J Appl Phycol
DOI 10.1007/s10811-012-9943-9

Microsatellite markers from expressed sequence tags (ESTs) of seaweeds in differentiating various *Gracilaria* species

Sze-Looi Song · Phaik-Eem Lim · Siew-Moi Phang · Weng-Wah Lee · Khanjanapaj Lewmanomont · Danilo B. Largo · Nurridan Abdul Han

Received: 8 August 2012 / Revised and accepted: 31 October 2012

Abstract *Gracilaria* is a red seaweed that has been cultivated worldwide and is commercially used for food, fertilizers, animal fodder, and phycocolloids. However, the high morphological plasticity of seaweeds often leads to the misidentification in the traditional identification of *Gracilaria* species. Molecular markers are important especially in the correct identification of *Gracilaria* species with high economic value. Microsatellite markers were developed from the expressed sequence tags of seaweeds deposited at the National Center for Biotechnology Information database and used for differentiating *Gracilaria changii* collected at various localities and two other *Gracilaria* species. Out of 33 primer pairs, only one primer pair gave significant results that can distinguish between three different *Gracilaria* species as well as *G. changii* from various localities based on the variation in repeated nucleotides. The unweighted pair group method using arithmetic mean dendrogram analysis grouped *Gracilaria* species into five main clades: (a) *G. changii* from Batu Besar (Malacca), Sandakan (Sabah), Bintulu (Sarawak), Batu Tengah (Malacca), Gua Tanah (Malacca), Middle Banks (Penang), Sungai (Sg.) Merbok (Kedah), Teluk Pelandok (Negeri Sembilan), Pantai Dickson (Negeri Sembilan), Sg. Kong-Kong (Johore), and Sg. Pulai (Johore); (b) *Gracilaria manilaensis* from Cebu, Philippines; (c) *G. changii* from Morib (Selangor); (d) *Gracilaria fisheri* from Pattani, Thailand; and (e) *G. changii* from Pantai Dickson (Negeri Sembilan), Gua Tanah (Malacca), Sg. Merbok (Kedah), Sg. Kong-Kong (Johore), and Sg. Pulai (Johore). This result shows that this primer pair was able to distinguish between three different species, which are *G. changii* from Morib (Malaysia), *G. fisheri* from Pattani (Thailand), and *G. manilaensis* from Cebu (Philippines), and also between different genotypes of *G. changii*. This suggested that the simple sequence repeat primer we developed was suitable for differentiating between different *Gracilaria* species due to the polymorphisms caused by the variability in the number of tandem repeats.

Keywords Correct identification · Genotypes · Red seaweeds · Rhodophyta · SSR markers

This paper was presented at the 8th Asia-Pacific Conference on Algal Biotechnology, Adelaide, Australia, 2012.

S.-L. Song · P.-E. Lim · S.-M. Phang
Institute of Ocean and Earth Sciences (IOES),
University of Malaya,
50603 Kuala Lumpur, Malaysia

S.-L. Song · P.-E. Lim (✉) · S.-M. Phang
Institute of Biological Sciences, University of Malaya,
50603 Kuala Lumpur, Malaysia
e-mail: phaikeem@um.edu.my

W.-W. Lee
ACGT Laboratories,
Lot L3-I-1, Enterprise 4, Technology Park Malaysia, Bukit Jalil,
57000 Kuala Lumpur, Malaysia

K. Lewmanomont
Department of Fishery Biology, Faculty of Fisheries,
Kasetsart University,
Chatuchak,
Bangkok 10900, Thailand

D. B. Largo
Department of Biology, University San Carlos,
Talamban,
Cebu City 6000, Philippines

N. A. Han
Institute of Fisheries Research Sarawak,
Jalan Perbadanan, Bintawa,
93744 Kuching, Sarawak, Malaysia

Introduction

Gracilaria is one of the largest genera in number of species of red seaweeds that has been cultivated worldwide and is

Published online: 20 November 2012

commercially used for food, fertilizers, animal fodder, and phycocolloids. In Malaysia, 20 species of *Gracilaria* have been reported, mainly inhabiting mangroves, sandy mudflats, and rocky shores (Lim and Phang 2004). The common species are: *Gracilaria changii* (Xia and Abbott), Abbott, Zhang, and Xia; *Gracilaria edulis* (Gmelin) Silva; *Gracilaria salicornia* (C. Agardh) Dawson; and *Gracilaria tenuistipitata* Chang and Xia (Phang 2006).

The taxonomy and systematics, especially of commercially important species, have been the focus of research, in addition to physiological aspects related to mass cultivation and the production of useful products (Chan et al. 2006). The high morphological plasticity and the lack of distinctive reproductive structures often lead to misidentification of *Gracilaria* species. Molecular markers are important especially in the correct identification of *Gracilaria* species with high economic value. The application of molecular approaches on seaweeds started in the 1980s, when the first molecular phylogenetic study was conducted on the red seaweeds (Maggs et al. 2007). This was followed by the development of genetic transformation techniques on seaweeds (Cheyney and Kurtzman 1992; Gan et al. 2003) and the functional genomics on the characterization of genes involved in carbohydrate synthesis (Zhou and Ragan 1995).

