

## Genetic variation in *Gracilaria tenuistipitata* (Rhodophyta) from northern Singapore and neighbouring countries

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**Abstract.** *Gracilaria tenuistipitata* Chang & Xia 1988 is a commercially important red alga species and has gradually become a model species for cultivation due to its rapid growth and high agar yield. *G. tenuistipitata* is found in Singapore but no information regarding the genetic variation of this species is available. The present study examined the genetic variability of this species collected at various localities: three from Singapore, two from Peninsular Malaysia, and one each from Thailand and Vietnam, using the mitochondrial *cox1* gene and microsatellite markers. The SSR (simple sequence repeat) marker separated the Singapore specimens into two different genotypes with *G. tenuistipitata* from Ubin Island (Singapore), Pattani (Thailand) and Quy Kim (Vietnam) in one clade while *G. tenuistipitata* from Lim Chu Kang and Pasir Ris Park (Singapore) and Peninsular Malaysia formed another clade. The mitochondrial *cox1* gene analyses showed that *G. tenuistipitata* from Singapore were grouped together with specimens from Middle Banks (Malaysia) while specimens from Batu Laut (Malaysia), Pattani (Thailand) and Quy Kim (Vietnam) were grouped in three distinct clades. These results are congruent with the TCS analysis in which five mitochondrial haplotypes (T1–T5) were displayed but no genetic variation was observed for all the specimens from Singapore and Middle Banks. This study demonstrated the low genetic diversity of *G. tenuistipitata* from Singapore despite the higher variability of *cox1* gene over the microsatellite markers.

**Key words.** *cox1* gene, genetic variation, *Gracilaria tenuistipitata*, SSR markers

### INTRODUCTION

The second largest genus amongst the red algae, *Gracilaria* species are distributed worldwide and cultivated for hydrocolloids in various countries including China, Vietnam, the Philippines, Indonesia and the Republic of Korea (McHugh, 2003). In Malaysia, several species of *Gracilaria* (e.g., *G. changii* and *G. tenuistipitata*) are consumed as salads as well as used for agar extraction (Phang, 2006). Agar has been extensively used in the food, cosmetic and pharmaceutical industries, as well as in microbiological research. *Gracilaria tenuistipitata* var. *liui* Zhang & Xia is consumed as a sea vegetable in the Philippines, and it is also intensively cultivated in China and Taiwan for food (Haglund & Pedersen, 1993; Tseng & Xia, 1999).

The high morphological plasticity of macroalgae can be detrimental to the proper identification of commercially important seaweeds such as *Gracilaria* (Yow et al., 2011), *Kappaphycus* (Tan et al., 2013) and *Sargassum* (Dixon & Huisman, 2010; Endo et al., 2013). Their morphology varies based on the adaptive response to changing environments. This can be problematic especially for species with high

economic value. For example, members of the family Gracilariaceae (Rhodophyta) are economically important for agar production and as abalone feed. However, their taxonomic and systematic positions are generally uncertain due to the lack of distinct morphological diagnostic characters and unreliable developmental characters of the female reproductive system (Wattier et al., 1997; Iyer et al., 2005). In a study by Iyer et al. (2005), small subunit rDNA and RuBisCO spacer sequences were employed to unravel the phylogenetic relationships of several members of the Gracilariaceae from South Africa. Their results showed that these markers were able to resolve the southern African gracilarioid complex into three species: *Gracilaria gracilis*, *Gracilariopsis longissima* and *Gracilariopsis funicularis* (Iyer et al., 2005). Hence, molecular information may be essential to distinguish between different species and to overcome the limits of morpho-species (John & Maggs, 1997). In addition, genetic analyses can reveal the extent of population structuring and give an indication of subgroup distinctiveness (Beebee & Rowe, 2008). To date, several genetic marker approaches have been used in the study of seaweeds, for example, restriction fragment length polymorphisms (RFLPs; Candia et al., 1999), random amplified polymorphic DNA (RAPD; Lim & Phang, 2004; Sim et al., 2007), and amplified fragment length polymorphisms (AFLPs; Niwa et al., 2004; Sun et al., 2005). However, there are always limitations arising from each technique.

Microsatellite markers, or simple sequence repeats (SSRs) are abundantly found in eukaryotic genomes and have high levels of polymorphism (Powell et al., 1996). These

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markers have been tested in various species of *Gracilaria*: *Gracilaria gracilis* (Wattier et al., 1997) and *Gracilaria chilensis* (Guillemin et al., 2005) for genetic variability and also to differentiate *Gracilaria changii* and other *Gracilaria* species collected from various localities (Song et al., 2013). A recent publication by Song et al. (2014) examined the intraspecific relationships of *G. tenuistipitata* from peninsular Malaysia, Thailand and Vietnam by utilising microsatellite markers developed from the chloroplast genome. Therefore, it will be interesting to compare the genetic variability of *G. tenuistipitata* from Singapore with those from neighboring countries.

