4.0 MATERIALS AND METHODS

4.1 *Sargassum* species

The following descriptions of species are based on the whole plant morphology and structure of receptacles. The very specific description of certain features has to be carried out in order to identify individual samples at the species level. Descriptions by Yoshida (1988b), Tseng and Lu (1988), Trono (1992) and Ajisaka (personal communication) are the basis for taxonomic determination of the *Sargassum* species in this study. All *Sargassum* specimens are collected from the intertidal coral reef flat bed at Cape Rachado and Teluk Kemang, Port Dickson, Negeri Sembilan.

4.1.1 *Sargassum oligocystum* Montagne

(Plate 1 - 5)

Holdfast discoid, small. Stem terete, short, with a warty surface, bearing up to a few primary branches. Primary branches stongly flattened, distinctly compressed, with a smooth surface, producing regularly alternately arranged secondary branches. Leaves large, linear-lanceolate to lanceolate or spatulate, simple, with an acute apex, margin dentate with teeth or entire, midrib distinct, reaching near apex, cryptostomata scattered or arranged in rows on both sides of midrib. Vesicles spherical or elliptical; cryptostomata scattered; stalk terete, shorter than the vesicles themselves (Plate 11).

Plant monoecious. Receptacles androgynous, terete to slightly compressed at upper portion, solitary or forked two to three times, warty, without spines or with a few spines, usually pseudozygocarpic with vesicles.

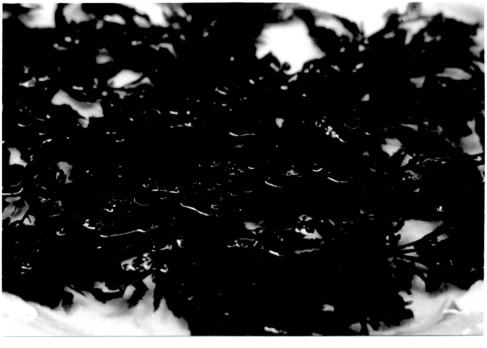


Plate 1: *Sargassum oligocystum* Montagne

Plate 2: *Sargassum oligocystum* Montagne PSM2865

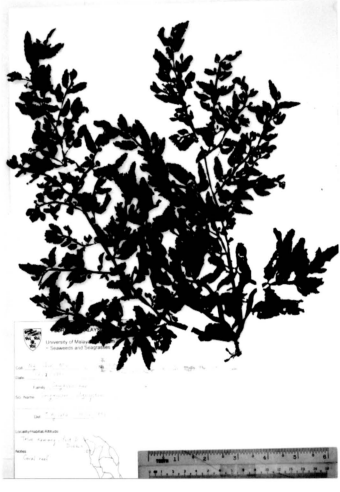


Plate 3: *Sargassum oligocystum* Montagne PSM2866

Plate 4: *Sargassum oligocystum* Montagne PSM2868

Plate 5: *Sargassum oligocystum* Montagne PSM2869

4.1.2 *Sargassum binderi* Sonder

(Plate 6 - 10)

Holdfast discoid. Stem terete, warty. Primary branches flattened or compressed, smooth, producing distichously secondary branches. Lower leaves large lanceolate, simple, with rounded apices, margin entire to dentate with small teeth, midrib indistinct, vanishing below their upper parts or evanescent, small crytostomata scattered. Upper leaves on the secondary branches and branchlets slender lanceolate, simple, with sharp apices, margin dentate with sharp teeth, midrib vanishing near apices, small crytostomata scattered. Vesicles spherical to elliptical, often mucronate at the apex; stalks flattened, usually longer than the vesicles (Plate 11).

Plant androgynous. Receptacles flattened, often twisted, with sharply dentate at their margin, simple to furcate once, racemosely arranged, clustered.