Over the years, a number of genetic marker systems have been developed for seaweed identification. These include allozymes (Intasuwan et al. 1993), restriction fragment length polymorphisms (Candia et al. 1999; Scholfield et al. 1991), random amplified polymorphic DNA (RAPD) (Lim et al. 2001; Sim et al. 2007), amplified fragment length polymorphisms (Niwa et al. 2004; Sun et al. 2005), microsatellites or simple sequence repeats (SSRs), and single nucleotide polymorphism (Liu and Cordes 2004). In Malaysia, molecular research has focused on *Gracilaria* species for studying genetic relationships using RAPD (Lim et al. 2001; Sim et al. 2007), development of genetic transformation system (Gan et al. 2003), genetic diversity using mitochondrial *cox*I gene (Yow et al. 2011), and molecular cloning and biochemical study (Siow et al. 2012). cDNA libraries have also been generated for the Malaysian *G. changii* (Teo et al. 2007) and *Sargassum binderi* (Wong et al. 2007).

Microsatellites, alternatively known as SSRs (Jacob et al. 1991), short tandem repeats (Craig et al. 1988), or simple sequence length polymorphisms (Rassmann et al. 1991), are tandem repeats of one to six nucleotides that are arranged throughout the genomes of most eukaryotic species (Powell et al. 1996). They are the markers of choice for a variety of applications in plant genetics and breeding because of their multiallelic nature, higher levels of polymorphism, codominant inheritance, and relative abundance (Sun et al. 2006). The conventional method of developing SSR markers is labor intensive, time-consuming, and costly (Kantety et al. 2002; Varshney et al. 2002; Yu et al. 2004), but now, abundance of expressed sequence tags (ESTs) sequences are available in online databases. The development of SSRs from ESTs databases has been reported in a few seaweeds such as *Laminaria digitata* (Billot et al. 1998), *Cladophoropsis membranacea* (van der Strate et al. 2000), *Fucus serratus* and *Fucus evanescens* (Coyer et al. 2002), *Ulva* (*Enteromorpha*) *intestinalis* (Alström-Paraport and Leskinen 2002), *Postelsia palmaeformis* (Whitmer 2002), *Ascophyllum nodusum* (Olsen et al. 2002), *Gracilaria chilensis* (Guillemin et al. 2005), *Saccharina* (*Laminaria*) *japonica* (Shi et al. 2007), and *Chondrus crispus* (Provan and Maggs 2012).

The objective of this study was to develop microsatellite markers from ESTs of *Gracilaria* species (*G. changii*, *Gracilaria gracilis* (Stackhouse) Steentoft, Irvine, and Farnham, and *Gracilariopsis lemaneiformis* (Bory de Saint-Vincent) Dawson, Acleto and Foldvik) using available bioinformatics tools and to test the suitability of the derived primer pairs in distinguishing *G. changii* from different populations and between three different *Gracilaria* species.

Materials and methods

A total of 117 *Gracilaria changii* specimens used in this study were collected from 12 different localities: (a) Morib, Selangor, Malaysia, (b) Middle Banks, Penang, Malaysia, (c) Sungai (Sg.) Merbok, Kedah, Malaysia, (d) Teluk Pelandok and Pantai Dickson, Negeri Sembilan, Malaysia, (e) Gua Tanah, Batu Besar and Batu Tengah, Malacca, Malaysia, (f) Sg. Pulai and Sg. Kong-Kong, Johore, Malaysia, (g) Sandakan, Sabah, Malaysia, and (h) Bintulu, Sarawak, Malaysia (Fig. 1). Ten *Gracilaria manilaensis* Yamamoto and Trono from Cebu, Philippines and ten *Gracilaria fisheri* (Xia and Abbott) Abbott, Zhang, and Xia from Pattani, Thailand were also included in this study. All specimens were identified based on the morphological descriptions from Xia and Abbott (1987) and Lim and Phang (2004). Female gametophytes (haploid) and tetrasporophytes (diploid) were identified by their reproductive organs. The samples were ground into powder in liquid nitrogen and DNA extraction was carried out using DNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's instructions with minor modifications. The extracted genomic DNA was kept at −20 °C before further analysis.

All *Gracilaria* species (*G. changii* (8,147), *G. gracilis* (200), and *Gp. lemaneiformis* (178)) ESTs used in this study were downloaded from the dbEST database at National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/dbEST) and saved in FASTA format. Perl scripts were developed to search for microsatellite tandem repeats in the genome sequence data using microsatellite search module (http://

