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Molecular differentiation of two morphological variants of *Gracilaria salicornia*

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Abstract

Xia in 1986 combined *Gracilaria salicornia*, *G. canaliculata* (*G. crassa*), *G. cacalia* and *G. minor* into one species: *G. salicornia*. Two morphological variants of *G. salicornia* were collected from different localities in Malaysia. Variant A collected from Morib, Selangor grew on the roots of *Avicennia*. The samples showed absence of main axis; segmented constrictions throughout; cylindrical or slightly compressed thalli. Variant B was collected from the mudflats of Tanjung Tuan, growing on rocks, coral or forming mats on the mud. Plants showed absence of main axis; segments were not constricted throughout the plant (if present only slightly articulated at the upper part), branching was dichotomous or irregular; cylindrical or slightly compressed thalli. The technique of Random Amplified Polymorphic DNA analysis (RAPD) was used to investigate molecular characteristics of the two variants. Out of sixty Operon primers that were screened, four primers, OPA 1, OPA 10, OPA 11 and OPK 7 were able to give polymorphism. The fingerprints generated were stable and reproducible on repeated analysis. The DNA fingerprints generated were visually analysed and clustering analysis was carried out using GelCompar 4.0. The matrix of similarities was based on the Dice coefficients (S_D) and the cluster analysis was carried out using the unweighted pair group method using arithmetic averages (UPGMA). DNA analysis showed that two primers (OPA 01, CAGGCCCTTC and OPK 07, AGCGAGCAAG) were able to differentiate the two variants.

Introduction

Over one hundred species of the commercially important agarophyte of *Gracilaria* (Gracilariaceae, Rhodophyta) are widely distributed throughout the tropical and temperate waters of the world. Proper classification is important for both cultivation and industrial exploitation of *Gracilaria.*

Gracilaria species are classified based on morphological features: branching mode, grade of constriction along thallus, shape of thallus and the reproductive structures. However, species delineation of *Gracilaria* is problematic due to the limitations of distinct morphological and reproductive characteristics (Bird, 1995). *Gracilaria salicornia* (C. Agardh) Dawson is one species with uncertain taxonomic position (Yamamoto, 1978). In 1986, Xia chose several features especially the presence of main axis and constrictions, to distinguish *G. salicornia*, *G. canaliculata* (= *G. crassa*), *G. cacalia* and *G. minor* but the features were found not constant. Therefore, Xia combined all these related species as synonyms in *G. salicornia*. Following that, Abbott (1988) suggested the following as synonyms of *G. salicornia*: *Corallopsis salicornia* (C. Agardh) Greville; *C. dichotoma* Ruprecht; *G. cacalia* J. Agardh; *G. cacalia* (J. Agardh) Dawson; *C. opuntia* J. Agardh; *C. salicornia* var. *minor* Sonder; *C. minor* (Sonder) J. Agardh; *G. minor* (Sonder) Chang et Xia; *G. crassa* Harvey ex J. Agardh; *G. canaliculta* (Kütz.) Sonder; *Sphaerococcus canaliculatus* Kütz.

Confusion in the classification of *Gracilaria salicornia* and its closely related species is due to the

absence of definitive morphological characters, made more difficult through phenotypic convergence and plasticity. Uncertainties in the classification can be overcome by using molecular techniques because they measure genetic rather than phenotypic changes (Donoghue & Sanderson, 1992). It is important to have a quick and reliable technique to confirm the identity of commercial species of *Gracilaria*. Proper classification would also allow the efficient exploitation of newly discovered species with valuable biotechnological products.

The random amplified polymorphic DNA (RAPD) technique has been used in the taxonomy and classification of seaweeds at the genus and species level, for example, *Gracilaria* species (González et al., 1996; Meneses, 1996); *Gelidium* species (Patwary et al., 1993); *Porphyra* (Dutcher & Kapraun, 1994) and *Sargassum* species (Ho et al., 1995a; b). *G. tenuistipitata* was separated from *G. chilensis* through RAPD (Meneses, 1996), although gene sequences of rDNA and *rubisco* spacer indicated high similarity (Bird, 1995). RAPD is applied in this study to understand the relationship between *Gracilaria salicornia* and its closely related species in Malaysia.