The mitochondrial cytochrome c oxidase subunit I gene (*cox1*) is a highly variable marker used for species differentiation and also to elucidate phylogenetic relationships at the genus and species levels in red algae (Saunders, 2005; Yang et al., 2008; Kim et al., 2012). This marker has also been found to be an ideal DNA barcoding marker (Robba et al., 2006). The *cox1* gene was successfully used to evaluate genetic diversity in *Gracilaria changii* (Yow et al., 2011). It was found that *cox1* performed better than *cox2* and *cox2-3* spacer as a DNA barcode for the red algae *Kappaphycus* and *Euचेuma* (Tan et al., 2012).

Here we attempt to augment the lack of information of a morphologically plastic but economically important species. We analysed the genetic variation of *Gracilaria tenuistipitata* populations from Singapore, west coast of Peninsular Malaysia, Thailand and Vietnam using *cox1* gene and microsatellite markers.

## MATERIAL AND METHODS

**Sample collection and DNA extraction.** A total of 21 *Gracilaria tenuistipitata* specimens were collected from three localities in Singapore: i) Ubin Island, ii) Lim Chu Kang, and iii) Pasir Ris Park; two different localities from the west coast of Peninsular Malaysia: i) Batu Laut, Selangor and ii) Middle Banks, Penang; one locality from Vietnam (Quy Kim, Hai Phong), and one locality from Thailand (Pattani) (see Fig. 1). The samples were thoroughly washed with seawater, followed by distilled water and ultra high quality (UHQ) water. The samples were then blot dried with a clean paper towel and kept in a sealed plastic bags with silica gel to absorb moisture. DNA extraction was carried out using a DNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's instructions with slight modifications. The extracted genomic DNA was stored at  $-20^{\circ}\text{C}$  for further analysis.

**Simple Sequence Repeat (SSR) analysis.** The eight microsatellite markers used in this study were developed by Song et al. (2014) from the chloroplast genome of *G. tenuistipitata* var. *liui* deposited in GenBank (Table 1, Song et al., 2014). Polymerase chain reaction (PCR) was performed using TaKaRa Ex Taq (TaKaRa Bio, Otsu, Japan) with  $10\times$  Ex Taq buffer, 0.2 mM dNTP mixture, 0.5 U TaKaRa Ex Taq polymerase, 0.3 mM of each primer, 3  $\mu\text{L}$  genomic DNA and UHQ water added to a total volume of 15  $\mu\text{L}$ . The

