CHAPTER 3: MATERIALS AND METHOD

3.1 SAMPLE COLLECTION

Three species of *Caulerpa* were collected at from the coral reefs areas at Pulau Redang, Terengganu. Specimens were distinguished using morphological characteristics to assign specimens to species and varieties, and each sample was processed and pressed into herbarium to be used for further morphological study. A small portion of the tips (approximately 3-4 cm) of the plants were excised and kept in sealed plastic bags with silica-gel for DNAs extraction.

3.2 SPECIMEN PROCESSING

The silica gel dried specimens were washed with seawater and distilled water to remove silt, sand, epiphytes and detritus. The washed samples were then air-dried and place in 1.5ml Eppendorf tube for DNA extraction.

3.3 GENOMIC DNA EXTRACTION

Genomic DNA was extracted in 1.5mL Eppendorf tubes using the DNeasy Plant Mini Kit (Qiagene, Germany) which is designed to provide a fast and simple way to isolate or extract DNA from plant tissue. The isolation steps were carried out according to the instructions provided by the manufacturer.

In DNeasy Plant Mini Kit procedure, *Caulerpa* samples were first ground with liquid nitrogen to fine powder by using a mortar and pestle. The maximum amount of

starting material was 20mg for dried samples. Then, the resulting fine powder was transferred to a 1.5ml microcentrifuge tube. In the following step, the plant was lysed by addition of 400µL of lysis Buffer AP1 and 4µL of RNase A Stock Solution. The mixture was incubated for twenty minutes in a waterbath at 65^{o}C.

130µL of precipitation buffer AP2 was added into the lysate. It was mixed and incubated for 5 minutes on ice. The lysate was centrifuged for 5 minutes at the speed of 12,000 rpm to precipitate the detergent, proteins and polysaccharides. The lysate was applied to the QIAshredder Mini Spin Column (lilac) sitting in a 2ml collection tube and was centrifuged for two minutes at the speed of 12,000 rpm. The cleared lysate was transferred into a new microcentrifuge tube.

Then, 1.5 volumes of binding Buffer AP3/E were added to the clear lysate and the mixture was mixed by gently inverting the microcentrifuge tube. Next, the mixture was applied to a DNeasy mini spin column and centrifuged for one minute. The DNA was bound to the membrane of the DNeasy Mini Spin Column and the flow-through was discarded.

During the washing step, contaminants such as proteins and polysaccharide were removed by using 500ml of AW Buffer with ethanol and centrifuged for 1 minute at 8,000 rpm. The flow-through was discarded and this was repeated once.

In elution process, the DNeasy column was transferred to a 1.5ml microcentrifuge tube. 50µL of preheated (65°C) AE Buffer was pipetted directly onto the DNeasy membrane. It was incubated for 5 minutes at room temperature and then centrifuged for 1 minute at 8,000 rpm to elute the genomic DNA from the DNeasy membrane.

3.4 DETERMINATION OF PURITY AND CONCENTRATION OF DNA USING SPECTROPHOTOMETRY

The purity and quantity of the isolated DNA was estimated using BioPhotometer (Eppendorf, Germany). 1µl DNA was mixed well with 49µl elution buffer from DNeasy Plant Mini Kit (Qiagen, Germany) in 0.2ml PCR tube before being transferred into disposable cuvette for optical density readings. The ratio of absorbance at 260nm and 280nm reflects the purity of DNA, where a pure DNA yields a ratio of 1.8 -2.0. A ratio less than that implies sample being contaminated by protein or carbohydrate; while a ratio more than 2.0 suggests that DNA sample contains RNA.

3.5. GENE SEQUENCING

The gene *tuf*A was utilized in this study to understand the phylogenetic relations of *Caulerpa* species for taxonomic purposes.

3.5.1. PCR Parameter for Amplification of *tuf*A Gene

Polymerase chain reaction (PCR) amplifications were performed using isolated DNA as template and primer of *tuf*A gene. The sequences of the forward and reverse primer are shown in Table 1. Amplification of DNA was performed on a total volume of 25µl containing 2.5µl 10X buffer, 0.2mM of each dNTP (dATP, dTTP, dCTP, and dGTP), 10pmol of respective forward and reverse primer and 1.5U *Taq* polymerase. PCR was carried out with an initial denaturation at 94ºC for 10 minutes, followed by 35 cycles of amplification (denaturing at 94ºC for 30 seconds, annealing at 52ºC for 30 seconds and extension at 72ºC for 2 minutes) with a final extension for 10 minutes at

72ºC. Optimization using different annealing temperatures of 45, 48, 50 and 52 ºC was conducted. A step-down PCR modification was carried out to optimize the amplification of DNA extracted from *Caulerpa* species.

Table 3.1: Synthetic primers used for sequencing *tuf*A region.

