CHAPTER 2: LITERATURE REVIEW

2.1 ALGAE

The algae are defined as thallophytes with the lack of the specialized reproductive structures found in plants. They share similar features with the plant division Bryophyta, such as absence of true roots, stems, and leaves.

Algae are autotrophic organisms other than plants, comprising a group of 30,000 to 40,000 different and described living organisms (Norton *et al.*, 1996). Several characteristics are used to classify algae such as the nature or type of pigments present, the carbon reserve, polymers produced, the cell wall structure and the type of motility. Every alga contains chlorophyll *a* and also other types of chlorophylls and pigments. The occurrence of these additional pigments is characteristic of particular algal groups (Madigan *et al.*, 2003). These pigments enable the algae to use light energy for photosynthesis.

Algae range in size from microscopic, unicellular organisms to huge seaweeds that can grow up to 300 ft (100 m) long. Inhibit the world's oceans, sea shores and coasts, freshwaters, and there are also terrestrial forms on soils, rocks and trees. Both prokaryotic and eukaryotic taxa are included in the algae.

There are three types of double- membrane- bound organelles in the eukaryotic algal cells; the nucleus, the chloroplast, and the mitochondrion. Most algal cells have only one nucleus whereas some others are multinucleate; among these multinucleate algal cells, some algae have many nuclei which are not separated by cell walls. They are described as "siphonaceous".

Algae regenerate by sexual, asexual, or by both reproduction ways. Large algae reproduce by spores; some red algae produce non-felagellated monospores which are walled spherical cells and are carried by water currents and upon germination produce a new organism. Some green algae produce aplanospores which do not have motility. Many smaller algae reproduce asexually through ordinary cell division or by fragmentation (Rangaswami and Bagyaraj, 2004).

The body of the vegetative form of algae is called “thallus” which for singled-cell algae, is just a single cell while for multi- celled algae, the thallus includes the entire organism.

Algae are not considered as a phylogenetic concept but have a significant role in economics. Since it is thought that all major phyla and divisions of animals and plants arose in the sea, it is there that scientists can find representatives of many ancient evolutionary lineages. Thus, it is essential to investigate the marine algae to understand the diversity and phylogeny of the plant world.

2.2 ALGAE CLASSIFICATION

There are altogether six major classes of algae, comprising of Rhodophyta (red algae), Phaeophyta (brown algae), Chrysophyta (golden algae), Bacillariophyta (diatoms), Chlorophyta (green algae) and Ulvophyceae (green algae) (Madigan *et al.*, 2003).

Nowadays, nomenclature is managed by the Nomenclature Codes, which allow names to be divided into an indefinite number of ranks. The standard botanical classification system used in the systematics of algae is as follows:

Phylum -phyta

Class -phyceae

Order -ales

Family -aceae

Genus

Species

The presence of photosynthetic pigments and absence of a vascular system or the presence of a chemical nature and the absence of a structural component make algae differentiated from other phyla.

Algae do not constitute a formal taxonomic group of organisms, but represent a loose collection of divisions or phyla with representatives. The divisions are distinguished from each other based on a combination of characteristics, such as photosynthetic pigments, starch-like reserve products, cell covering, and other aspects of cellular organization. (Van den Hoek *et al*., 1995; Sze, 1998; Graham and Wilcox, 2000). However the divisions are based primarily on the types of photosynthetic pigment.

The classification of algae is changing rapidly because new taxonomic information is being discovered. Many more than three groups are now recognized, each sharing a common set of pigment types. Algae are not closely related to each other in an evolutionary sense.

- Bacillariophyta (diatoms)
- Charophyta (stoneworts)
- Chlorophyta (green algae)

- Chrysophyta (golden algae)
- Cyanobacteria (blue-green algae)
- Dinophyta ("fire algae", dinoflagellates)
- Phaeophyta (brown algae)
- Rhodophyta (red algae)

2.3 CHLOROPYTA (Green Algae)

Chlorophyta is formed from two Greek root words that mean green (chloros); and plant (phyto). The reference is to the typical colour of members of the phylum. The division Chlorophyta consists of four classes such as Prasinophyceae, Chlorophyceae, Ulvophyceae and Charophyceae (Sze, 1986).