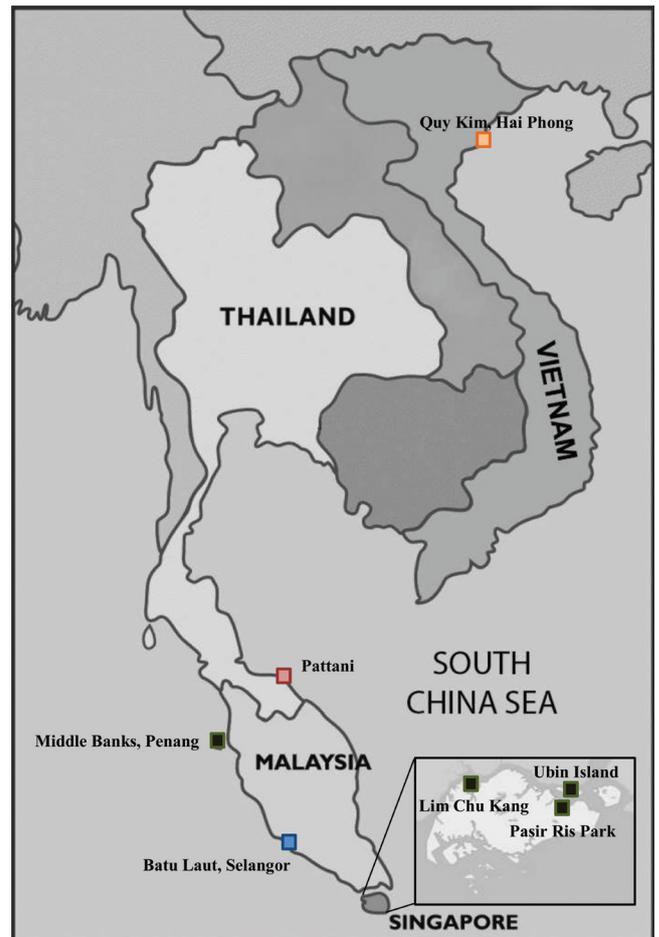


Fig. 1. Collection sites of *Gracilaria tenuistipitata* in Southeast Asia. Haplotypes from seven locations are represented by different colours. For SSR analysis, samples from Pattani (Thailand), Quy Kim (Vietnam) and Ubin Island (Singapore) correspond to clade A (see Figs. 3, 4), whilst samples from Batu Laut and Middle Banks (Malaysia) and Lim Chu Kang and Pasir Ris Park (Singapore) correspond to clade B. (maps adapted from <http://commons.wikimedia.org/wiki/File:Singapore-OutlineMap-20050606.png>; <http://ww2.valdosta.edu/~accreshaw/global.html>).

amplification profiles were as follows: 5 min denaturation step at  $94^{\circ}\text{C}$ , followed by 35 cycles of 1 min at  $94^{\circ}\text{C}$ , 1 min at  $52^{\circ}\text{C}$ , 2 min elongation at  $72^{\circ}\text{C}$ , and a final extension at  $72^{\circ}\text{C}$  for 5 min. The forward primer of each primer pair was fluorescently [69-carboxyfluorecine (FAM)] labeled. The optimised annealing temperature for PCR amplifications was  $52^{\circ}\text{C}$  and this optimum temperature was used for all specimens. The amplified products were separated on a 1.0% TAE agarose gel pre-stained with SYBR SAFE (Invitrogen, USA). Amplified products were sent for fragment analysis (First Base Sdn. Bhd.) to detect alleles using an automated DNA sequencer.

SSR products were scored using a binary matrix method with "1" (presence) and "0" (absence) based on the SSR pattern that was amplified by primer-pair GT5. DNA fingerprints of *G. tenuistipitata* from different localities in Singapore were constructed and analysed together with the results obtained for Batu Laut, Middle Banks (Malaysia), Quy Kim (Vietnam) and Pattani (Thailand) in Song et al.

(2014) using GelQuest software (Hepperle, 2002) based on the size standard template ABI GeneScan 50-500 followed by similarity matrix cluster analysis based on the UPGMA (unweighted pair group method using arithmetic means) using ClusterVis software version 1.4.2 (Hepperle, 2002). The resulting UPGMA dendrogram was visualised and edited using the TreeMe software (Hepperle, 2004).

**cox1 analysis.** The *cox1* gene were amplified using the primers *cox1* 43F and *cox1* 1549R following Freshwater and Rueness (1994), Gavio and Fredericq (2002), Geraldino et al. (2006) and Yang et al. (2008). The parameters used for PCR amplification were as described in the SSR analysis. PCR products were purified using a LaboPass PCR purification kit (Cosmo Genetech, Seoul, Korea) prior to automated DNA sequencing service (First Base Sdn. Bhd.) using the ABI 3730xl DNA analyzer (Applied Biosystems, Foster City, CA). Sequence data were edited and assembled using ChromasPro v.1.5 (Technelysium Pty Ltd., Australia) software followed by multiple sequence alignment with ClustalX (Thompson et al., 1997) program. The resulting alignment was subsequently trimmed using BioEdit v.7.0.5.3 (Hall, 1999). The phylogenetic inference using unweighted

pair-group method of arithmetic averages (UPGMA) based on Kimura-2-Parameter (K2P) model was computed with PAUP\* v4.0b10 (Swofford, 2002). FigTree v.1.4.0 was used to view and edit the UPGMA dendrogram (Rambaut, 2009). No outgroup was defined and the UPGMA dendrogram was midpoint rooted.

The haplotype networks (gene genealogies) of the specimens were constructed using TCS 1.13 (Clement et al., 2000) by calculating the minimum number of mutational steps in which the sequences can be joined with >95% confidence.

## RESULTS

**Morphological observations.** No distinct differences were observed for *G. tenuistipitata* collected from various localities. Several thalli 5 to 15 cm high originated from a small discoid holdfast. They were cartilaginous and terete throughout. Main axes were 0.5–1.0 mm wide with irregularly and densely arranged first-order branches 5 to 10 cm long by 0.2 to 0.3 mm wide. The first-order branches bore shorter and more slender branches which branched up to three orders (Fig. 2a–g).

