or the number of haplotypes of the populations of *G. changii* compared with our earlier results using the *cox*1 gene (Yow et al. 2011). Hence, we would like to propose the *cox*1 marker as a suitable genetic marker to be used for future studies on the understanding of genetic diversity and evolutionary relationships of *G. changii* from wider geographical regions with larger sample size.

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