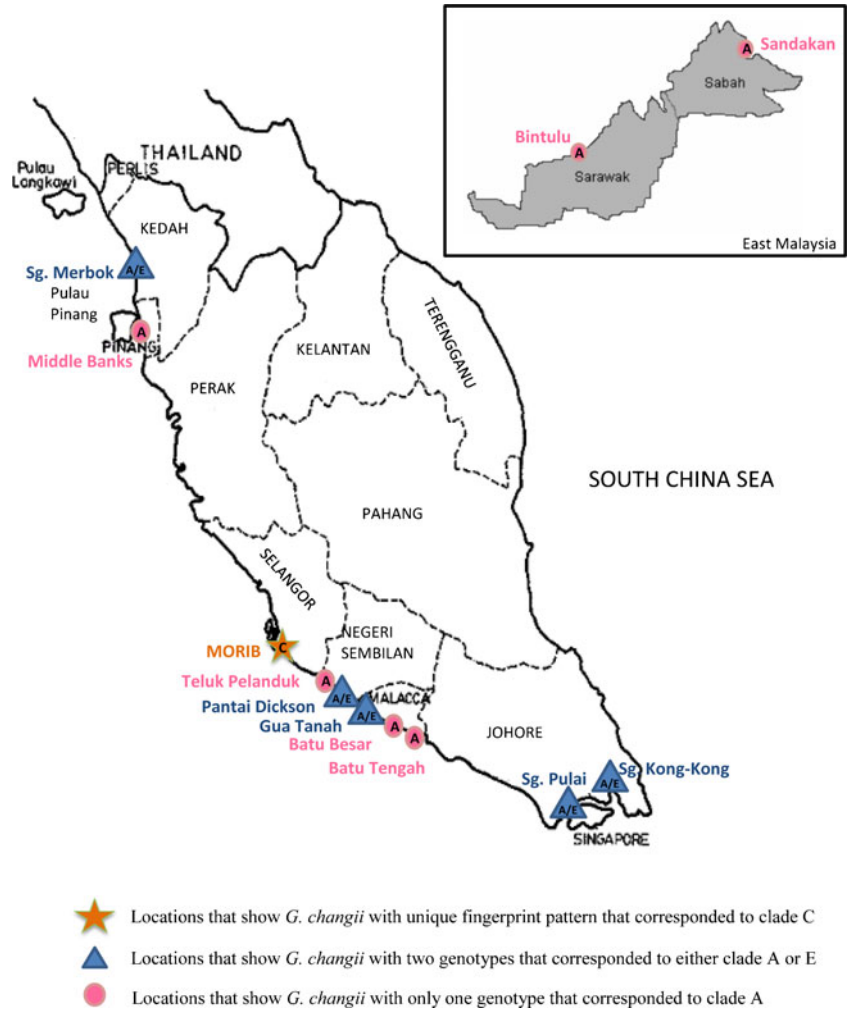

Fig. 1 Map showing the different locations in Malaysia where *Gracilaria changii* was collected (adapted from http://www.fao.org/docrep/field/009/ag160e/AG160E09.htm and http://www.fatbirder.com/links_geo/asia/malaysia.html)

pgrc.Ipk-gatersleben.de/misa/). CAP3 software (Huang and Madan 1999) was used to assemble the sequences into contigs as the EST sequences were in redundant form. In order to avoid homoplasy, only perfect SSRs (with no substitutions interrupting the core motif) and nucleotides with repeated patterns of length two or more than two were used in this study. Mononucleotides were excluded from further analysis. Primers were designed using Primer3 (Rozen and Skaletsky 2000). The parameters used for the design of SSR primers were defined in such a way that the primer annealing temperatures varied from 48 °C to 55 °C with primer length within 20–24 bp, GC content between 50 % and 70 %, and the expected product size between 280 bp and 350 bp.

Polymerase chain reactions (PCRs) were performed in a total volume of 15 μL containing 1.5 μL 10× PCR buffer (Takara Biotechnology, Dalian, China), 0.2 mM dNTP mix (Takara), 0.5 U *Taq* polymerase (Takara), 0.3 mM of each primer pairs, and 25–50 ng genomic DNA with UHQ water added to a total volume of 15 μL. The amplification reaction conditions were as follows: 5 min denaturation step at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 52 °C, 2 min elongation at 72 °C, and a final extension at 72 °C for 5 min. Forward primer of each primer pair was fluorescently [69-carboxy-fluorecine (FAM)] labeled. The optimized annealing temperature for PCR amplifications was 52 °C and this optimum temperature was used to test all the specimens. The amplification products were separated on 3.0 % MetaPhor® agarose (FMC or Cambrex Corporation, USA) at 75 V for 75 min and visualized by SYBR Safe staining (Invitrogen, USA). Amplification products were sent for fragment analysis to detect alleles using an automated DNA sequencer.

Primer pairs that showed good amplification in all the *Gracilaria* species were considered as usable. SSR products amplified by each primer pair were scored using binary matrix method with "1" (presence) and "0" (absence). These DNA fingerprints of *Gracilaria* specimens from different locations were constructed using GelQuest software (Hepperle 2002) based on the size standard template ABI GeneScan 50–500 and followed with similarity matrix cluster analysis based on the unweighted pair group method using arithmetic mean (UPGMA) using ClusterVis software version 1.4.2 (Hepperle 2002).

Table 1 Primer pairs derived from expressed sequence tags (ESTs) of *Gracilaria* species (*G. changii*, *G. gracilis*, and *Gp. lemaneiformis*)

