CHAPTER 3:MATERIALS AND METHODS

3.1 FIELD COLLECTION

Gracilaria (Gracilariaceae, Rhodophyta) species were obtained from various localities around Peninsular Malaysia and Thailand. Table 4 shows the list of species and collection sites. Photographs show the sample collection sites at Carey Island, Sungai Pulai and Phuket, Thailand (Figures 1-3). Figure 4 shows the location of each site. The freshly collected samples were cleaned (with gloves on) using seawater and distilled water, subsequently. Then, they were left to dry in a clean air-conditioned room. The dried samples were ground to powder with liquid nitrogen and stored at –20°C until use.

Figure 1: Carey Island (Sample collection site in a Malaysian-mangrove)

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Figure 1: Carey Island (Sample collection site in a Malaysian-mangrove)

Figure 2: Sungai Pulai (Sample collection site in a Malaysian-mangrove and estuary)

Figure 3: Phuket (Sample collection site in a mangrove in Thailand)

Table 2: The list of samples collected from different places around Malaysia and Thailand.

LOCATION	SAMPLES
Ao Nambo, Phuket, Thailand	*Gracilaria changii* (Xia et Abbott) Abbott, Zhang et Xia
Takbai, Thailand	*Gracilaria changii* (Xia et Abbott) Abbott, Zhang et Xia
Ban Merbok, Kedah, Malaysia	*Gracilaria changii* (Xia et Abbott) Abbott, Zhang et Xia
Morib, Kuala Selangor, Malaysia	*Gracilaria changii* (Xia et Abbott) Abbott, Zhang et Xia, *Gracilaria edulis* (Gmelin) Silva , *Gracilaria salicornia* (C.Agardh) Dawson
Carey Island, Kuala Selangor, Malaysia	*Gracilaria changii* (Xia et Abbott) Abbott, Zhang et Xia
Sungai Pulai, Johor, Malaysia	*Gracilaria changii* (Xia et Abbott) Abbott, Zhang et Xia
Port Dickson, Malaysia	*Sargassum oligocystum* Montagne

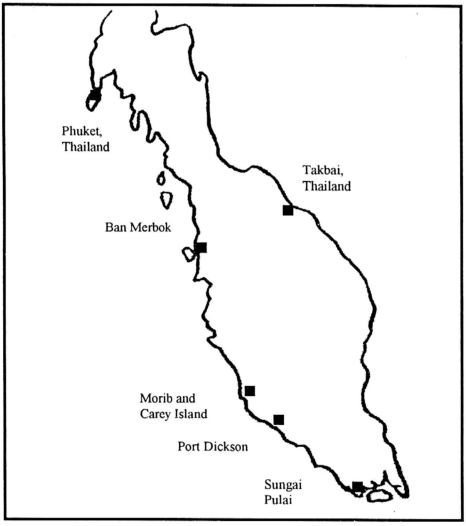

Figure 4: Map of Peninsular Malaysia and Southern Thailand showing collection sites of *Gracilaria* species.

3.2 DEOXYRIBONUCLEIC ACID (DNA) ISOLATION PROTOCOLS

The DNA isolation method used by Sinnappah (1994) was employed to isolate the DNA from the *Gracilaria* samples. A modification of this method was also carried out.

3.2.1 Protocol 1

Cetyltrimethylammonium bromide (CTAB) and phenol-chloroform combination extraction method

(Sinnappah, 1994)

Protease treatment

Buffer and solutions:

Protease buffer	0.1 M Tris-base, pH 8.0 0.1 M Na_2EDTA 0.25M NaCl 100μg/ml proteinase K
Phenol:Chloroform	1:1
10% CTAB	10% (w/v) CTAB 0.7 M NaCl

(The CTAB portion of this protocol was adapted from Murray and Thompson, 1980.)

Tris-EDTA(TE)	10mM Tris-base, pH 8.0 1 mM Na_2EDTA

Methodology

Liquid nitrogen-ground dry sample material (3g) was suspended in 30 ml of ice cold protease buffer (the proteinase K was added fresh). N-Lauroyl-sarcosine sodium salt (Sigma) was added to make it a 1% final concentration of the initial buffer volume (20% stock solution was prepared). Fifty milliliters screwtop-capped polypropylene tubes (Nalgene) were used for all extraction steps. The mixture was gently swirled in a 65°C water bath for about an hour. One-half volume of sevac was added and mixed well. Then the mixture was centrifuged at 3,000 rpm in a bench-top centrifuge for 5 minutes at room temperature. The aqueous (upper) layer was transferred to a fresh tube.