The green algal genus *Caulerpa* is a widespread benthic inhabitant of intertidal and subtidal zones of (sub)-tropical coastal waters (Lisette *et al.*, 2003). The green algae have a wide ecological range, growing in the oceans, freshwaters and moist terrestrial habitats. The Chlorophyta are primarily freshwater; 90% are freshwater and only about 10% of the algae are marine (Smith, 1955). Some orders are predominantly marine (Caulerpales, Dasycladales, Siphonocladales), whereas others are predominantly freshwater (Coleochaetlaes, Ulotrichales) or exclusively freshwater (Oedogoniales, Zygnematales). Lee (1999) reported the green algae in the warmer tropical and semi-tropical waters tend to be similar everywhere in the world. In the colder marine waters, the species have markedly different in the waters of the Northern and Southern hemispheres. The warmer waters near the equator have acted as a geographical barrier for the evolution of new species and genera.

The Chlorophyta, or green algae, have pigments similar to those of higher plants; chlorophylls *a* and *b* are present, and the main carotenoid is lutein (Lee, 1999). Generally, green algae are distinguished by their pigments of photosynthetic, carbohydrate reserve, chloroplast structure and flagella (Sze, 2000). Colour of green algae is due to the fact that the chlorophylls *a* and *b* of green algae are usually not concealed by large amounts of other pigments (Linda and Lee, 2000). Other pigments of the chloroplast of green algae are α, β and γ carotenes and several xanthophylls (Goodwin, 1974).

Chlorophyta are common habitants of both freshwater and marine habitats, but they are chiefly freshwater algae (mainly Chlorophytes) and are more closely related to the higher plants than other marine algae. There are a small number of terrestrial species. This group contains the largest number of divisions.

In 1985, Bold and Wynne presented a very conservative classification scheme that is little changed from that of Smith (1950) and ignored the vast body of ultrastructural data that had accumulated over the past three decades (Pickett-Heaps and Marchant, 1972; Pickett-Heaps, 1975; Mattox and Stewart, 1984). In the system of Graham and Wilcox (2002) and Van den Hoek *et al*. (1995), the Chlorophyta as a phylum is much abbreviated from systems like those of Margulis and Schwartz (1998).

Classification according to Hoek, Mann and Jahns (1995):

- *Bryopsidophyceae*
- *Chlorophyceae*
- *Pleurastrophyceae* (Pleurastrales and Prasiolales)
- *Prasinophyceae*
- *Trentepoliophyceae*
- *Ulvophyceae*

- *Zygnematophyceae*

The family Caulerpaceae has been studied by Coppejans and Beeckman (1989, 1990); the genus *Caulerpa* is being morphmetrically investigated by de Senerpont Domis (Rijksherbarium Leiden, Netherlands) while genetic analysis is being performed by Olsen.s team (Rijksuniversiteit Groningen, Netherlands). The families Bryopsidaceae (Bryopsis, Trichosolen) and Codiaceae (Codium) have been studied by Van den Heede (1994), Coppejans and Van den Heede (1996) and Van den Heede and Coppejans (1995).

The family Halimedaceae was preliminarily studied by Verellen (1990). The genus *Halimeda* was studied monographically on a worldwide scale by Dargent (1998), Dargent and Coppejans (1998) using morphometrics, in collaboration with Kooistra from the Smithsonian Institute, Panama, and examined materials from a genetic perspective.

2.4 *Caulerpa*

The genus *Caulerpa* was first established by Lamouroux in 1809, and since then it is estimated that some 75 species of *Caulerpa* have been described (Weber-van Bosse, 1898; Calvert *et al*., 1976; Price *et al*., 1998). In Malaysia, there are at least six species of *Caulerpa* (*Caulerpa racemosa, Caulerpa lenitillifera, Caulerpa serrulata, Caulerpa prolifera, Caulerpa taxifolia* and *Caulerpa verticillata*) found in Cape Rachado, Port Dickson (Phang, 1998).