No.	Primer name	Microsatellite repeats	Primers (5′ – >3′)	Tm (°C)
P3	GC_Contig1289	$(CCA)_8$	F: CATATAATTGGGCACAAGAG R: CATCAGACGCTTGAATAGAT	53
P5	GC_Contig752	$(GCG)_{11}$	F: ATTGAATGCATTACCAATGT R: TGAATGACTGGTTTCAGATT	52
P6	GC_Contig969	$(GTGA)_7$	F: GATTGATCGGAGCATAGC R: TTTCTTTTGTAAAGCATTGG	54
P7	GC_Contig1209	$(CG)_6$	F: CCACCAGAACGGAGTTAT R: ACAACAGTAGCAATCAGCTT	53
P8	GC_Contig1477	$(TG)_{12}$	F: CTGCACGAGAGTGAGAAG R: GCTTGCTCATCAATGATAC	52
P9	GC_Contig1364	$(AGA)_5$	F: GAGCGATGATATCAAGAAAA R: GAGCGATGATATCAAGAAAA	53
P10	GC_Contig546	$(GGC)_5$	F: ACTGGTATGGGTTTCGAG R: ATCTTTACCTCTCCTGTCGT	53
P11	GC_Contig673	$(GA)_6$	F: AGACAGAAGGGAGAAGAGG R: AGAGGAGAACTGACAAGGAT	53
P12	GC_Contig761	$(GAC)_5$	F: GAACATCTTCACTCTCAACG R: TTCTCCTTCAATAGTGGTGA	53
P13	GC_Contig1171	$(GT)_9$	F: TTTCTGTTTTCCTTGTGTGT R: ACAGCGCACCTACTTAATAC	53
P14	GC_Contig1110	$(GC)_6$	F: GTCAGTTCATATGCCGAGT R: GGCTCTTTATATGAGAGGGT	53
P15	GC_Contig1241	$(AGC)_5$	F: ACAAGGACCCTAACTACGAC R: ATCAAATCTGCAGAAAAGC	53
P16	GC_Contig1232	$(CCG)_8$	F: CATATCATAGCCGTTTTACG R: TGTTAGAGCTGGTGGTAAAG	53
P17	GC_Contig792	$(AT)_6$	F: GGTGATGTGTACACCTTTGT R: GTATGTGCAATGAGGTTTTC	53
P18	GC_Contig814	$(CGG)_9$	F: TGTGAATCAGACGGTGTC R: GTAAAGCGTAGCGTTCAGT	53
P19	GC_Contig645	$(GAC)_{12}$	F: GTGAACTAGGTAGTTTGGCA R: CAAGAAATGACAAGCAAGAT	52
P20	GC_Contig569’	$(GGC)_5$	F: GTTTTTCCACCATATCCAG R: ACCACCAGGCAGATACTC	52
P21	GC_Contig607	$(GCA)_5$	F: CCTCTTCTCAATATGAGCG R: AGATTGGTAGAGGAGGTACG	53
P22	GC_Contig607’	$(CCG)_{10}$	F: CCTCTTCTCAATATGAGCG R: AGATTGGTAGAGGAGGTACG	53
P23	GC_Contig703	$(CT)_8$	F: AAACACTCTCCGAACAACTA R: AATATTGCGAAAATGATAGC	52
P24	GC_Contig574	$(AAC)_9$	F: GTACATGCTCCAAACTTCC R: GTTCATGAGCAGCTTGAG	53
P25	GC_Contig1353	$(GTG)_5$	F: TGAAGATTTGTATTTGGTGG R: GCGTGTACTAATGAGGCTT	53
P26	GC_Contig1386	$(CAA)_7$	F: CGACTACTCCAAGACGTTC R: GTTTTCAACTACCTTTGCTG	53
P27	GC_Contig1220	$(CGC)_5$	F: AAATACTGGTCACGGAAGA R: CTGTTAGCGAATGATTTAGG	53
P28	GC_Contig1254	$(CCT)_7$	F: ACAAGTGGAGAAGCAGAAG R: GAAGGCAGAAAATAGAACCT	52
P29	GC_Contig1129	$(CGC)_5$	F: ACATGTCTTCGCTTGCTC R: CTGTGGTTGAAGACGAAAC	54
P30	GC_Contig698	$(GCA)_6$	F: CTTTCTGTTTTGAGCTCTGT R: ATGCTGTTACTGCGAATG	52

Table 1 (continued)

No.	Primer name	Microsatellite repeats	Primers (5′ – >3′)	Tm (°C)
P31	GC_Contig1211	$(CGC)_5$	F: ACAGTGTGTTGTACTCCTGC R: AGTGTTGCAAGTGAAGACTG	53
P32	GC_Contig800	$(CGC)_8$	F: CATACGCTCCGTCAACTA R: GAAGTCGAAGTCTGGCAA	53
P33	GC_Contig972	$(GGC)_6$	F: ATAACACCAGTTCAGTGCC R: TTTTTAAAATTAAGGTGCCA	53
P34	GC_Contig583	$(GGC)_5$	F: GTACATGGGATCACGTCTT R: AAGTGGAGTGGACAAATGTA	53
P35	GC_Contig569	$(GGC)_5$	F: CTTATCCTTGATCATACCGA R: CTGGATATGGTGGAAAAAC	52
P36	GC_Contig785	$(ACA)_5$	F: CCTTCAACCACTACAAACAG R: GTCGTCTTCATCATCTTCAC	53

Tm annealing temperature

When selecting the allele size for the cluster analysis, in order to avoid background noise, only the peak which was 50 % or higher than the highest peak, was selected for each sample. The allele size range was set to include all the selected alleles. Cluster analysis was performed and the dendrogram generated based on the presence and absence of the band. The resulting UPGMA dendrogram was visualized and edited using TreeMe software (Hepperle 2004).

Results

A total of 8,535 *Gracilaria* species ESTs were screened for redundancy and resulting in 5,369 of non-redundant *Gracilaria* species ESTs. From these, 1,273 SSRs were identified in 1,027 ESTs. Only perfect SSRs were used in this study, thus the 1,273 EST-SSRs contained 880 perfect EST-SSRs with 584 (46 %) mononucleotide, 170 (14 %) dinucleotide, 120 (9.5 %) trinucleotide, 3 (0.3 %) tetranucleotide, and 3 (0.3 %) haxanucleotide.

Not all SSRs were suitable for primer design; some SSRs were located too close to the end of the flanking region for primer design, or the base composition of the flanking sequence was unsuitable (Sun et al. 2006), or the EST sequences containing microsatellites are too short (Varshney et al. 2002). In order to obtain useful primer pairs for PCR amplification, a few parameters (e.g., oligonucleotide melting temperature, size of the primer pairs, GC content, and PCR product size) were defined during the designing of primer pairs. Out of 880 perfect SSRs, only 45 (3.5 %) primer pairs were designed based on the parameters defined earlier. Of these, 33 primer pairs with 25 (75 %) trinucleotide, 7 (21 %) dinucleotide, and 1 (3 %) tetranucleotide (Table 1) were then used for amplification. Among the trimeric repeats that were synthesized, the motifs GGC were the most common, followed by CGC, GAC, and GCA. However, we observed no obvious pattern in the base composition of dimeric repeats in our products.

