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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

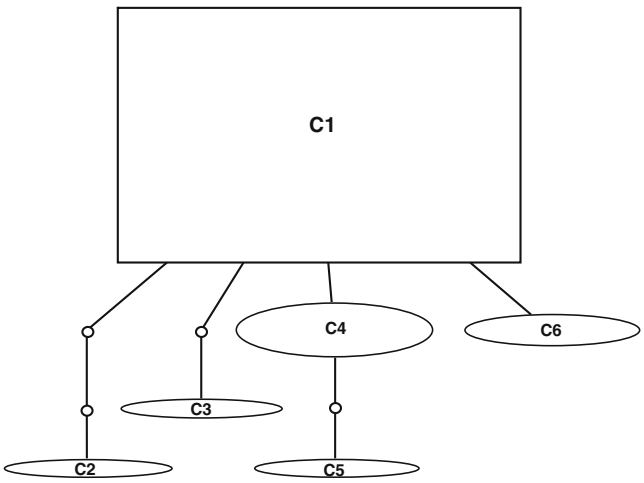

Fig. 1 Statistical parsimony networks for *cox*1 haplotypes of *G. changii*. *Lines* represent parsimonious connections between haplotypes with a probability higher than 95%, with each representing one mutational step, and the *small circles* indicate missing haplotype. The size of square or oval corresponds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

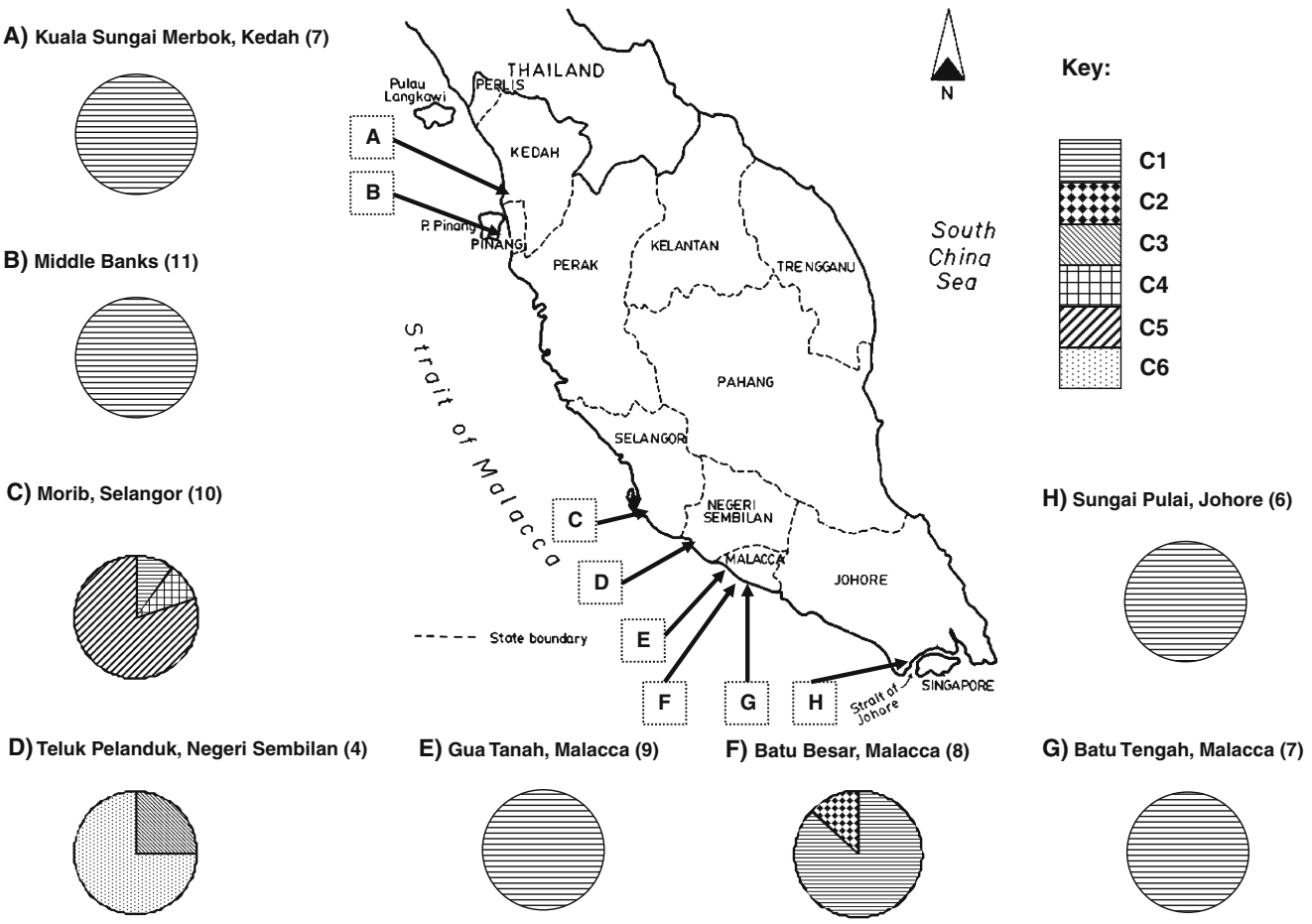

Fig. 2 Haplotype distribution (C1–C6) of *cox*1 for *G. changii* along the west coast of Peninsular Malaysia. The number of examined samples is shown in *parentheses* (map of west coast Peninsular Malaysia adapted from http://www.fao.org/docrep/field/009/ag160e/AG160E09.htm)

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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