Many *Caulerpa* species are of economic importance (Chamberlain, 1998; Payri *et al.*, 2000; Littler and Littler, 2003). In Thailand, *C. racemosa* var. *corynephora* and *C. racemosa* var. *macrophysa*, are consumed as a fresh salad, and *C. lentillifera* has

been used in bioremediation in ponds by association with shrimp aquaculture. *C. taxifolia* is commonly sold for use in home aquaria throughout the world mostly in the Mediterranean, around San Diego, California, and in Australia (Jousson *et al*., 1998; Meisnesz *et al*., 2001; Wiedenmann *et al*., 2001; Wiliams and Grosholz, 2002).

Caulerpa is a macroalgae that belongs to the Bryopsidophyceae (Van den Hoek *et al*., 1995), a class of algae with a coenocytic thallus organization and is defined by the presence of trabeculae in the form of projecting cylindrical extensions of cell wall material passing through the central lumen of the siphons (Lamouroux 1809, Bold and Wynne 1985).

Research has shown that upright shoots on the same stolon may have a different morphology and that these upright shoots, if found separated, may be distinguished as different subspecific taxa or even different at the species level (Ohba and Enomoto, 1987). These units, called metameres (White, 1979), can potentially regenerate new gamets after a frond or stipe is cut.

This genus shows a complex external morphology: thalli are differentiated into a cylindrical, prostrate, creeping stolon (rhizome), branched anchoring rhizoids and upright branches (assimilators) that bear distinctive branchlets termed ramuli which are used in species identification. The genus *Caulerpa* is characterized by the absence of transverse cell walls and the presence of internal cell wall ingrowths called trabeculae, providing the thallus with mechanical support (Menzel, 1987). In spite of *Caulerpa* unicellularity, *Caulerpa* species exhibit a complex morphology and high morphological plasticity due to environmental factors and temporal variation especially prevalent among tropical species (Coppejans, 1992), making species boundaries difficult to define.

Caulerpa growth is influenced by environmental factors including wave exposure, water current, light intensity, depth, season, and grazing pressure (Svedelius, 1906; BØrgesen, 1907; Peterson, 1972; Calvert, 1976; Ohba and Enomoto, 1987; Ohba *et al.*, 1992; Carruthers *et al*., 1993; Meinesz *et al*., 1995; Gacia *et al*., 1996; Collado-Vides and Robledo, 1999; Collado-Vides, 2002). This genus attracted considerable research interest due to the capability of its many species, which are predominantly tropical and subtropical, expanding into more temperate environments (Meinesz and Hesse, 1991; Piazzi *et al*., 1994; Dalton, 2000; Kaiser, 2000).

Some taxonomic entities of *Caulerpa* species are morphologically distinctive with their well-defined boundaries while other entities show morphological plasticity which results in an unstable classification of varieties and formae (Ohba *et al*., 1992; Prud'homme van Reine *et al*., 1996; Pillmann *et al*., 1997; Fama` *et al*., 2002). Coppejans and Prud'homme van Reine (1992) made use of the term ''ecad'' to describe intermediate growth forms, in addition to many varieties, subspecies and forms. Sectional division among taxa (Agardh, 1872; Webervan Bosse, 1898) is predominantly supported by differences in assimilator morphology. These assimilators, however, can be highly plastic and are strongly influenced by the environment (Gilbert, 1941; Calvert, 1976; Ohba *et al*., 1992). Therefore, species boundaries, species relationships, and sectional divisions require further investigation.

2.4.1 Life cycle of *Caulerpa*

There are signs that all *Caulerpa* species have a haplontic life-cycle, in which a multinucleate, coenocytic and most probably haploid gametophyte alternates with a microscopic zygote stage. Reproduction of *Caulerpa* species can be sexual or asexual (anisogamous); when sexual, biflagellated gametes are liberated through branch papillae.