The 117 *G. changii* from different localities, 10 *G. manilaensis* from Cebu, Philippines, and 10 *G. fisheri* from Pattani, Thailand were analyzed with the 33 useable EST-SSR primer pairs. In this study, one primer pair that is P3 showed good amplification and polymorphism between *Gracilaria* species in 3 % Metaphor agarose gel electrophoresis is discussed. The other 32 primer pairs either produced null alleles or did not show polymorphism among the *Gracilaria* specimens tested; hence, they are excluded from data analyses. There is no difference in the SSR results for female gametophytes and tetrasporophytes. Amplification with primer pair P3 generated products of five amplified fragment sizes that are 326 bp, 329 bp, 331 bp, 334 bp, and 337 bp and samples with similar peaks of base size fell into the respective column (Table 2).

The dendrogram (Fig. 2) for primer pair P3 showed that the *Gracilaria* species were grouped into five main clades: (a) clade A, *G. changii* from Batu Besar, Batu Tengah and Gua Tanah (Malacca), Sandakan (Sabah), Bintulu (Sarawak), Middle Banks (Penang), Sg. Merbok (Kedah), Teluk Pelandok and Pantai Dickson (Negeri Sembilan), Sg. Kong-Kong, and Sg. Pulai (Johore); (b) clade B, *G. manilaensis* from Cebu, Philippines; (c) clade C, *G. changii* from Morib, Selangor; (d) clade D, *G. fisheri* from Pattani, Thailand; and (e) clade E, *G. changii* from Pantai Dickson (Negeri Sembilan), Gua Tanah (Malacca), Sg. Merbok (Kedah), Sg. Kong-Kong, and Sg. Pulai (Johore). All five clades were supported with similarity coefficient of 0.5, respectively. The support value of 0.5 is the 50 % similarity between two clades.

Table 2 Allele sizes amplified by primer pair P3 from specimens of Malaysian *Gracilaria* species, *Gracilaria manilaensis*, and *Gracilaria fisheri* listed by species and location. The number in bracket after the allele size is the number of samples

Species name	Locations	Amplified allele size	
Gracilaria changii	Morib, Selangor, Malaysia	337 bp (10)	
	Middle Banks, Penang, Malaysia	331 bp (10)	
	Batu Besar, Malacca, Malaysia	331 bp (10)	
	Batu Tengah, Malacca, Malaysia	331 bp (10)	
	Gua Tanah, Malacca, Malaysia	331 bp (4)	334 bp (6)
	Sg. Merbok, Kedah, Malaysia	331 bp (4)	334 bp (4)
	Teluk Pelandok, Negeri Sembilan, Malaysia	331 bp (10)	
	Pantai Dickson, Negeri Sembilan, Malaysia	331 bp (8)	334 bp (2)
	Sg. Kong-Kong, Johore, Malaysia	331 bp (1)	334 bp (8)
	Sg. Pulai, Johore, Malaysia	331 bp (9)	334 bp (1)
	Sandakan, Sabah, Malaysia	331 bp (10)	
	Bintulu, Sarawak, Malaysia	331 bp (10)	
Gracilaria manilaensis	Cebu, Philippines	329 bp (10)	
Gracilaria fisheri	Pattani, Thailand	326 bp (10)	

Discussion

In *Porphyra* species, approximately 6.02 % of *Porphyra haitanensis* ESTs contained SSRs (Xie et al. 2009) and 2.1 % for *Porphyra yezoensis* (Sun et al. 2006). In this study, about 1.24 % of all the *Gracilaria* species (*G. changii*, *G. gracilis*, and *Gp. lemaneiformis*) ESTs contained SSRs. This is a lower percentage compared to *Porphyra* species, barley (3.4 %), rice (4.7 %), and wheat (3.2 %), but higher than the maize (1.5 %; Kantety et al. 2002).