Materials and methods

Sample collection

Two morphological variants of *G. salicornia* were collected from two different localities in Malaysia. Variant A was collected from Morib, Selangor while Variant B was collected from Tanjung Tuan, Negeri Sembilan. Individual plants were kept in separate plastic bags without water, transported to the laboratory in an icebox within fours hours after collection. Samples were kept frozen at –20 °C.

DNA extraction

The algal samples were washed in filtered seawater to remove all epiphytes and debris. The washed samples were air-dried in the air-conditioned culture room or by using silica gel. The air-dried samples (1–3 g) were ground with liquid nitrogen using mortar and pestle until powder form for DNA extraction.

The sample was suspended in 30 mL ice cold protease buffer (0.1 M Tris base, pH 8.0; 0.1 M Na_2EDTA; 0.25 M NaCl; 100 μg ml^{-1} proteinase K; 1% N-Laurosyl-sarcosine sodium salt). The mixture was gently swirled in a 65 °C water bath for about an hour. One half volume of sevac (24:1: chloroform: isoamyl alcohol) was added and mixed well. The mixture was centrifuged at 3000 × g for 10 min, at 4 °C. The upper aqueous layer was transferred to a new polypropylene tube.

One-tenth volume of 10% w/v CTAB (cetyltrimethylammonium bromide) was added to each aqueous phase and residue waste and incubated for one hour at 65 °C with gentle shaking. Sevac solution was added into each tube and mixed thoroughly for 5 min at room temperature. The sample was then centrifuged (3000 × g) for 10 min. The aqueous layer from each tube was transferred to a new tube and the extraction step with 10% w/v CTAB was repeated. The final aqueous phase from CTAB treatment was transferred to a clean tube and an equal volume of phenol/sevac (1:1) solution was added and mixed thoroughly by inverting the tubes manually for 15–20 min. The mixture was then centrifuged (3000 × g) for 20 min. The aqueous layer was transferred to a clean tube and the sevac treatment was repeated for another two times.

Ice cold isopropanol (2X volume) was added to the final clear aqueous phase (which sometimes might appear slightly pink or yellow in colour) to precipitate the DNA. The DNA was either spooled or spun down at top speed for 30 min. The pellet DNA was washed with washing buffer (70% v/v ethanol, 10 mM ammonium acetate). The DNA pellet was then air-dried and dissolved in TE (Tris-EDTA) buffer. The quantity and quality of the extracted DNA was determined spectrophotometrically.

Random amplified polymorphic DNA (RAPD)

Polymerase chain reaction (PCR) amplification was performed in a final volume of 25 μl containing 1.5 unit of *Taq* polymerase (GIBCO BRL), 2.5 μl of 10 X *Taq* DNA polymerase buffer, 0.2 mM of dNTP (dATP, dGTP, dCTP, dTTP), 10 pmol primer (Operon Technologies, California, USA), 25ng of genomic DNA and 2.0 mM $MgCl_2$. Amplification was performed in a Biometra UNO II thermocycler programmed at 95 °C for two minutes denaturation and followed by 45 cycles of one minute denaturation at 94 °C, one minute annealing at different temperature (depend on the type of primer) and two minutes of extension at 72 °C and further extension at 72 °C for five minutes. Twelve microlitres of the reaction products were separated by electrophoresis through 1.8% agarose gels and stained with ethidium bromide. Agarose gels were photographed with a MP-4 Polaroid camera over a UV