One-tenth volume of 10% CTAB was added to the tube of supernatant and incubated at 65°C for 30 minutes with occasionally shaking. The tube was cooled to room temperature before adding an equal volume of sevac and mixed well. Then, it was centrifuged at top speed in a bench-top centrifuge for 5 minutes at room temperature. The aqueous (upper layer) phase was transferred to a clean tube. The CTAB treatment was repeated once more for 30 minutes. In a clean tube, the final aqueous phase of the CTAB treatment was treated with an equal volume of phenol/sevac (1:1). The mixture was centrifuged at 3000 rpm in a bench top centrifuge for 5 minutes at room temperature. The aqueous phase was transferred to a clean tube. Sevac treatment was repeated two more times.

Ice cold isopropanol (2.2 volumes) was added to the final clear aqueous phase (which sometimes may appear slightly pink or yellow in colour) to precipitate the DNA. The DNA can either be spun down by centrifugation or spooled out. The pellet DNA was washed with wash buffer (70% ethanol) overnight. The pellet was then air-dried and dissolved in a preferred volume (1.0 ml) of TE buffer depending on the amount of DNA.

3.2.2 Protocol 2 (Flow-chart is shown in Figure 5)

(Modification of Protocol 1)

Protease treatment

Buffer and solutions:

Protease buffer	0.1 M Tris-base, pH 8.0 0.1 M Na_2EDTA 0.25M NaCl 100μg/ml proteinase K
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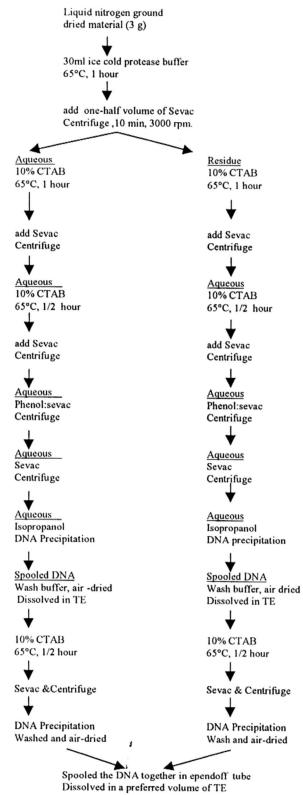
Both the residue in the former tube and the fresh aqueous solution (supernatant) were treated with one-tenth volume of 10% CTAB for one hour at 65°C. Sevac solution was added into each tube and mixed well. The supernatant was then centrifuged at top speed in a bench-top centrifuge for 5 minutes at room temperature. The aqueous (upper layer) phase was transferred to a clean tube. The 10% CTAB treatment was repeated with an incubation of 30 minutes at 65°C. The final aqueous phase of the CTAB treatment was transferred to a fresh tube and treated with an equal volume of phenol/sevac (1:1). It was mixed well by rotating the tube for 15 minutes manually. The mixture was centrifuged at 3000 rpm in a bench top centrifuge for 5 minutes at room temperature. The aqueous

phase was transferred to a clean tube. Sevac treatment was repeated two more times.

Ice cold isopropanol (2.2 volumes) was added to the final clear aqueous phase (which sometime may appears slightly pink or yellow in colour) to precipitate the DNA. The DNA can either be spun down by centrifugation or spooled out. The pellet DNA was washed with wash buffer (70% ethanol) for overnight. The pellet was then air-dried and dissolved in a preferred volume (5.0 ml) of TE buffer depending on the amount of DNA.

The DNA solution was further purified by subjecting to the 10 % CTAB treatment for half an hour and proceeding to the Sevac treatment. Precipitation of DNA was done using ice cold isopropanol. The DNA obtained (combining the DNA obtained from both the residue and the aqueous layer mentioned in the protease treatment stage) was washed with the 70% ethanol, dried and dissolved in a preferred volume (0.7-1.0 ml) of TE (depending on the amount of DNA).

