CHAPTER 5: DISCUSSION

5.1 TAXONOMIC STUDIES BASED ON MORPHOLOGY

The genus *Caulerpa* is characterized by a lack of transverse cell walls and the presence of internal cell wall ingrowths called trabeculae, which provides the thallus with mechanical support (Menzel, 1987). Thought it is unicellularity, the *Caulerpa* plant body shows a complex external morphology, they will differentiated into creeping stolons, rhizophores with rhizoid clusters, and erect assimilators. The assimilators usually bear numerous branchlets termed ramuli. It is based on the morphology of these assimilators that *Caulerpa* species are named (Weber-van Bosse, 1898; Svedelius, 1906; Børgesen, 1907; Nizamuddin, 1964; Calvert, 1976; Ohba and Enomoto, 1987; Coppejans and Meinesz, 1988; Coppejans, 1992; Coppejans and Prud'homme van Reine, 1992). Although the definition of these criteria was complex, all specimens collected in this study were classified according to the assimilators' morphology.

Caulerpa serrulata var. *serrulata* and *Caulerpa serrulata* var. *boryana* collected from Pulau Redang can be classified as a distinct species on its own without much doubt since its morphological features clearly sets it apart from the rest of the *Caulerpa* specimens found in Pulau Redang. The morphological identification was easy without any confusion with *Caulerpa serrulata* var. *serrulata*'s branches spiral upward while *C. serrulata* var. *boryana*'s branches are linear and serrated on their edges.

Studies have shown that the different varieties of *Caulerpa racemosa* are due to environmental influences (Svedelius, 1906; Børgesen, 1907; Peterson, 1972; Coppejans and Meinesz, 1988; Coppejans, 1992; Coppejans and Prud'homme van Reine, 1992). According to Taylor (1960), *Caulerpa racemosa* is a popular, ubiquitous species that is among the most variable in its variable genus. Studies done by Eubank (1946),

Coppejans (1992) and Meinesz *et al*. (1995) supported Taylor's description by emphasizing that considerable environmental plasticity and temporal variation in the morphology of tropical species in particular make species boundaries difficult to define. Under differing light conditions, *Caulerpa racemosa* and *C. lentillifera* show variations in morphology. Normally, under favourable light conditions – which are good illumination for *Caulerpa racemosa* and somewhat less strong illumination for *Caulerpa lentillifera* – both bear many vesicles densely arranged around the axis of the assimilator, giving it a cylindrical appearance. However, when growing in dim light, both develop assimilators which bear much less vesicles that are arranged in one plane only, giving the assimilator a flattened look; this is the so-called 'var. *lamourouxii*' (Turner) Weber-van Bosse of *Caulerpa racemosa* and a parallel, very similar form in *Caulerpa lentillifera* (Lipkin, 1971). The close association between light intensity and morphology has already been demonstrated by other studies (Peterson, 1972; Calvert, 1976; Ohba and Enomoto, 1987; Collado-Vides, 2002). However, there are not much study being conducted on the varieties of *Caulerpa serrulata*, hence molecular analyses was conducted to examine the different varieties is due to the response from the environment or there is genetic basis.

5.2 SAMPLE COLLECTION AND SPECIMEN PROCESSING

Upon collection of the samples from Pulau Redang, Port Dickson, they were treated immediately by drying in silica-gel to prevent any decrease in DNA quality. *Caulerpa* specimens collected for this study were found on corals and sandy areas. The occurrence of the *Caulerpa* specimens in such habitats renders contaminations due to salt, epiphytes, animals, sand and mud unavoidable. Hence, in order to attain high purity of DNA, the specimens must be stringently selected and carefully washed. Sand, mud

and parasites were scrapped off the plants gently by using forceps. Plants that were heavily contaminated were disposed. *Caulerpa* has been long recognized to harbour endosymbiotic and epiphytic bacteria, which may be associated with various metabolic functions including nitrogen fixation and / or the synthesis of various toxic compounds (Meusnier *et al.*, 2001). Moreover, ubiquitous epiphytic bacteria have been observed on the upper surfaces of many types of seaweed (Provasoli and Pintner, 1980; Shiba, 1992). Since the bacterial epiphytes cannot be spotted by the naked eye, the *Caulerpa* specimens used for this study were checked randomly under the microscope to minimized unnecessary contamination of the DNA extracted. Hence, after an initial round of washing, the specimens were rinsed with distilled water and deionised (UHQ) water to prevent any bacterial contamination.

The samples were then placed in plastic bags with silica gel which plays a vital role in the drying of specimens and the reduction of water content. This is essential as water will form ice crystals which will disrupt DNA during the liquid nitrogen grinding procedure in DNA extraction.