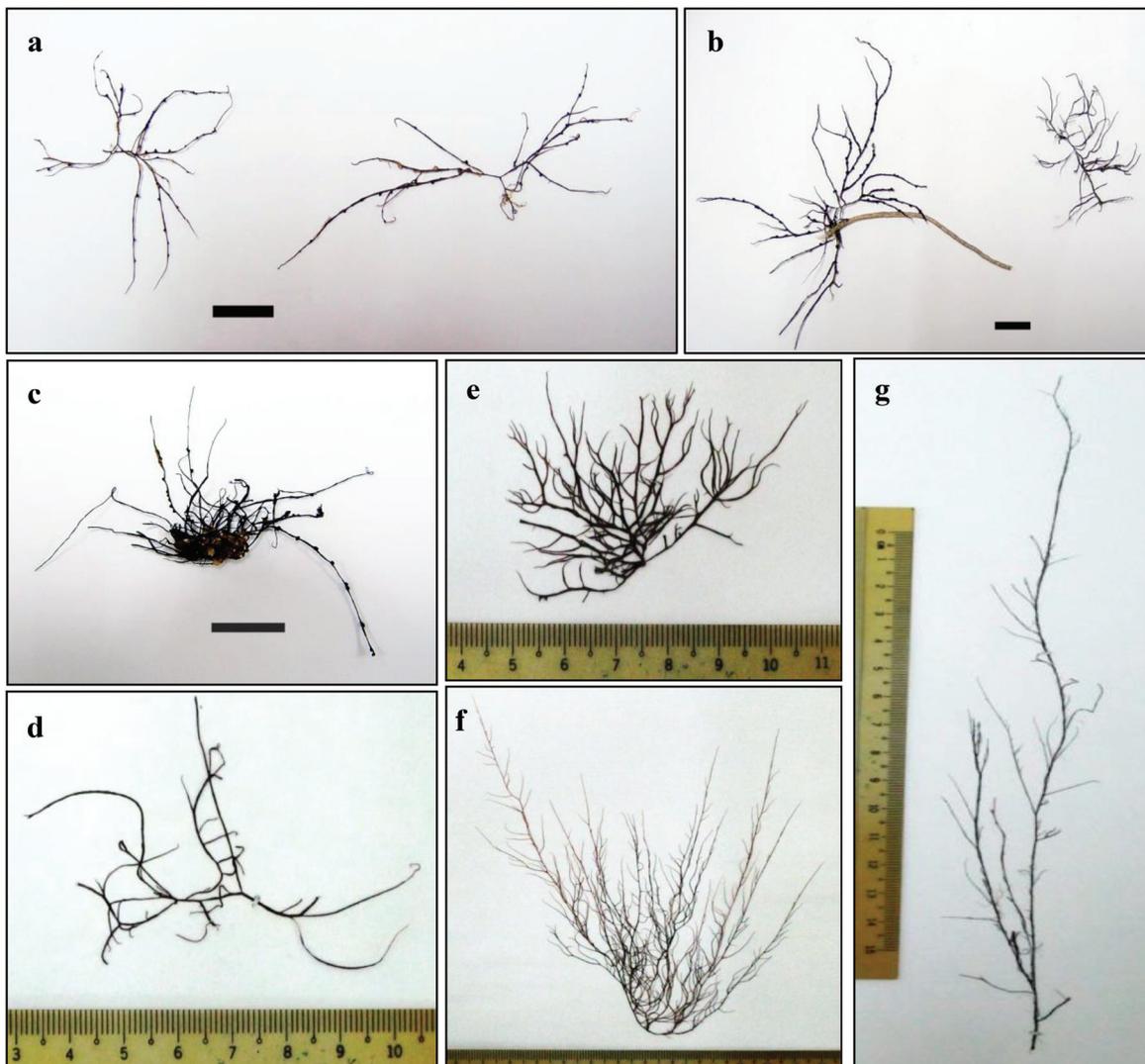


Fig. 2. *Gracilaria tenuistipitata*: a, Lim Chu Kang, Singapore; b, Pasir Ris Park, Singapore; c, Ubin Island, Singapore; d, Middle Banks, Malaysia; e, Batu Laut, Malaysia; f, Quy Kim, Vietnam; g, Pattani, Thailand. Scale bars = 1cm

Table 1. Collection sites and haplotypes of *Gracilaria tenuistipitata* identified using *cox1* gene.