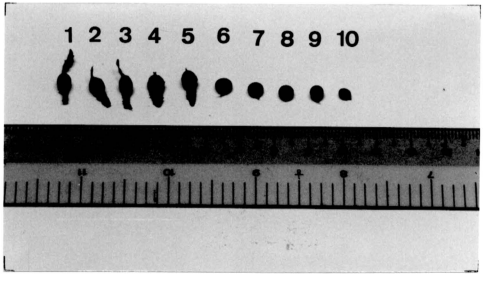
Plate 6: *Sargasusm binderi* Sonder

Plate 7: *Sargassum binderi* Sonder PSM2872

Plate 8: *Sargassum binderi* Sonder PSM2873

Plate 9: *Sargassum binderi* Sonder PSM2874

Plate 10: *Sargassum binderi* Sonder PSM2875



1-5 : Vesicles of *Sargassum binderi* Sonder
6-10: Vesicles of *S. oligocystum* Montagne

Plate 11: Vesicles of *Sargassum binderi* Sonder and *S. oligocystum* Montagne

4.2 Preparation of samples

Clean healthy plants, free of epiphytes and with receptacles where possible were collected. Only clean, healthy leaves and young shoots were used for DNA extraction. The samples were washed using diluted seawater (3 seawater : 1 tap water). A baby tooth brush with soft and fine plastic bristles was used to brush away sand, silt, debris, contaminants and epiphytes such as diatoms, fungus and bacteria which were attached to the surface of the leaves. The leaves were then transferred to a basin containing diluted seawater (2 seawater : 1 tap water). A second cleaning process was carried out. Finally, the cleaned samples were rinsed once with ultra pure deionised water to totally get rid of contaminants. The samples were then stored at -20°C.

4.3 DNA extraction

4.3.1 Protocol 1

Three grams of frozen seaweed were ground into fine powder in liquid nitrogen in a chilled mortar and pestle. The powder was scraped directly into 50 ml of 1x CTAB (cetyltriammonium bromide) [1% (w/v) CTAB, 50 mM Tris (pH 8.0), 10 mM EDTA (pH 8.0), 0.7 M NaCl, 1% PVP] extraction buffer and then shaken vigorously in order to complete the hydration of the tissues. The solution was then incubated for 15 min at 65°C. The liquid was transferred into a new tube and an equal volume of chloroform-isoamylalcohol (24:1) was then added. The solution was mixed to form an emulsion and the tube was centrifuged at 10,000 r.p.m. for 1 min. The top phase was transferred to another clean tube and one tenth volume of 65°C 10% CTAB [10% (w/v) CTAB, 0.7 M NaCl] was added. A second chloroform-isoamylalcohol extraction and centrifugation was performed. One volume of the top phase was added to 2 volumes of CTAB precipitation buffer [1% CTAB, 50 mM Tris (pH 8.0), 10 mM EDTA (pH 8.0)] followed by gentle but thorough mixing. The tube was placed on ice for 30 min and then centrifuged at 12,000 r.p.m. for 3 min. The pellet was rehydrated in 100 µl high salt TE buffer [10 mM Tris (pH 8.0), 1 mM EDTA (pH 8.0), 1 M NaCl] and heated to 65°C for 10 min to facilitate rehydration of some pellets. After rehydration was completed, the DNA was precipitated with 2 volumes of ice-cold 95% ethanol and left at -20°C overnight. The DNA was washed in 70%

cold ethanol, partially dried and dissolved in 10 µl of TE [0.01 M Tris (pH 8.0), 0.001 M EDTA (pH 8.0)]. This method was a modification from Murray and Thompson (1980) and Rogers *et al.* (1989).