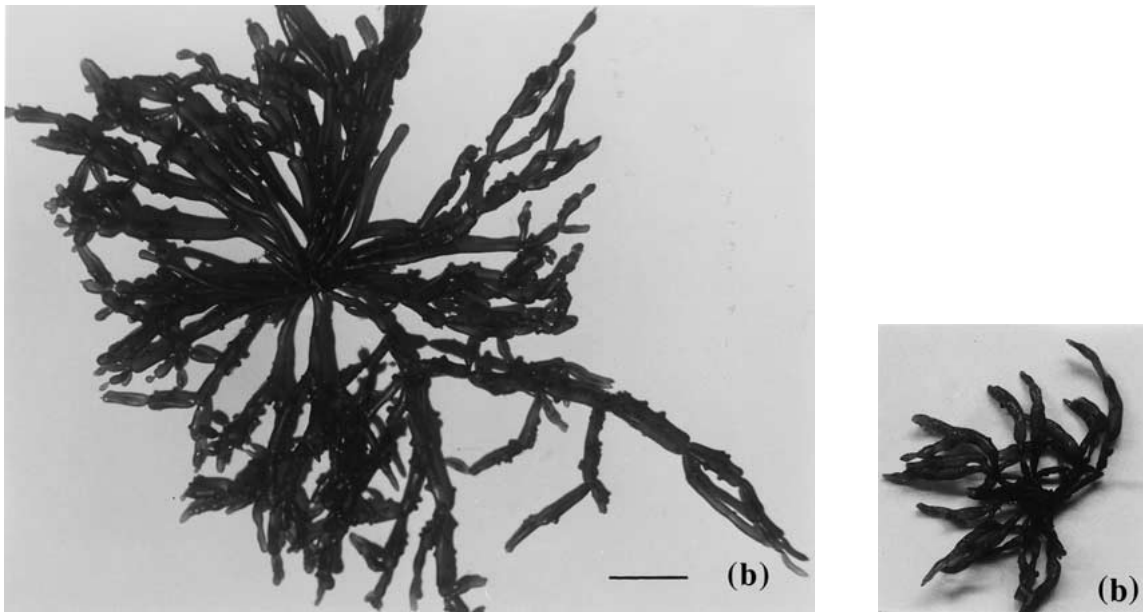

Figure 1. a) Variant A collected from Morib, Selangor. b) Variant B collected from Tanjung Tuan, Port Dickson. Scale bar = 1.5 cm.

transilluminator (312nm) using Polaroid 665 instant pack film.

RAPD analysis

RAPD data analysis was carried out using the Gelcompar software version 4.0 (Kortrijik, Belgium). The calculation of the matrix of similarities was based on the Dice coefficients (S_D) and the clustering was carried out using the unweighted pair group method using arithmetic averages (UPGMA).

$$S_D = \text{ coefficient of similarity, } S_D = \frac{2(n_{xy})}{n_x + n_y}$$

n_x = total number of DNA fragments from sample X
n_y = total number of DNA fragments from sample Y
n_{xy} = number of DNA fragments identical in the two samples.
If S_D is equal to 1, this indicates that the two samples are similar, but if S_D = 0, then the two samples are totally dissimilar.

Results

Morphology

The collected samples were divided into two variants based on the branching mode and presence of constriction on the thallus. Variant A (Figure 1a) which was collected from Morib, Selangor grew on the roots of *Avicennia* in a mangrove forest. The samples showed absence of main axis, thalli were segmented and constricted throughout, branches were cylindrical or slightly compressed. The colour ranged from greenish to brownish. Variant A is *Gracilaria salicornia* (C. Agardh.) Dawson C.J. based on Xia (1986).

Variant B (Figure 1b) was collected from Tanjung Tuan, Negeri Sembilan. The natural habitat for this variant is a muddy area where the plants grow on rocks, corals or formed mats on the mud. No main axis was observed and the thalli were not constricted throughout the plants (if present only slightly articulated at the upper parts). The branching was dichotomous or irregular; the branches were cylindrical or slightly compressed. The colour ranged from brownish to reddish. Variant B is *G. crassa* Harvey ex J. Agardh = *G. canaliculata* (Kütz.) Sonder, based on Xia (1986).