Collection Site	Collection Date	Isolate	GenBank Accession Number	Haplotype
Ubin Island, Singapore	30 October 2012	413	KJ569759	T1
Lim Chu Kang, Singapore	27 October 2012	406	KJ569760	T1
Pasir Ris Park, Singapore	27 October 2012	398	KJ569761	T1
Batu Laut, Selangor, Malaysia	10 September 2009	275	KJ569758	T2
Middle Banks, Penang, Malaysia	20 March 2011	278	KJ569757	T1
Pattani, Thailand	21 October 2009	219	KJ569756	T3
Quy Kim, Hai Phong, Vietnam	28 December 2008	233	KJ569755	T5

**Molecular analyses.** All eight primer-pairs (GT1–GT8) derived from the chloroplast genome of *G. tenuistipitata* var. *liui*, showed good amplification on the specimens tested but were monomorphic except for primer-pair GT5. Two defined amplified fragment sizes, 327 bp and 329 bp were obtained from primer-pair GT5, and samples with similar base size peaks fell into the same defined amplified fragment size. The generated SSR dendrogram (Fig. 3) indicated that the *G. tenuistipitata* specimens were grouped into two main clades: (a) *G. tenuistipitata* from Pattani (Thailand), Quy Kim, Hai Phong (Vietnam) and Ubin Island (Singapore); (b) *G. tenuistipitata* from Batu Laut and Middle Banks (Malaysia) and Pasir Ris Park and Lim Chu Kang (Singapore). Both clades were supported with similarity coefficients of 0.5.

The *cox1* UPGMA dendrogram (Fig. 4) showed that all *G. tenuistipitata* were grouped into two main clades, Clade A and Clade B. Clade A was divided into three sub-clades: *G. tenuistipitata* from Middle Banks and Batu Laut (Malaysia), Ubin Island, Lim Chu Kang and Pasir Ris Park (Singapore) in Clade A1 with a moderate support value (62%); *G. tenuistipitata* from Pattani (Thailand) and Quy Kim, Hai Phong (Vietnam) in Clade A2 (57%) while Clade A3 was represented by *G. tenuistipitata* from Orissa, Chilika Lake, India (Accession number: EF434924). Clade B consisted of *G. tenuistipitata* from Qingdao, China (Accession number: KC782893). Clade A1 was further resolved into two sub-clades: *G. tenuistipitata* from Middle Banks (Malaysia), Ubin Island, Lim Chu Kang and Pasir Ris Park (Singapore) formed Clade A1a with a high support value (82%) whereas *G. tenuistipitata* from Batu Laut (Malaysia) was nested in Clade A1b with a moderate support value (65%). Likewise, Clade A2 was resolved into two sub-clades with *G. tenuistipitata* from Quy Kim, Hai Phong (Vietnam) in Clade A2a with 86% support value while *G. tenuistipitata* from Pattani (Thailand) formed the Clade A2b with a moderate support value of 78%.

Five haplotypes, namely T1, T2, T3, T4 and T5 were identified based on the *cox1* gene analysis using a statistical parsimony network on 21 specimens collected from seven different localities (Table 1, Fig. 5). *G. tenuistipitata* from Middle Banks, Penang (Malaysia) showed the same haplotype, T1

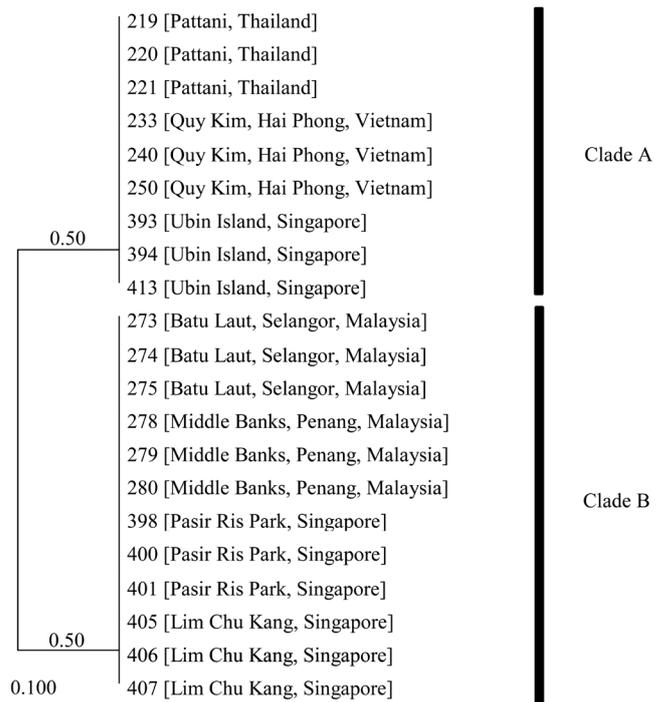


Fig. 3. Dendrogram of *Gracilaria tenuistipitata* from different localities generated using the unweighted pair group method with arithmetic average (UPGMA) by primer-pair GT5.