The inferred anisogametes develop inside the coenocytic thalli. Gametogenesis is a relatively non-obvious process involving the migration of the cytoplasm into a net-like lattice of unspecialized gametangia concentrated at the terminal ends of the fronds. Approximately two days before gamete release, migration of the cytoplasm occurs resulting in transparency of the stolons. Within 12 hours before gamete release, many species usually acquire a yellowish-green appearance. Light green sections of gametangia release the small microgametes, whereas brownish-orange-coloured gametangia release somewhat larger macrogametes. Siphonous shedding tubes, 5-15mm long, develop 12-36 hours preceding gamete release (Prud'homme and Trono, 2001) (Figure 2.1).

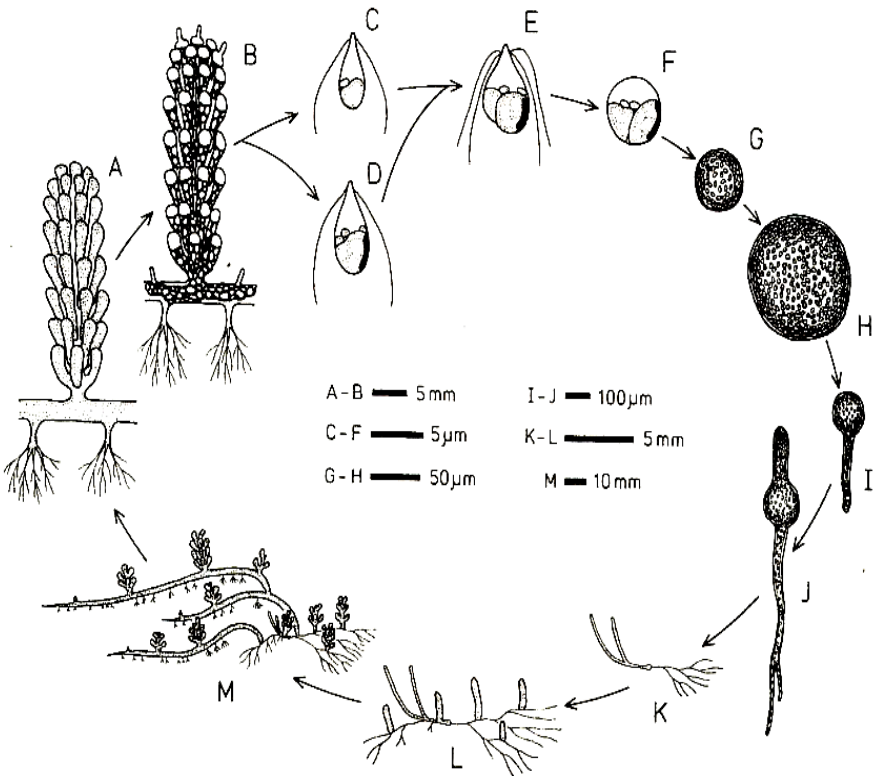

Figure 2.1: Life history of *C. racemosa* var. *laetevirens* (Adapted from Enomoto and Ohba, 1987).

(A. Vegetative plant; B. Fertile Plant with protoplasmic networks; C. Male gamete; E. Planozygote; F. Settled zygote; G. Spherical body, 3 weeks after settling; H. Enlarged spherical body, after 5 weeks; I. Germination, germling with a primary germ tube, after 6 weeks; J. Germling with a primary and a secondary germ tube; K. Protonema-like plant; L. Creeping filaments with erect shoots; M. Juvenile plant with assimilators).

2.4.2 Secondary Metabolites Of *Caulerpa*

Caulerpa species produce variable concentrations of secondary metabolites such as caulerpin, caulerpenyne, caulerpicin and other terpenoids (Doty and Aguilar-Santos, 1970; Amico *et al*., 1978; Maiti *et al.*, 1978; Paul, 1985), but the first compound to be identified from *Caulerpa* was caulerpicin, by Doty and Aguilar-Santos (1966).