DNA isolation

The yield of DNA extracted from all samples ranged from 138–206.5μgg^{-1} and the purity (OD_{260}:OD_{280}) ranged from 1.685–1.863. Sixty random primers (Operon Technologies, California, USA) were screened for DNA amplification using template DNA from the two variants of *Gracilaria salicornia*. Four random primers: OPA 1 (CAGCCCTTC), OPA 10 (GTG-

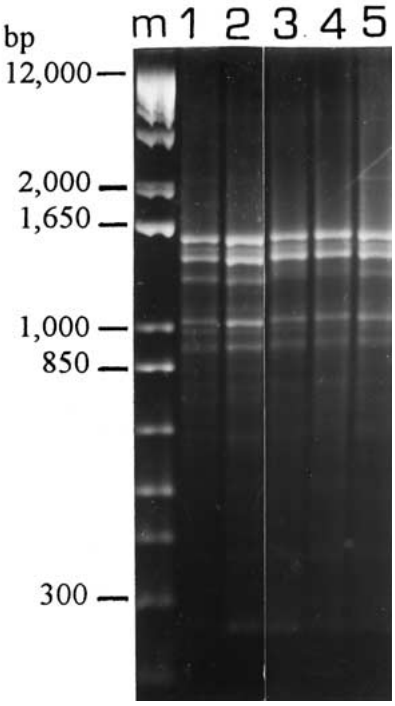

Figure 2. Assessment of reproducibility of RAPD performed on multiple, identical DNA samples from Variant A (MB 16) using OPA 11 (lanes 1–5).

ATCGCAG), OPA 11 (CAATCGCCGT) and OPK 7 (AGCGAGCAAG) were able to generate reproducible amplification products. To assess the reproducibility of RAPD, PCR amplifications were performed on multiple identical samples. The DNA fingerprints that were generated by PCR showed good reproducibility although some minor variability was noted (Figure 2).

Random amplified polymorphic DNA(RAPD)

The optimised conditions for each of the primers are shown in Table 1. In general, the size of amplicons ranged from 300 bp to 5000 bp for all the four primers (Figure 3). The number of fragments generated ranged from two to twelve (Figures 3). There were two common bands, 1,600 bp and 1550 bp generated by primer OPA 11 (Figure 3) from both the Variants A and B. Primers OPA 1 and OPK 7 gave one common band each, 2000 bp and 850 bp, respectively.

Relationship between variants

The degree of similarity, S_D (coefficient of similarity) between the two variants was calculated by using the GelCompar 4.0. Different primers gave different values of S_D. The ranges of S_D values varied from one primer to the other primer (Table 2). The percentage of $S_D > 0.5$ were very low for all the primers (Table 2). Out of the four primers OPK 7 gave the lowest value of S_D between the two variants.

Cluster analysis was performed to generate dendrograms constructed by each of the four primers. Out of the four primers, OPK 7 (Figure 4) was the best primer to differentiate the two variants, followed by OPA 1 in which only MB 20 (Variant A) was included in the cluster of samples from Tanjung Tuan (Variant B). The dendrogram generated by primer OPA 10 divided the samples into two clusters in which Group 2 comprised of Variant B samples only and Group 1 consisted of Variant A samples and two samples of Variant B. OPA 11 (Figure 5) generated two clusters in which Group 2 consisted of one sample of Variant B and the rest were Variant A, while group 1 only comprised Variant B samples. MB18 was obtained as an outgroup.

Discussion

Variant A (*G. salicornia*) was distinctly separated from Variant A (*G. crassa*) by the presence of regular constrictions along the thallus. The difficulties in using morphological features as discussed by Xia (1986) were not encountered in these samples.