as specimens from Ubin Island, Lim Chu Kang and Pasir Ris Park (Singapore). Haplotype T2 was detected in Batu Laut, Malaysia and differed from haplotype T1 by one mutation change: guanidine to adenine at position 190. Haplotype T3 from Pattani, Thailand varied from haplotype T1 with three mutation changes: cytosine to thymine at position 225, thymine to cytosine at position 1125 and guanidine to adenine at position 1203. Haplotype T4 which was represented by *G. tenuistipitata* from Orissa, Chilika Lake, India (Accession number: EF434924) had four mutation changes: cytosine to thymine at position 225, thymine to adenine at position 978, thymine to cytosine at position 1125 and thymine to cytosine at position 1140. Haplotype T5 from Quy Kim, Vietnam varied from haplotype T3 by two mutation changes with substitution of adenine to guanidine at position 174 and thymine to cytosine at position 1179 (Table 2).

Table 2. Variation sites in DNA sequences of *Gracilaria tenuistipitata* corresponding to mitochondrial haplotypes from different localities (see Fig. 1). The DNA sequence of *G. tenuistipitata* from Orissa, Chilika Lake, India (Accession number: EF434924) was downloaded from GenBank (<http://www.ncbi.nlm.nih.gov/>).

Haplotype	Collection Site	Variation sites in DNA sequence							
		174	190	225	978	1125	1179	1203	1140
T1	Singapore (Ubin Island, Lim Chu Kang, Pasir Ris Park) and Malaysia (Middle Banks, Penang)	A	G	C	T	T	T	G	T
T2	Batu Laut, Malaysia	A	A	C	T	T	T	G	T
T3	Pattani, Thailand	A	G	T	T	C	T	A	T
T4	EF434924 (Orissa, Chilika Lake, India)	A	G	T	A	C	T	G	C
T5	Quy Kim, Hai Phong, Vietnam	G	G	T	T	C	C	A	T