4.3.2 Protocol 2

CTAB isolation buffer (15 ml) [4% (w/v) CTAB (Aldrich), 1.4 M NaCl, 0.2% (w/v) 2-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl, (pH 8.0)] was heated in a 50 ml centrifuge tube in a 60 °C water bath. Three grams of frozen seaweed sample were ground to a fine powder as in Protocol 1. The powder was scraped directly into the preheated buffer and swirled gently to mix. The sample was then incubated at 65 °C for 45 min with occassional gentle swirling. An equal volume of chloroform-isoamyl alcohol (24:1 v:v) was added and mixed gently but thoroughly. The tube was then centrifuged at 1600 r.p.m. at -4 °C for 2 min. The top phase was transferred to a clean centrifuge tube. 2/3 volumes of ice cold isopropanol was added into the tube and gently mixed to precipitate the nucleic acids. A glass hook was used to spool out the DNA. The DNA was then washed with 76% ethanol (with 10 mM ammonium acetate). The tube was centrifuged at 3200 r.p.m. for 10 min after a minimum of 20 min of washing at -20 °C. The supernatant was carefully poured off and the pellet was allowed to air dry at room temperature. The pellet was then resuspended in 0.5 ml TE [10 mM Tris-HCl, 1 mM EDTA (pH 8.0)]. This method was a modification from Doyle and Doyle (1990).

4.4 Gel electrophoresis

Gel electrophoresis was carried out using 0.8% agarose gel (Life Technologies) in 1X TBE (SSS mM Tris-borate, SSS mM EDTA). For each sample about 20 µl crude DNA was put into the well together with 1 µl 100 bp or 1 µl 1 kb marker. Electrophoresis was performed at a constant voltage of 70 V for 3 hours. The gel was then stained with 0.2 mg ml^{-1} ethidium bromide and observed under a Spectroline Model TVC-312A variable intensity UV transilluminator (312 nm). Photograph was taken by a MP-4 polaroid camera system over the UV transilluminator (312 nm) using 665 instant pack films.

4.5 Spectrophotometry

The purity of the isolated DNA was measured using a Shimadzu UV-visible recording spectrophotometer at 260 nm and 280 nm. The $A_{260/280}$ ratio was given as below:

$$A_{260/280} \text{ ratio} = A_{260} (A_{280})^{-1}$$

The amount of the isolated DNA can be calculated by the formula below:

DNA amount ($\mu g\ ml^{-1}$) = A_{260} x dilution factor x 50

(Spectrophotometric conversions : 1 A_{260} unit = 50 $\mu g\ ml^{-1}$ of double-stranded DNA)

4.6 Polymerase Chain Reaction (PCR)

4.6.1 Optimisation of Random Amplified Polymorphic DNA - Polymerase Chain Reaction (RAPD-PCR)

RAPD-PCR amplifications were carried out in 0.5 µl microfuge tube which contained 2.5 µl 10x PCR buffer [20 mM Tris-HCL (pH 8.4) and 500 mM Kcl], dNTP [dATP, dCTP, dGTP and dTTP], random primer [Operon Kits A, K and N, Operon Technologies, California, USA], genomic DNA, $MgCl_2$, Taq polymerase and sterilised distilled water to make up to a total volume of 25 µl. For negative control of PCR, the same condition without DNA templates was carried out.

Optimisation of RAPD-PCR amplification was carried out as shown in Table 4.

Parameter	Variation
DNA	25 ng, 50 ng, 100 ng
Primer	5 pmol, 10 pmol
dNTP	100 µM, 200 µM
Taq DNA polymerase	1 unit, 2 unit
$MgCl_2$	1.5 mM, 2.0 mM, 2.5 mM
Number of cycles	30, 35, 40, 45, 50, 55, 60
Annealing temperature	30°C, 33°C, 36°C, 40°C, 43°C

Table 4: Optimisation of RAPD-PCR parameters

The amplification was performed in a Perkin-Elmer DNA Thermal Cycler 480 programmed for 55 cycles of 1 min denaturation at 94 °C, 1 min annealing at 30 °C, (when screening for primers, OPK 7 and OPK 9), 2 min extension at 72 °C. The annealing temperature was increased slowly to 33 °C (OPN 16), 36 °C (OPN6) and 40 °C (OPA 13) in order to avoid any mispriming that may happen.

The amplification products of 15 µl in volume were separated by electrophoresis using 1.4% agarose gel and stained with 0.2 mg ml^{-1} ethedium bromide, under constant voltage of 70 V for 3.5 hours. The gel was then photographed with a MP-4 Polaroid camera system over a UV transilluminator (312 nm) using Polaroid 665 instant pack film.

4.6.2 Screening of primers

Operon Kits A, K and N were used for this study. A total of 60 random primers were screened for DNA amplification.