Primers	Direction	Sequence
*tuf*AF	Forward	5'-TGA AAC AGA AMA WCG TCA TTA TGC-3'
*tuf*AR	Reverse	5'-CCT TCN CGA ATM GCR AAW CGC-3'

The PCR parameter applied for amplification *tuf*A gene by Step-down PCR are summarised as followed:

Table 3.2: Applied PCR parameters

	Steps	Temperature, °C	Time, min	Number of cycles
1	Initial Denaturation	94	3	-
2	Denaturation	94	1	3
	Annealing	52	1	
	Extension	72	1	
	Denaturation	94	1	25
	Annealing	46	1	
	Extension	72	1	
3	Final Extension	72	10	-

3.5.2 Determination of the Yield and Quality of the Amplified Product

The PCR products were checked by running electrophoresis on 1 %(w/v) agarose gel pre-stained with ethidium bromide in TAE buffer. 2µl 1kb commercial DNA ladder was used to compare with the DNA band produced, for identification of the correct amplified DNA region. The gel was viewed under ultraviolet. Thick and bright DNA bands showed that the PCR amplification yields sufficient DNA.

3.5.3 Purification of Amplified Products and Sequencing

The amplified DNA band was excised from the agarose gel under UV light and the weight of the gel was determined. Further purification was conducted using QIAquick Gel Extraction Kit (Qiagen, Germany) for sequencing purposes. Three volumes of Buffer QG were added to one volume of the gel. The mixture was incubated at 50°C for 10 minutes to solubilize the agarose and bind the DNA. The tube was vortex every two to three minutes during the incubation to ensure the gel was properly dissolved. The colour of the mixture was constantly checked to ensure it was yellow (similar to Buffer QG without dissolved agarose). If the colour of the mixture was orange or violet, 10µL of 3M sodium acetate, pH5.0 was added and mixed. The colour of the mixture should turn yellow. The adsorption of DNA to the QIAquick membrane is efficient only at pH $\leq$ 7.5. Buffer QG contains a pH indicator which is yellow at pH $\leq$ 7.5, and orange or violet at higher pH, allowing easy determination of the optimal pH for DNA binding.

After incubation, the sample was applied to the QIAquick column and centrifuged for one minute at 13,000 rpm to bind DNA. The flow-through was then discarded and QIAquick column was placed back in the same collection tube. 500µl of

QG Buffer was added to QIAquick column and centrifuged for one minute at 13,000 rpm again. This is to ensure that all traces of agarose were removed.

After that, the flow-through was discarded and the pellet was washed by adding 750µl of PE Buffer into column and centrifuged for one minute at 13,000 rpm. The flow-through was discarded and the QIAquick column was centrifuged for another one minute at 13,000 rpm. These washing steps removed residual salt contaminants. The column was placed into a new 1.5ml appendoff tube.

For the final elution step, 50µL of Buffer EB (10 mM Tris-Cl, pH 8.5) was added to the center of the QIAquick membrane for complete elution of bound DNA and the column was let stand for 1 minute to increase DNA concentration. The column was then centrifuge for one minute at 13,000 rpm. Finally, the purified DNA can be kept for further use. Purified DNA samples were sent for automated DNA sequencing service by Laboratory of First Base.

3.5.4 Analysis of Sequencing Data

3.5.4.1 Sequence alignment

The DNA sequences were edited using CHROMAS and BioEdit (Tom Hall, 1999). The edited sequences from this study were preliminarily aligned using the CLUSTAL X program (Thompson *et al*., 1994) and subsequently manually aligned.

3.5.4.2 Phylogenetic analysis

The aligned sequences were subjected to maximum-parsimony (MP) and maximum-likelihood (ML) analyses using PAUP* 4.0b10 (Swofford 2002). The MP tree was constructed using the heuristic search option, 100 random sequences additions, tree bisection reconnection (TBR) branch swapping, and unordered and unweighted characters. Bootstrap percentage (BP) was computed with 1000 replications. A list of sequences of *Caulerpa* species included in the analyses is as shown in Table 3.3. In addition to the DNA sequences obtained from this study, other sequences of *Caulerpa* were downloaded from NCBI (National Centre for Biotechnology Information) (Table 3.3) to be included for the phylogenetic analyses.

Bayesian analysis was performed using MrBayes 3.1.1 (Huelsenbeck and Ronquist, 2001), using GTR model with gamma distribution. The program was set to start with a random starting tree, analysis using four chains of Markov chain Monte Carlo iterations simultaneously for 2×10^6 generations and sampling the data every 100 generations. The likelihood scores stabilized after 200,000 generations. Hence, for construction of consensus tree, a burn-off of 200,000 generations was used.

Table 3.3: Isolates of *Caulerpa* species: taxon, geographical origins, collectors and accession numbers.