5.3 MOLECULAR ANALYSES

5.3.1 Deoxyribonucleic Acid (DNA) Extraction

The coenocytic nature of *Caulerpa* that permits extraction of thousands of nuclei after disrupting a single cell (Staves and La Claire, 1985), lack significant quantities of polyphenolic compounds that interferes with nucleic acid purification from brown algae and many higher plants (John, 1992) and less abundant in polysaccharides that may hinder isolation of DNA make extraction of *Caulerpa* DNA fairly easy in comparison to other types of algae.

As such, the isolation of *Caulerpa* DNA using DNeasy Plant Mini Kit (Qiagen, Germany) was sufficient to obtain high purity of DNA for PCR amplifications of specific genes for sequencing purposes. Nevertheless, to ensure high yields of DNA in terms of quantity and quality were obtained each time, the dried samples were ground until powder form. High DNA purity is essential to obtain reliable results especially on the reproducibility of polymerase chain reaction (PCR) amplification. The purity of DNA is essential for the reproducibility of RAPD fingerprints. In the study done by Mizukami *et al.* (1998), results of RAPD were not reproducible even though low purity of DNA can produce RAPD fingerprints. DNA purification is therefore very important to obtain reliable results.

5.3.2 Polymerase Chain Reaction (PCR) for Amplification of tufA Gene

For the purpose of phylogenetic studies, amplifications of *rbc*L gene, *tuf*A gene or *ycf*10-*chl*B chloroplast spacers were available alternatives. However, *tuf*A gene was selected for this study as previous studies by Ludwig *et al*. (1990), Delwiche *et al.* (1995) and Baldauf *et al.* (1996) had shown that phylogeny inferred from *tuf*A sequences was useful in resolving phylogenetic relationships even at species levels. Furthermore, *tuf*A gene had proven to be more variable (giving higher resolution on the phylogenetic trees) compared to *rbc*L gene.

For the optimization of PCR amplification parameters, repeated trial runs of PCR amplification had shown that the optimum annealing temperature was 52℃ as used in this study.

5.4 PHYLOGENETIC ANALYSES OF *Caulerpa* SPECIES BASED ON *tufA* GENE

This study represents an estimate of phylogenetic relationships within the genus *Caulerpa*, based on the analysis of *tuf*A sequences. The analysis revealed the existence of species-poor ancient lineages and a rapidly diversifying clade. The highest genetic divergence between the outgroup used, *Caulerpella ambigua* and all *Caulerpa* species supports the taxonomic distinction of *Caulerpella* proposed by Prud'homme van Reine and Lokhorst (1992). *Caulerpella ambigua* differs from *Caulerpa* by its nonholocarpic mode of reproduction, although it shares most anatomical characters with its sister genus, for example, presence of trabeculae, coenocytic thalli, stoloniferous habit with rhizoids and branched vertical axes.

MP and BI trees have very similar topologies with exception of some variations in bootstrap values for a few clades, although BI analysis provides a stronger support for some major clades. Based on the phylogenetic analyses, it is confirmed that the *Caulerpa serrulata* var. *serrulata* and *Caulerpa serrulata* var. *boryana* are the same species despite of the differences in the morphological appearance. Hence we accept the null hypothesis, H_0: The *Caulerpa serrulata* var. *boryana* and *Caulerpa serrulata* var. *serrulata* are similar species.

CHAPTER 6: CONCLUSIONS

Due to the rampant morphological plasticity exhibited by *Caulerpa* species, morphologically-based identification of the specimens collected from Pulau Redang, Terengganu proved to be questionable. The classification of the remaining species remains rather vague as whether the differences in external morphology are due to plasticity of the species or genetic based. Hence, molecular approach is needed to clarify any uncertainties in the classification of species based on morphology.

Based on the combination of morphological and molecular analyses, hence we accept the null hypothesis, H_0: the *Caulerpa serrulata* var. *boryana* and *Caulerpa serrulata* var. *serrulata* are similar species. This is due to the distinct morphological characteristics that set both *Caulerpa serrulata* var. *boryana* and *Caulerpa serrulata* var. *serrulata* and also the independent clade which they formed in the phylogenetic trees generated.

DNA extraction protocol using DNeasy plant mini kit is suffice to isolate the DNA from the various specimens as the genus *Caulerpa* do not contain much polyphenolic compounds and polysaccharides that may hinder DNA extraction. This method proved not only to be less time consuming than the conventional method but it is also just as efficient since DNA of high quality and quantity was successfully isolated.

*tuf*A gene proved to be an excellent candidate for taxonomic studies as previous studies had also shown it was useful in resolving phylogenetic relationships and produces more variable results as opposed to other more conservative genes, such as the *rbc*L gene.

This study emphasizes the crucial role played by physical characteristics of assimilators in the identification of species within the genus *Caulerpa*. These assimilators, however, can be highly plastic and seem under strong control of the environment (Gilbert, 1941; Calvert, 1976; Ohba *et al.*, 1992). Therefore, species boundaries, species relationships and sectional divisions are dubious. This is where phylogenetic analysis comes in to confirm identities and genetic relationships among species.