Figure 5: Flow-chart of Protocol 2
(DNA EXTRACTION)



3.3 SPECTROPHOTOMETRIC DETERMINATION

Spectrophotometer from Pharmacia Biotech (Novaspec II) was used to measure the optical density at 260nm and 280nm. A 10x dilution was carried out with all the DNA samples before the spectrophotometric determination (the reading was taken according to the supplier instruction manual book). The purity of the nucleic acid sample can be estimated by calculating the ratio between the optical density readings at 260nm and 280nm (OD_{260}/OD_{280}). High purity of DNA will give a ratio of 1.8 to 2.0. An OD of 1.0 at 260nm is equivalent to 50μg/ml of double-stranded DNA (dsDNA).

3.4 RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD)

3.4.1 PCR Parameters

Polymerase chain reaction (PCR) amplifications were performed in 25μl volumes containing :

DNA	25ng
Primer(OPA)	10pmol(Operon Technologies)
10x buffer	1x (GibcoBRL)
25mM dNTP	100mM (GibcoBRL)
50mM $MgCl_2$	2mM (GibcoBRL)
5U/μl *Taq* polymerase	1U (GibcoBRL)

Amplification was performed in a Perkin-Elmer DNA Thermal Cycler 480. Table 3 shows the list of primers used in screening suitable primers for the taxonomic study of *Gracilaria*. Different annealing temperatures were tried at 33°C, 36°C, 38°C and 40°C to optimise the PCR conditions for primers OPA3, OPA10, OPA11 and OPA13. The reproducibility of this technique was checked by running differently RAPD reactions on the same DNA sample of *Gracilaria changii* collected from Morib with OPA 10.

Fourteen microlitres of the reaction products were separated by electrophoresis through 2.0% agarose gels at 45 volts and stained with ethidium bromide (0.2mg/ml). Agarose gels were photographed with a MP-4 polaroid camera system over a UV transilluminator (312nm) using Polaroid 665 instant pack film.

3.4.2 Random Amplified Polymorphic DNA (RAPD)

Random amplified polymorphism of the DNA samples were compared. Primers used were OPA3, OPA10, OPA11 and OPA13. Figure 6 shows the program used for RAPD analysis.

For every batch of RAPD amplification, a negative control consisting of all the PCR mixture except the template DNA was carried out. This served as a control to check if any contamination in the PCR mixture.

Table 3 : List of random primers from the OPA kit
(according to the Operon suppliers instruction booklet)

Primer	sequence 5' to 3'	molecular weight
OPA-01	CAGGCCCTTC	2955
OPA-02	TGCCGAGCTG	3035
OPA-03	AGTCAGCCAC	2988
OPA-04	AATCGGGCTG	3059
OPA-05	AGGGGTCTTG	3090
OPA-06	GGTCCCTGAC	2995
OPA-07	GAAACGGGTG	3108
OPA-08	GTGACGTAGG	3099
OPA-09	GGGTAACGCC	3044
OPA-10	GTGATCGCAG	3059
OPA-11	CAATCGCCGT	2979
OPA-12	TCGGCGATAG	3059
OPA-13	CAGCACCCAC	2933
OPA-14	TCTGTGCTGG	3041
OPA-15	TTCCGAACCC	2939
OPA-16	AGCCAGCGAA	3037
OPA-17	GACCGCTTGT	3010
OPA-18	AGGTGACCGT	3059
OPA-19	CAAACGTCGG	3028
OPA-20	GTTGCGATCC	3010

Figure 6:PCR PARAMETERS

Denature 94°C 5 minutes
↓
Denature 94°C 1 minute
Anneal 38°C 1 minute
Extend 72°C 2 minutes
↓
Extension 72°C 2 minutes
↓
stored at 4°C

3.4.3 RAPD Data Analysis

RAPD data analysis was carried out using the Gelcompar software 4.1. The calculation of the matrix of similarities was based on the Dice coefficients (S_D) and the dendrogram was carried out using the unweighted pair group method using arithmetic averages (UPGMA).

$S_D=2n_{xy}/(n_x+n_y)$, where

S_D= the coefficient of similarity

n_x = the total number of DNA fragments from sample X

n_y = the total number of DNA fragments from sample Y

n_{xy} = the number of DNA fragments that were identical in the two samples.

If the S_D= 1.0, it indicates that the two samples were identical and the two samples are totally dissimilar when the S_D= 0.