According to various researches [Ogden, 1976; Vadas, 1977; Ogden and Lobel, 1981; McConnell *et al.*, 1982; Norris and Fenical, 1982; Fenical, 1982; Paul and Fenical, 1983; Lewis, 1985] it was hypothesized that secondary metabolites produced by tropical marine macrophytes may function as chemical defences to inhibit grazing by herbivores.

2.4.2.1 Caulerpin

Caulerpin is a compound from *Caulerpa racemosa*, a cyclo-octatetraene ring which contains a pigment (Ayyad and Badria, 1994; Clavijo *et al*., 1996), and displays anti-tumor activity in vitro (Ayyad and Badria, 1994).

Caulerpin, a dimer derivate of indole-3-acrylic acid might function as a growth regulator in these species and is accountable for the peppery taste and is considered toxic for microorganisms, fish and humans. It has been recognized in Hawaii and in the Philippines and is known to enter into marine food chains (Doty and Aguilar-Santos, 1970). In some individuals, the compounds of caulerpin and caulerpicin can function as mild anaesthetics and may cause dizziness, numbness of the tip of the tongue, weakening of the extremities and difficulty in breathing.

Caulerpin was first isolated from *C. racemosa* and *C. taxifolia* which were collected in Sri Lanka (Maiti *et al*., 1978). It was first thought that caulerpin was

afeeding deterrent but later it was shown that caulerpenyne was the metabolite responsible for deterrent effect on herbivores (McConnell *et al*., 1982; Paul *et al*., 1987; Wylie and Paul, 1988). However, these compounds are only present in fresh samples, although some diterpenes still can be isolated from dried samples.

Bioactivity of caulerpin is not as high as caulerpenyne in laboratory bioassays (Doty and Aguilar-Santos, 1970; Maiti *et al*., 1978; McConnell *et al*., 1982).

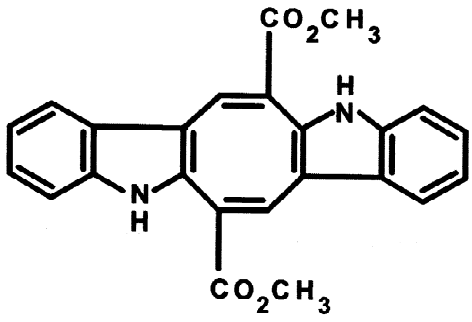

Figure 2.2: Structure of caulerpin (Adapted from Madl and Yip, 1999.)

2.4.2.2 Caulerpenyne

Caulerpenyne is the most plentiful cytotoxic sesquiterpenoid which is released among the many secondary metabolites produced by the genus of *Caulerpa*. This terpenoid has been investigated to evaluate their ecological role in chemical defence against herbivores (Mc Connel *et al*., 1982; Paul and Fenical, 1986 a, b; Pesando *et al*., 1996; Leme´e *et al*., 1997).

Since caulerpin has no deterrent effect on herbivores according to McConnell *et al*. (1982), Paul *et al*. (1987), Wylie and Paul (1988), therefore, the focus was placed on the biologically active caulerpenyne (Paul and Fenical, 1986; Targett *et al*., 1986). It is demonstrated by McConnell *et al*. (1982); Hodgson (1984), and Paul (1985),

caulerpenyne has biological activities such as antimicrobial, antineoplastic, ichthyotoxic and feeding deterrent effects in laboratory assays. Caulerpenyne has anticancer, antitumour and antiproliferative properties, as well (Fischel *et al*., 1995; Parent-Massin *et al*., 1996; Barbier *et al*., 2001).

Caulerpenyne from *Caulerpa taxifolia* has an antiproliferative activity on tumor cell line SK-N-SH and modifies the microtubule network (Barbier *et al*. 2001), and as lypophilic compounds to penetrate cellular membranes for the first step in the interaction with and the subsequent inhibition of electron transporters, as cytochrome P450.

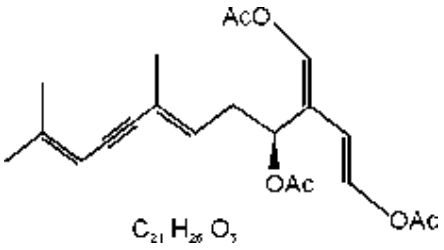

Figure 2.3: Structure of caulerpenyne (Adapted from Madl and Yip, 1999.)