The DNA isolation of *Gracilaria salicornia* involved both CTAB and phenol-chloroform. The percentage of CTAB used was high compared to the DNA extraction for higher plants because *Gracilaria* is an agar producer and is rich in polysaccharides. 10% of CTAB (w/v) was used to remove the polysaccharide which inhibit amplification. Ho et al. (1995a) used CTAB only in the extraction DNA from *Sargassum* and obtained the highest purity (OD_{260}: OD_{280}, 0.83–1.25) compared to the other three protocols used. The *Sargassum* used here contained lower polysaccharide content than *Gracilaria*. In the protocol used in this paper, incubation in the protease buffer was a crucial step in which the mixture was gently swirled throughout the incubation period to ensure that the sample had a wide surface area (not clumped together) to interact with the solution. This is to ensure that the materials were properly digested to facilitate the following steps to remove all the contaminants that will affect the purity of the DNA. We found that it is not by increasing the quantity of the starting material that will increase the yield and quality of DNA but the efficiency of

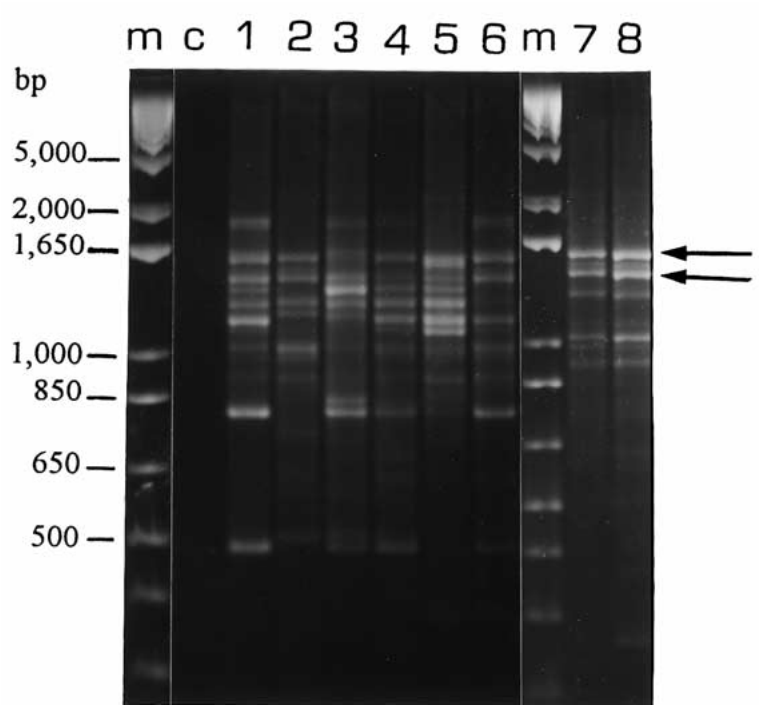

Figure 3. PCR amplification of genomic DNA from *Gracilaria* spp. with primer OPA 11. (m, Marker, 1kb plus DNA marker; c, control (without template DNA in the PCR amplification); lane 1, TT 23; lane 2, TT 24; lane 3, TT 25; lane 4, TT 26; lane 5, TT 27; lane 6, TT 28; lane 7, MB 15; lane 8, MB 16). TT = Tanjung Tuan; MB = Morib; ⟵ indicates the common band.

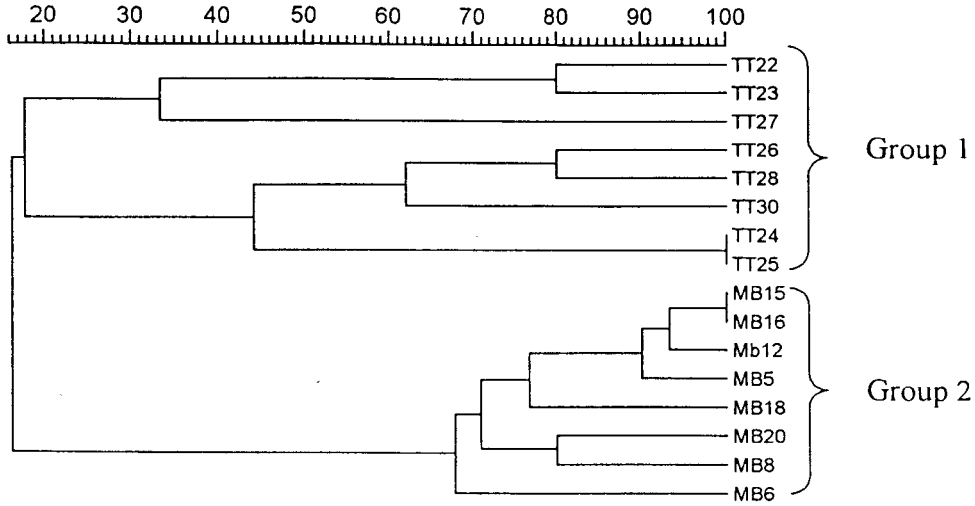

Figure 4. Dendrogram generated by primer OPK 7. MB = samples from Morib; TT = samples from Tanjung Tuan.