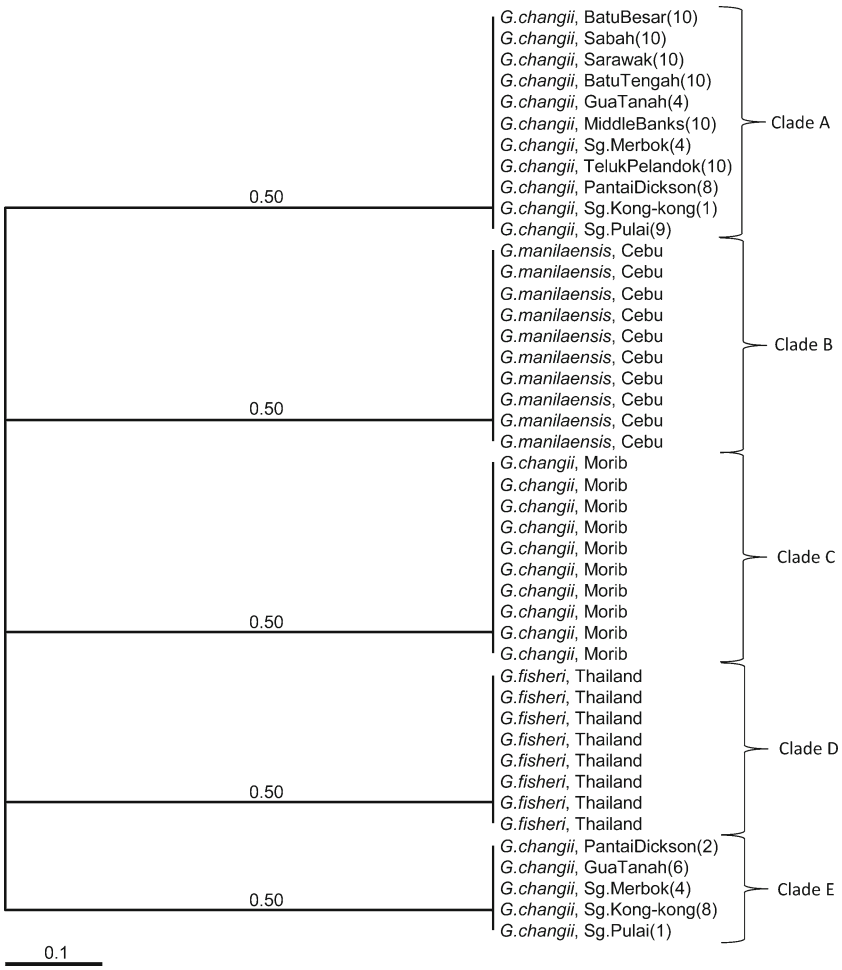

Fig. 2 Dendrogram generated using the unweighted pair group method with arithmetic average (UPGMA) of *Gracilaria* species from different localities. The *number in bracket* after the locality is the number of samples used for analysis. Samples from Morib corresponded to clade C; samples from Batu Besar, Batu Tengah, Middle Banks, Sg. Kong-Kong, Sg. Pulai, Sabah, and Sarawak corresponded to clade A; and samples from Pantai Dickson, Gua Tanah, Sg. Merbok, Sg. Kong-Kong, and Sg. Pulai corresponded to either clade A or E

The taxonomic and systematic problems within the members of Gracilariaceae have not been resolved 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. 2004). For example, the distinction between *G. changii*, *G. manilaensis*, and *G. fisheri* using morphological and anatomical characters is very difficult as the only difference between these three species is the proliferous branching in *G. manilaensis* and *G. fisheri* while less proliferous in *G. changii*. The difference between *G. manilaensis* and *G. fisheri* is mainly the male reproductive structure in which *G. manilaensis* has *verrucosa* type of spermatangia that are crowded and separated by modified vegetative cell walls while there is no modified vegetative cell walls in *G. fisheri* (Chang and Xia 1988; Xia and Abbott 1987; Abbott et al. 1991; Yamamoto and Trono 1994; Phang and Lewmanomont 2001). Whether the branching mode is proliferative or is not very subjective and the occurrence of male spermatangia is rare, therefore, it is very hard to differentiate them morphologically. Thus, molecular markers are important in this context. This study has shown that primer pair P3 was able to distinguish these three different *Gracilaria* species into five different clades (clade B, *G. manilaensis*, and clade D, *G. fisheri*, are clearly differentiated from the three clades of *G. changii* (clades A, C, and E)) and hence can be used as molecular marker to distinguish the three species.

This study also showed that the population of *G. changii* from Morib, Selangor, Malaysia (clade C) has their unique allele that can distinguish *G. changii* from the other populations (clades A and E). Although the populations of *G. changii* from Sabah and Sarawak are located in east Malaysia, they shared a similar genotype with some of the populations in west coast of Peninsular Malaysia.

Of the 45 EST-SSRs, 33 primer pairs with repeats of two or more nucleotides were chosen for analyses as they were shown to be more polymorphic (Varshney et al. 2002). Although EST-SSRs markers are less polymorphic compared to genomic SSRs (Scott 2001), they have higher level of transferability among related species as shown in this study.

In conclusion, our results have shown that the primer pair P3 was able to differentiate between three different species, which are *G. changii* from various localities in Malaysia (clades A, C, and E), *G. manilaensis* from Cebu, Philippines (clade B), and *G. fisheri* from Pattani, Thailand (Clade D). *G. changii* particularly from Morib, Selangor, Malaysia (clade C) can be differentiated from the other localities (including Sabah and Sarawak, east Malaysia) using this primer pair. Besides, there are three genotypes of *G. changii* observed from this analysis: (a) *G. changii* from Batu Besar, Batu Tengah, Gua Tanah, Middle Banks, Teluk Pelandok, Sg. Merbok, Pantai Dickson, Sg. Kong-Kong, Sg. Pulai, Sabah, and Sarawak (clade A); (ii) *G. changii* from Morib (clade C); and (iii) *G. changii* from Pantai Dickson, Gua Tanah, Sg. Merbok, Sg. Kong-Kong, and Sg. Pulai (clade E).

Development of molecular markers, particularly the microsatellite markers in distinguishing different populations and across related species, is essential to select valuable strains of the species for cultivation. The microsatellite marker that has been developed in this study was able to solve the confusion between three different *Gracilaria* species that are morphologically indistinguishable. Further studies in developing microsatellite markers from seaweeds with high economic value such as *Gracilaria* spp. will be most beneficial to the seaweed industry.

Acknowledgments This work was supported by the University of Malaya Research Grant Scheme: Development of Simple Sequence Repeats (SSRs) from Expressed Sequence Tags (ESTs) of Seaweeds (RG025-09SUS) and Postgraduate Research Fund (PS304/2009B and PS269/2010B), University of Malaya. We would like to thank Yow Yoon-Yen in providing the *Gracilaria* samples.