No.	Taxon	Origin	Collector	Accession no.
1	*Caulerpella ambigua* (Okamura) Prud'homme van Reine & Lokhorst	Texas Flower Gardens, USA	B. Wysor	AJ417963
2	*C. flexilis* J.V. Lamouroux	Jervis Bay, Australia	J. Zuccarello	AJ417970
3	*C. mexicana* Sonder ex Kützing	Cuba	J. Montoya	AJ417951
4	*C. mexicana*	Content Keys, Florida, USA	B. Wysor	AJ417952
5	*C. scalpelliformis* (R. Brown ex Turner) C. Agardh	Cape Banks, Australia	J. Zuccarello	AJ417971
6	*C. scalpelliformis* var. *denticulate* (Decaisne) Weber-van Bosse	Damour, Lebanon	A. Meinesz	AJ417972
7	*C. taxifolia*	Guayacan Island, Puerto-Rico	D. Ballantine	AJ417938
8	*C. taxifolia*	N. Kwa-Zulu Natal, South Africa	S. Fredericq	AJ417939
9	*C. brachypus* Harvey	Cangaluyan, Pangasinan	L. de Sénerpont Domis	AJ417934
10	*C. cactoides* (Turner) C. Agardh	Jervis Bay, Australia	J. Zuccarello	AJ417969
11	*C. geminata* Harvey	Coffs Harbour, Australia	J. Zuccarello	AJ417968
12	*C. microphysa* (Weber-van Bosse) Feldmann	Texas Flower Gardens, USA	B. Wysor	AJ417961
13	*C. racemosa* var. lamourouxii (Turner) Weber-van Bosse	Uken, Japan	T. Hanyuda	AJ417954
14	*C. racemosa* var. *occidentalis* (J. Agardh) Børgesen	Livorno, Italy	L. Piazzi	AJ417955
15	*C. racemosa* var. *macrophysa* W.R. Taylor	Galeta, Panamá	W. Kooistra	AJ417947
16	*C. racemosa* var. *peltata*	Isla Naos, Panamá	B. Wysor	AJ417949
17	*C. racemosa* var.*turbinata* (J. Agardh) Eubank	Dahab, Egypt	A. Meinesz	AJ417957
18	*C. racemosa* ecad laet-turb-pelt	Pangasinan, NW Luzon Island, Philippines	L. N. de Senerpoint Domis	AJ512415

Table 3.3, continued

19	*C. racemosa* ecad mucronata	Pangasinan, NW Luzon Island, Philippines	L. N. de Senerpoint Domis	AJ512417
20	*C. racemosa* ecad mucronata	Pangasinan, NW Luzon Island, Philippines	L. N. de Senerpoint Domis	AJ512418
21	*C. racemosa* rac-cor-lam	Pangasinan, NW Luzon Island, Philippines	L. N. de Senerpoint Domis	AJ512413
22	*C. racemosa* rac-cor-lam	Pangasinan, NW Luzon Island, Philippines	L. N. de Senerpoint Domis	AJ512467
23	*C. serrulata* var. *serrulata*	Pangasinan, NW Luzon Island, Philippines	L. N. de Senerpoint Domis	AJ512411
24	*C. sertularioides* f. *brevipes*	Pangasinan, NW Luzon Island, Philippines	L. N. de Senerpoint Domis	AJ512420
25	*C. sertularioides* f. *longipes*	Pangasinan, NW Luzon Island, Philippines	L. N. de Senerpoint Domis	AJ512421
26	*C. serrulata*	Colón, Panamá	B. Wysor	AJ417933
27	*C. racemosa* 1	Port Dickson, Negeri Sembilan	Lim Phaik Eem	Unpublished data
28	*C. racemosa 2*	Port Dickson, Negeri Sembilan	Lim Phaik Eem	Unpublished data
29	*C. racemosa* 3	Port Dickson, Negeri Sembilan	Lim Phaik Eem	Unpublished data
30	*Caulerpa racemosa* sp1, cory1	Langkawi, Kedah	Lim Phaik Eem	Unpublished data
31	*Caulerpa racemosa* sp1, cory2	Langkawi, Kedah	Lim Phaik Eem	Unpublished data
32	*C. racemosa var. racemosa, CR1*	Port Dickson,Negeri Sembilan	Lim Phaik Eem	Unpublished data
33	*C. racemosa var. macrophya,* C1_1	Pulau Redang, Terengganu	Lim Phaik Eem	Unpublished data
34	*C. racemosa var. macrophya,* C2_2	Pulau Redang, Terengganu	Lim Phaik Eem	Unpublished data
35	*C. racemosa var. macrophya,* C2_1	Pulau Redang, Terengganu	Lim Phaik Eem	Unpublished data
36	*C. racemosa var. racemosa,* CR2	Port Dickson,Negeri Sembilan	Lim Phaik Eem	Unpublished data
37	*C. serrulata var. serrulata,* Cser6_2	Pulau Redang, Terengganu	Maryam Dalili	Current Study
38	*C. serrulata var. serrulata,* Cser6_1	Pulau Redang, Terengganu	Maryam Dalili	Current Study

Table 3.3, continued

39	*C. serrulata var.boryana*, CSer6_3	Pulau Redang, Terengganu	Maryam Dalili	Current Study
40	*Caulerpa lentilifera*, CL1	Port Dickson,Negeri Sembilan	Lim Phaik Eem	Unpublished data
41	*Caulerpa lentilifera*, CL2	Port Dickson,Negeri Sembilan	Lim Phaik Eem	Unpublished data