The primers and their sequences are shown in Table 5.

Primer size	Primer	Primer Sequences	% of G/C
10	OPA 01	CAGGCCCTTC	70
10	OPA 02	TGCCGAGCTG	70
10	OPA 03	AGTCAGCCAC	60
10	OPA 04	AATCGGGCTG	60
10	OPA 05	AGGGGTCTTG	60
10	OPA 06	GGTCCCTGAC	70
10	OPA 07	GAAACGGGTG	60
10	OPA 08	GTGACGTAGG	60
10	OPA 09	GGGTAACGCC	70
10	OPA 10	GTGATCGCAG	60
10	OPA 11	CAATCGCCGT	60
10	OPA 12	TCGGCGATAG	60
10	OPA 13	CAGCACCCAC	70
10	OPA 14	TCTGTGCTGG	60
10	OPA 15	TTCCGAACCC	60
10	OPA 16	AGCCAGCGAA	60
10	OPA 17	GACCGCTTGT	60
10	OPA 18	AGGTGACCGT	60
10	OPA 19	CAAACGTCGG	60
10	OPA 20	GTTGCGATCC	60
10	OPK 01	CATTCGAGCC	60
10	OPK 02	GTCTCCGCAA	60
10	OPK 03	CCAGCTTAGG	60
10	OPK 04	CCGCCCAAAC	70
10	OPK 05	TCTGTCCAGG	60
10	OPK 06	CACCTTTCCC	60
10	OPK 07	AGCGAGCAAG	60
10	OPK 08	GAACACTGGG	60
10	OPK 09	CCCTACCGAC	70
10	OPK 10	GTGCAACGTG	60
10	OPK 11	AATGCCCCAG	60
10	OPK 12	TGGCCCTCAC	70
10	OPK 13	GGTTGTACCC	60
10	OPK 14	CCCGCTACAC	70
10	OPK 15	CTCCTGCCAA	60
10	OPK 16	GAGCGTCGAA	60
10	OPK 17	CCCAGCTGTG	70
10	OPK 18	CCTAGTCGAG	60
10	OPK 19	CACAGGCGGA	70
10	OPK 20	GTGTCGCGAG	70
10	OPN 01	CTCACGTTGG	60
10	OPN 02	ACCAGGGGCA	70
10	OPN 03	GGTACTCCCC	70
10	OPN 04	GACCGACCCA	70
10	OPN 05	ACTGAACGCC	60
10	OPN 06	GAGACGCACA	60
10	OPN 07	CAGCCCAGAG	70
10	OPN 08	ACCTCAGCTC	60
10	OPN 09	TGCCGGCTTG	70
10	OPN 10	ACAACTGGGG	60
10	OPN 11	TCGCCGCAAA	60
10	OPN 12	CACAGACACC	60
10	OPN 13	AGCGTCACTC	60
10	OPN 14	TCGTGCGGGT	70
10	OPN 15	CAGCGACTGT	60
10	OPN 16	AAGCGACCTG	60
10	OPN 17	CATTGGGGAG	60
10	OPN 18	GGTGAGGTCA	60
10	OPN 19	GTCCGTACTG	60
10	OPN 20	GGTGCTCCGT	70

Table 5: Sequences of RAPD primers

4.7 Data Analysis

According to the formula of Nei and Li (1979), the results obtained from RAPD-PCR data can be compared by pairwise band comparison of each sample. The coefficient of similarity, F value was obtained according to the formula below:

$$F = 2 n_{xy}(n_x + n_y)^{-1}$$

where : n_x is the total number of DNA fragments from sample X,

n_y is the total number of DNA fragments from sample Y,

n_{xy} is the number of DNA fragments that were identical in two samples X and Y

4.8 Clustering by the UPGMA method

The clustering method used was the "Unweighted Pair-Group Average" method (UPGMA) by Sokal and Michener (1958) by a NTSYS computer package (NTSYS version 1.80, Kianian, S. 1993) and the result of the clustering process was represented in the form of a dendrogram.