References

Abbott IA, Zhang J, Xia BM (1991) *Gracilaria mixta*, sp. nov. and other western Pacific species of the genus (Rhodophyta: Gracilariaceae). Pac Sci 45:12–27

Alström-Paraport C, Leskinen E (2002) Development of microsatellite markers in the green algae *Enteromorpha intestinalis* (Chlorophyta). Mol Ecol Notes 2:581–583

Billot C, Rousvoal S, Estoup A, Epplen JT, Saumitou-Laprade P et al (1998) Isolation and characterization of microsatellite markers in the nuclear genome of the brown alga *Laminaria digitata* (Phaeophyceae). Mol Ecol 7:1778–1780

Candia A, González MA, Montoya R, Gómez P, Nelson W (1999) Comparison of ITS RFLP patterns of *Gracilaria* (Rhodophyceae, Gracilariales) populations from Chile and New Zealand and an examination of interfertility of Chilean morphotypes. J Appl Phycol 11:185–193

Chan CX, Ho CL, Phang SM (2006) Trends in seaweeds research. Trends Plant Sci 11:165–166

Chang CF, Xia BM (1988) On two new *Gracilaria* (Gigartinales, Rhodophyta) from South China. In: Abbott IA (ed) Taxonomy of economic seaweeds with reference to some Pacific and Caribbean species Vol. 2, California Sea Grant College Program. pp 127–129

Cheyney D, Kurtzman A (1992) Progress in protoplast fusion and gene transfer in red algae. Abstracts XIVth International Seaweed Symposium. p 68

Coyer JA, Veldsink JH, Jones K, Stam WT, Olsen JL (2002) Characterization of microsatellite loci in the marine seaweeds, *Fucus serratus* and *F. evanescens* (Heterokontophyta; Fucaceae). Mol Ecol Not 2:35–37

Craig J, Fowler S, Burgoyne LA, Scott AC, Harding HWJ (1988) Repetitive deoxyribonycleic acid (DNA) and human genome variation: a concise review relevant to forensic biology. J Forensic Sci 33:1111–1126

Gan S-Y, Qin S, Rofina YO, Yu D, Phang S-M (2003) Transient expression of lacZ in particle bombarded *Gracilaria changii* (Gracilariales, Rhodophyta). J Appl Phycol 15:345–349

Guillemin M-L, Destombe C, Faugeron S, Correa JA, Valero M (2005) Development of microsatellites DNA markers in the cultivated seaweed, *Gracilaria chilensis* (Gracilariales, Rhodophyta). Molec Ecol Notes 5:155–157

Hepperle D (2002) Fingerprinter©. A software for the analysis, bin-assignment and 01 matrix construction of molecular fingerprints (AFLPs). Win32-Version Distributed by the author via: http://sciencedo-mixde

Hepperle D (2004) TreeMe©. A software for visualization, manipulation, layouting and labeling of phylogenetic trees. Win32-Version Distributed by the author via: http://wwwsequentixde

Huang X, Madan A (1999) CAP3: a DNA sequence assembly program. Genome Res 9:868–877

Intasuwan S, Gordon ME, Daugherty CH, Lindsay GC (1993) Assessment of allozyme variation among New Zealand populations of *Gracilaria chilensis* (Gracilariales, Rhodophyta) using starch-gel electrophoresis. Hydrobiologia 260/261:159–165

Iyer R, Clerck OD, Bolton J, Coyne V (2004) Morphological and taxonomic studies of *Gracilaria* and *Gracilariopsis* species (Gracilariales, Rhodophyta) from South Africa. S Afr J Bot 70:521–539

Jacob HJ, Linderpaintner K, Lincoln SE, Kusumi K, Bunker RK, Mao YP, Ganten D, Dzau VJ, Lander ES (1991) Genetic mapping of a gene causing hypertension in the stoke-prone spontaneously rat. Cell 67:213–224

Kantety RV, La Rota M, Matthews DE, Sorrells ME (2002) Data mining for simple-sequence repeats in expressed sequence tags from barley, maize, rice, sorghum, and wheat. Plant Mol Biol 48:501–510

Lim PE, Phang SM (2004) *Gracilaria* species (Gracilariales, Rhodophyta) of Malaysia including two new records. Malays J Sci 23:71–80

Lim PE, Thong KL, Phang SM (2001) Molecular differentiation of two morphological variants of *Gracilaria salicornia*. J Appl Phycol 13:335–342

Liu ZJ, Cordes JF (2004) DNA marker technologies and their applications in aquaculture genetics. Aquaculture 238:1–37

Maggs CA, Verbruggen H, De Clerck O (2007) Molecular systematics of red algae: building future structures of firm foundations. In: Brodie J, Lewis J (eds) Unravelling the algae: the past, present, and future of algal systematics. CRC Press, Boca Raton, pp 103–121

Niwa K, Kikuchi N, Iwabuchi M, Aruga Y (2004) Morphological and AFLP variation of *Porphyra yezoensis* Ueda form, *narawaensis* Miura (Bangiales, Rhodophyta). Phycol Res 52:180–190

Olsen JL, Sadowski G, Stam WT, Veldsink JH, Jones K (2002) Characterization of microsatellite loci in the marine seaweed *Ascophyllum nodosum* (Phaeophyceae Fucales). Molec Ecol Notes 2:33–34

Phang SM (2006) Seaweed resources in Malaysia: current status and future prospects. Aquati Ecosyst Health Manag 9:185–202