Table 1. Summary of optimised conditions for the 4four primers selected for further amplification of genomic DNA of *Gracilaria salicornia*

Optimised condition	Primer			
	OPA 1	OPA 10	OPA 11	OPK 7
Mg^{2+}	2.0 mM	2.5 mM	2.0mM	2.0 mM
Taq polymerase (a)	1.5 U	1.5 U	1.5 U	1.5 U
dNTP	0.2 mM	0.2 mM	0.2 mM	0.2 mM
Annealing temperature	36 °C	34 °C	40 °C	38 °C
DNA	25 ng	25 ng	25 ng	25 ng
Primer	10 pmol	10 pmol	10 pmol	10 pmol
Ramp time (b)	dT= 1 °C s^{-1}	dT= 4 °C s^{-1}	dT= 1 °C s^{-1}	dT= 4 °C s^{-1}

(a) GIBCO BRL; (b) Biometra UNO II.

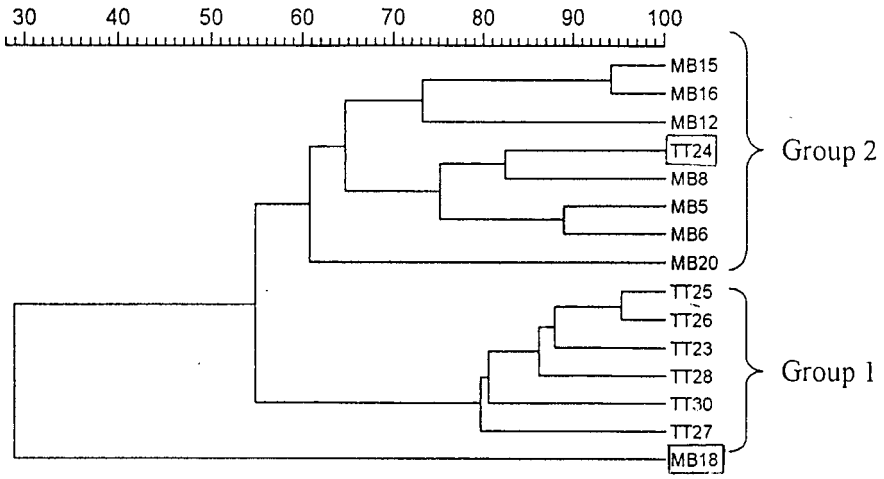

Figure 5. Dendrogram generated by primer OPA 11. MB = samples from Morib; TT = samples from Tanjung Tuan.

handling this step of extraction. Thorough washing is necessary to remove biological contaminants such as epiphytes and debris. In addition, the adelphoparasites, *Congracilaria* spp. (Yamamoto & Phang, 1997) were removed while plants containing endoparasites (George, 1998) were discarded after microscopic inspection. This was to ensure minimal contamination of the *Gracilaria* with DNA from other organisms.