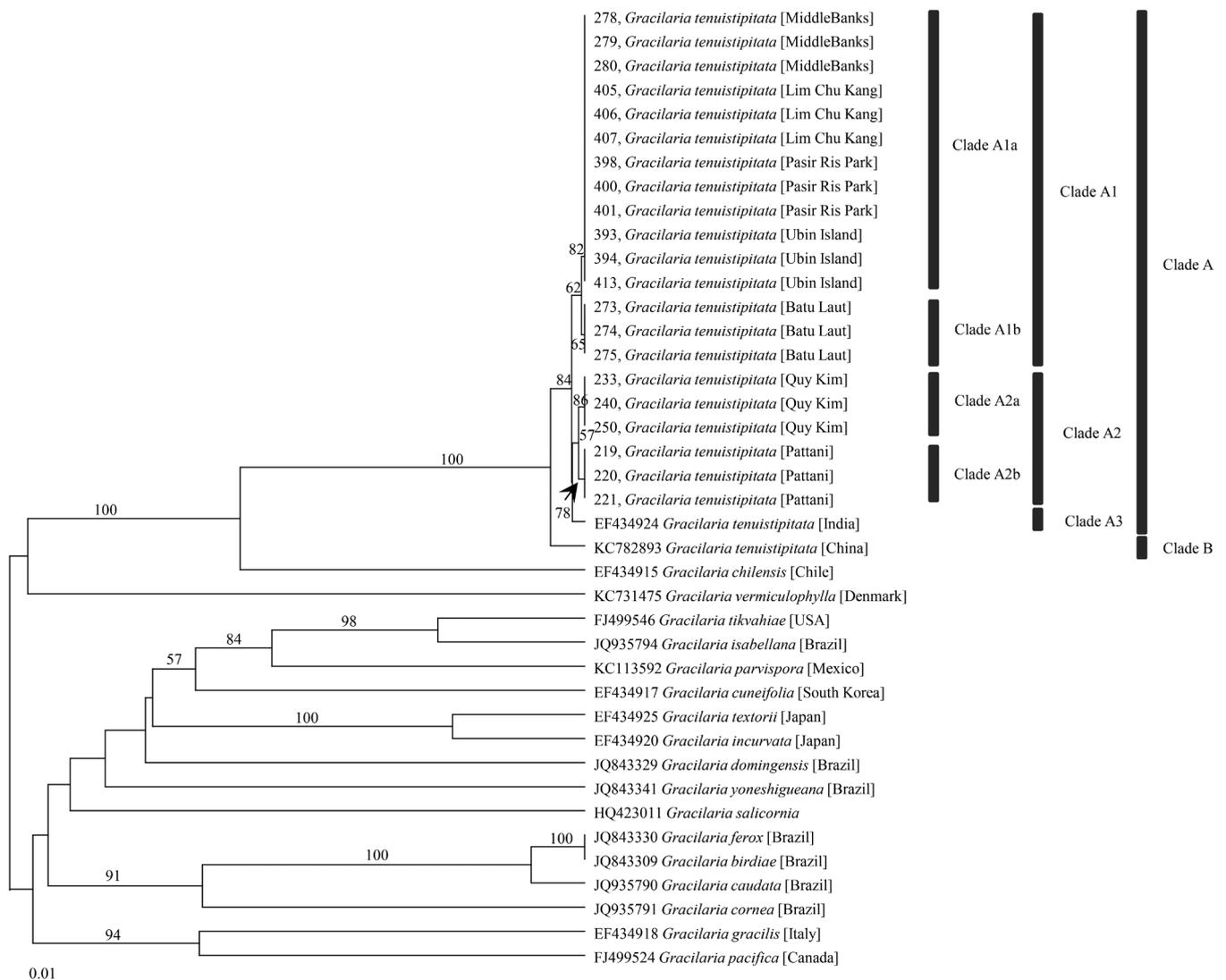


Fig. 4. UPGMA dendrogram of *Gracilaria tenuistipitata* and other *Gracilaria* species inferred from *cox1* gene. Numbers at the nodes are arranged as bootstrap value.

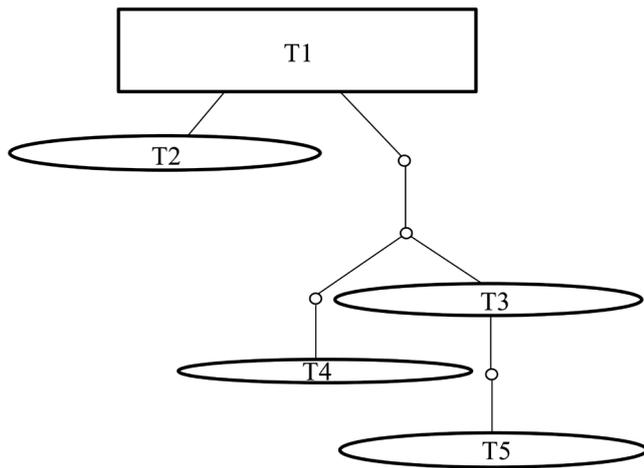


Fig. 5. Statistical parsimony networks for *cox1* haplotypes of *Gracilaria tenuistipitata*. The small circles indicate missing haplotype and the lines represent parsimonious connections between haplotypes with a probability higher than 95%, with each representing one mutational step. The size of rectangle or oval corresponds to the haplotype frequency.

## DISCUSSION

In the latest checklist of the algae of Singapore by Pham et al. (2011), there were a total of 1,054 species, varieties and forms of algae classified into nine phyla and 189 families. Of the 126 species in Rhodophyta, 20 species of *Gracilaria* were recorded for Singapore inclusive of *G. tenuistipitata*. During the Johor Straits workshop (part of the Comprehensive Marine Biodiversity Survey of Singapore), *G. tenuistipitata* was observed at Singapore's main island (Pasir Ris Park and Lim Chu Kang) as well as in Ubin Island, a small island situated in the north east of Singapore.

Based on the generated SSR dendrogram (Fig. 3), *G. tenuistipitata* from Pasir Ris Park and Lim Chu Kang were grouped in a clade with specimens from Batu Laut and Middle Banks (west coast of Peninsular Malaysia) while *G. tenuistipitata* from Ubin Island grouped together with specimens from Pattani (Thailand) and Quy Kim (Vietnam). The primer-pair GT5 was able to differentiate between *G. tenuistipitata* collected from Ubin Island and the main island of Singapore. In order to enlarge the genetic diversity study on *G. tenuistipitata*, analysis was carried out with the inclusion of specimens from Kuah (Langkawi Island, Malaysia) and Kelantan (Malaysia) using primer-pair GT5. Table 3 shows that *G. tenuistipitata* from Pasir Ris Park and Lim Chu Kang (Singapore) shared the same defined amplified fragment size (327 bp) with specimens from Batu Laut, Middle Banks and Kuah (west coast of Peninsular Malaysia). *G. tenuistipitata* from Pattani (Thailand), Quy Kim (Vietnam) and Ubin Island (Singapore) exhibited a similar amplified fragment size of 329 bp while specimens from Kelantan (west coast of Peninsular Malaysia) demonstrated a unique amplified fragment size of 333 bp. The results indicated that specimens from the west coast of Peninsular Malaysia in the Strait of Malacca shared a similar genetic allele with those from Singapore's main island. On the other hand, specimens from Ubin Island

have a unique allele that can be distinguished from those of Singapore's main island.