Phang SM, Lewmanomont K (2001) *Gracilaria changii* (B. M. Xia & I. A. Abbott) I. A. Abbott, C. F. Chang & B. M. Xia. In: Reine P, Trono GC Jr (eds) Plant resources of South-East Asia, no. 15(1). Cryptogams: algae. Backhuys, Leiden, pp 178–180

Powell W, Machray GC, Provan J (1996) Polymorphism revealed by simple sequence repeats. Trends Plant Sci 1:215–222

Provan J, Maggs CA (2012) Unique genetic variation at a species' rear edge is under threat from global climate change. Proc Roy Soc B 279:39–47

Rassmann K, Schlötterer C, Tautz D (1991) Isolation of simple-sequence loci for use in polymerase chain reaction-based DNA fingerprinting. Electrophoresis 12:113–118

Rozen S, Skaletsky HJ (2000) In: Krawetz S, Misener S (eds) Primer3 on the WWW for general users and for biologist programmers. Humana Press, Totowa, pp 365–386

Scholfield CI, Gacesa P, Price JH, Russell SJ, Bhoday R (1991) Restriction fragment length polymorphisms of enzymically-amplified small-subunit rRNA-coding regions from *Gracilaria* and *Gracilariopsis* (Rhodophyta)—a rapid method for assessing 'species' limits. J Appl Phycol 3:329–334

Scott KD (2001) Microsatellites derived from ESTs and their comparison with those derived from by other methods. In: Henry RJ (ed) Plant genotyping: the DNA fingerprinting of plants. CAB International, Wallingford, pp 225–237

Shi Y, Yang G, Liu Y, Liao M, Li X et al (2007) Development of 18 polymorphic microsatellite DNA markers of *Laminaria japonica* (Phaeophyceae). Molec Ecol Notes 7:620–622

Sim MC, Lim PE, Gan SY, Phang SM (2007) Identification of random amplified polymorphic DNA (RAPD) marker for differentiating male from female and sporophytic thalli of *Gracilaria changii* (Rhodophyta). J Appl Phycol 19:763–769

Siow RS, Teo SS, Ho WY, Mohd. Yunus Abd S, Phang SM, Ho CL (2012) Molecular cloning and biochemical characterization of galactose-1-phosphate uridylyltransferase from *Gracilaria changii* (Rhodophyta). J Phycol 48:155–162

Sun JW, Jin MD, Zhou CJ, Yang QK, Weng ML et al (2005) Identification of *Porphyra* lines (Rhodophyta) by AFLP DNA fingerprinting and molecular markers. Plant Mol Biol Rep 23:251–262

Sun J, Liu T, Guo B, Jin D, Weng M et al (2006) Development of SSR primers from EST sequences and their application in germplasm identification of *Porphyra* line (Rhodophyta). Europ J Phycol 41:329–336

Teo SS, Ho CL, Teoh S, Lee WW, Tee JM et al (2007) Analyses of expressed sequence tags (ESTs) from an agarophyte, *Gracilaria changii* (Gracilariales, Rhodophyta). Europ J Phycol 42:41–46

van der Strate HJ, Olsen JL, van de Zande L, Edwards KJ, Stam WT (2000) Isolation and characterization of microsatellite loci in the benthic seaweed, *Cladophoropsis membranacea* (Cladophorales, Chlorophyta). Mol Ecol 9:1442–1443

Varshney RK, Thiel T, Stein N, Langridge P, Graner A (2002) In silico analysis on frequency and distribution of microsatellites in ESTs of some cereal species. Cell Mol Biol Lett 7:537–546

Wattier R, Dallas JF, Destombe C, Saumitou-Laprade P, Valero M (1997) Single locus microsatellites in Gracilariales (Rhodophyta): high level of genetic variability within *Gracilaria gracilis* and conservation in related species. J Phycol 33:868–880

Whitmer AC (2002) Microsatellite markers for the intertidal kelp *Postelsia palmaeformis* (Heterokontophyta; Laminariales). Molec Ecol Notes 2:469–471

Wong TKM, Ho CL, Lee WW, Rahim RA, Phang SM (2007) Analyses of expressed sequence tags (ESTs) from *Sargassum binderi* (Phaeophyta). J Phycol 43:528–534

Xia BM, Abbott IA (1987) New species of Polycavernosa Chang & Xia (Gracilariaceae, Rhodophyta) from the western Pacific. Phycologia 26:405–418

Xie CT, Chen CS, Ji DH, Xu Y (2009) Characterization, development and exploitation of EST-derived microsatellites in *Porphyra haitanensis* Chang et Zheng (Bangiales, Rhodophyta). J Appl Phycol 21:367–374

Yamamoto H, Trono GC Jr (1994) Two new species of *Gracilaria* from the Philippines. In: Abbott IA (ed) Taxonomy of economic seaweeds with reference to some Pacific species, vol 4. California Sea Grant College, La Jolla, pp 95–101

Yow YY, Lim PE, Phang SM (2011) Genetic diversity of *Gracilaria changii* (Gracilariaceae, Rhodophyta) from west coast, Peninsular Malaysia based on mitochondrial cox1 gene analysis. J Appl Phycol 23:219–226

Yu JK, Dake TM, Singh S, Benscher D, Li W, Gill B, Sorrells ME (2004) Development and mapping of EST-derived simple sequence repeat markers for hexaploid wheat. Genome 47:805–818

Zhou YH, Ragan MA (1995) Cloning and characterization of the nuclear gene and cDNAs for triosephosphate isomerase of the marine red algae *Gracilaria verrucosa*. Curr Genet 28:317–323