RAPD fingerprints were reproducible when repeated analysis using multiple, identical samples was carried out. It was found that the optimised conditions for different primers were affected by the concentrations of template DNA, annealing temperatures, concentrations of Mg^{2+} and also the ramp time. The ramp time, which is the time taken to change from one temperature to the next, also affected the reproducibility and intensity of the band profiles. In this study different primers were shown to work best at different annealing temperatures, that is 34 °C, 36 °C, 38 °C and 40 °C. The concentration of Mg^{2+} also affected the intensity of the bands obtained and it was found that the best concentration of Mg^{2+} for the RAPD was at 2.0 mM for OPA 1, OPA 10 and OPK 7 and 2.5 mM for OPA 11. In this study, for example the

Table 2. Range of S_D values between RAPD profiles of Variants A and B, for four primers (OPA 1, OPA 10, OPA 11 and OPK 7)

Primer	OPA 1	OPA 10	OPA 11	OPK 7
S_D	0.333–0.588	0.200–0.696	0.143–0.889	0.000–0.400

Percentage of coefficient similarity between the two variants:
OPA 1, $S_D > 0.5 = 35.94\%$.
OPA 10, $S_D > 0.5 = 20.31\%$.
OPA 11, $S_D > 0.5 = 34.92\%$.
OPK 7, $S_D > 0.5 = 1.56\%$.

optimum ramp time for primer OPK 7 was 4 °Cs^{-1}, but if the ramp time was changed to 1°Cs^{-1}, there was no amplification. This phenomenon was reported by Schweder et al. (1995).

RAPD is a useful tool for determining whether the two morphological structures of the two variants are due to plasticity (habitat differences) or genetic differences. The low Dice coefficient of similarity, S_D, showed that the two variants were not identical. However, within the same variant, there were some samples in which the S_D = 1, such as MB5, MB6 and MB 8 which shown to be identical by OPA 1; MB 15 and MB 16 (OPA 10); TT 24 and TT25 (OPK 7); MB 15 and MB 16 (OPK 7). Cluster analysis showed that OPK 7 (Figure 4) was the best primer to differentiate the two variants, followed by OPA 1. Overall, the four primers were able to separate the two variants into two distinct clusters. Very few samples were grouped out of the cluster such as with primer OPA 10, samples from Tanjung Tuan (TT 25 and TT 24) were clustered together with samples from Morib; while with primer OPA 11 (Figure 5), TT 24 from Tanjung Tuan was clustered together with samples from Morib; and MB 18 was clustered as an outgroup from the two variants. Some genetic differences within each variant was observed (range of S_D, Table 2). This accounts for the presence of one variant in the cluster of the other variant. Different primers gave different percentage of similarity (S_D) for the two clusters. The percentage similarity between two separated clusters (that is the two variants) for primer OPA 1, OPA 10, OPA 11 and OPK 7 were 48%, 41%, 54% and 16% respectively. The four primers produced $S_D > 0.5$ and their efficiency in separation of the two variants, in decreasing order is: OPK 7 > OPA 10 > OPA 11 > OPA 1. Out of sixty primers tested only four primers were able to generate polymorphic fingerprints. Of the four primers, OPK 7 (lowest S_D) was the best primer to separate the two variants. In another study of *Gelidium vagum* Okamura, 165 primers were used to screen their usefulness in generating polymorphism but only 37 primers were able to generate polymorphic fingerprints (Patwary et al., 1993). Thus the higher numbers of primers being tested, the higher possibility of getting the best primers for the study of genetic relationship of *Gracilaria salicornia* and closely related species.

In conclusion, RAPD is a fast and useful technique in the study of the genetic relationship of *Gracilaria salicornia* and closely related species. The RAPD-DNA fingerprints were reproducible when precautions were taken with parameters such as concentrations of template DNA, Mg^{2+}, *Taq* polymerase and annealing temperature as reported by other researchers (Devos & Gale, 1992).

RAPD analysis showed that the morphological differences of Variant A and Variant B are genetically based. Branching and constriction in the thallus are important taxonomic criteria. Variant A (*G. salicornia*) grow attached to the roots of *Avicennia* while Variant B (*G. crassa*) grow in between rocks and corals in muddy area. The latter habitat is harsh and does not offer a uniform surface like the mangrove roots, hence the irregular branching and constricted thalli of Variant B. Over time these differences have resulted in genetic differences. Further studies using RAPD on the two other species: *G. minor* and *G. cacalia* which were also combined into *G. salicornia*, and gene sequencing are being conducted to complete the taxonomic clarification of *G. salicornia* and closely related species.

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