Based on the *cox1* gene analysis, all the *G. tenuistipitata* from Singapore were grouped in a same clade with specimens from Middle Banks (Malaysia) while *G. tenuistipitata* from Pattani (Thailand), Quy Kim (Vietnam) and Batu Laut (Malaysia) were grouped in three distinct clades. These results are in congruence with the TCS analysis in which five haplotypes (T1–T5) were obtained from the 21 specimens tested, inclusive of the isolate of *G. tenuistipitata* from India denoted as haplotype T4. Genetic isolation was observed for specimens from Batu Laut (Malaysia), Pattani (Thailand), Quy Kim (Vietnam) and Orissa, Chilika Lake (India; accession number: EF434924). However, no genetic variation was observed between *G. tenuistipitata* from Singapore and Middle Banks (Malaysia) despite their geographical separation. Therefore, we can infer that populations of *G. tenuistipitata* from Singapore and Malaysia are more similar in their genetic make-up as compared to *G. tenuistipitata* from other countries such as Thailand, Vietnam and India in which three to five mutation changes were detected. In addition, the genetic diversity of Malaysian populations of *G. tenuistipitata* was slightly more varied as exhibited by two haplotypes obtained (Fig. 5), whereas the Singaporean populations of *G. tenuistipitata* did not show any genetic variation (only one haplotype obtained).

The results of this study suggested that the *cox1* gene is more variable compared to the microsatellite markers developed from the chloroplast genome of *G. tenuistipitata*. With the *cox1* gene we were able to resolve five different haplotypes (Fig. 5), whereas microsatellite markers (SSR) could only resolve only two genotypes. In fact, the first study on intra- and inter-specific phylogenetic relationships of *Gracilaria vermiculophylla* using mitochondrial *cox1* gene has shown this marker to be highly variable and suitable for species identification (Yang et al., 2008). In 2011, Yow et al. studied the genetic diversity of *Gracilaria changii* using *cox1* gene and their results demonstrated this marker to be suitable and reliable for studies of intraspecific relationships and genetic variation in *Gracilaria*. Although the SSR marker only revealed two different genotypes in this study, the primer-pair GT5 was able to distinguish the specimens collected from Ubin Island and the main Island of Singapore. This was not resolved by the phylogenetic and TCS analysis using *cox1* gene.

In conclusion, these preliminary results indicated that the genetic diversity of *G. tenuistipitata* within Singapore is low as no variation was observed using the *cox1* gene and only two genotypes were resolved using the SSR marker. The genetic make-up of *G. tenuistipitata* from Singapore was largely similar to *G. tenuistipitata* from Malaysia as compared to those from Thailand and Vietnam. Nevertheless, inclusion of more individuals and greater geographical coverage are expected to enhance our understanding of the population genetic structure of *G. tenuistipitata* from Singapore and its neighbouring countries.

Table 3. Alleles of *Gracilaria tenuistipitata* from nine localities (see Fig. 1) analysed using primer-pairs GT5.

Location	Primer GT5		
	Allele (327bp)	Allele (329bp)	Allele (333bp)
Lim Chu Kang, Singapore	1	0	0
Pasir Ris Park, Singapore	1	0	0
Ubin Island, Singapore	0	1	0
Batu Laut, Selangor, Malaysia	1	0	0
Middle Banks, Penang, Malaysia	1	0	0
Quy Kim, Hai Phong, Vietnam	0	1	0
Pattani, Thailand	0	1	0
Kelantan, Malaysia	0	0	1
Kuah, Pulau Langkawi, Malaysia	1	0	0

1 = Presence of SSR product

0 = Absence of SSR product

### ACKNOWLEDGEMENTS

The Johor Straits marine biodiversity workshop on Pulau Ubin, Singapore was organised by the National Parks Board and National University of Singapore and held from 15 October to 2 November 2012 at Outward Bound School. The workshop, as part of the Comprehensive Marine Biodiversity Survey (CMBS) was supported by generous contributions from Asia Pacific Breweries Singapore, Care-for-Nature Trust Fund, Shell Companies in Singapore and The Air Liquide Group. We also wish to thank the management and staff of the Outward Bound School for kindly accommodating our special needs for a successful workshop. We are grateful to Dang Diem Hong and Anchana Prathep for providing us some of the samples used in this study. Special thanks are due to Ng Poh Kheng and Yow Yoon Yen for technical support and advice. This study was funded in part by the MoHE-HIR grant (H-50001-00-A000025) and Postgraduate Research Fund (PV082-2011B), University of Malaya.

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