2.4.2.3 Caulerpicin

Caulerpicin is the first secondary metabolite which was identified from *Caulerpa* by Doty and Aguilar Santos (1966), and is the minor component of *Caulerpa* sp. which appears to be a mixture of several minor compounds. According to the infrared spectrum of caulerpicin, this compound is a long- chain saturated hydroxy amide.

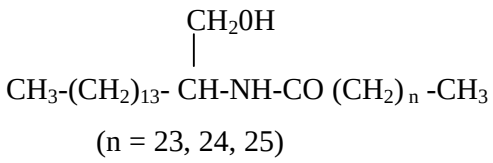

Figure 2.4: Chemical structure of Caulerpicin

2.4.2.4 Fatty Acids

Several species of *Caulerpa* are known to generate many saturated (e.g. palmitic acid and stearic acid) and unsaturated fatty acids (e.g. myristoleic acid). Cholesterol is present in appreciable amounts of all the sterols (Prud'homme and Trono, 2001). The presence of cytotoxic and antimitotic activity in methanol/toluene extracts of living specimens is documented for *Caulerpa prolifera* (Forssk) J.V. Lamour., while mosquito larvicidal activity is recorded for *Caulerpa scalpelliformis* (R.Br. exTurner) C. Agardh.

2.5 *Caulerpa serrulata* (Forsskål) J.Agardh

Synonyms

Fucus serrulatus Forsskål (1775)

Caulerpa freycinetii f. *serrulata* (1898)

Caulerpa freycinetii C.Agardh (1823)

Chauvinia freycinetti (C.Agardh) Trevisan (1849)

Caulerpa serrulata is a green alga which was originaly described by Forsskål (1775) as *Fucus serrulatus* Forsskål from Mokha, Yemen (Forsskål, 1775; Silva, Basson and Moe, 1996). Agardh in 1837, proposed *Caulerpa serrulata* (Forsskål)

J.Agardh as new combination for the species (Agardh, 1837). Subsequent studies depicted that *Caulerpa freycinetii* f. *serrulata, Caulerpa freycinetii* C.Agardh and *Chauvinia freycinetti* (C.Agardh) Trevisan are synonym with *Caulerpa serrulata* (Forsskål) J.Agardh (Silva, Basson and Moe, 1996).

Caulerpa serrulata is a tropical species and usually found in shallow rocky substrates, and covering under sands in sheltered environments with less than 5m depth.

Morphologically, *Caulerpa serrulata* is dark green, upright branches are distributed on the horizontal stolon at 2-11 mm distance; grow up to 6 cm tall; the cylindrical erect branches commonly become flattened from the lower 1/4-1/3 towards the tip; band-shaped branches divides dichotomously to sub dichotomously, leafy branches surrounded with rough teeth all throughout their margin are about 3 mm wide; the earlier ends of leafy branches are mostly curved or in one variety, the entire band-shaped blades are strongly twisted into spirals.

Distribution of *C. serrulata* was reported from Africa (Silva *et al*., 1996; Papenfuss, 1968), Indian Ocean (Silva *et al*., 1996), Pacific Islands (Skelton *et al*., 2004), South-west Asia (Silva *et al*., 1996; Papenfuss, 1968; Sahoo *et al*., 2001), South-east Asia (Verheij and Prud'homme van Reine 1993; Silva *et al*., 1996 and Phang *et al*., 2010), Australia and New Zealand (Lewis, 1987; Cribb, 1996; Phillips, 1997; Phillips, 2002; Kraft, 2007). In South- East Asian countries the species have been already reported from Indonesia (Verheij and Prud'homme van Reine, 1993; Silva, Basson and Moe, 1996), Malaysia (Silva, Basson and Moe, 1996), Philippines (Silva, Meñez and Moe, 1987), Singapore (Teo and Wee, 1983; Silva, Basson and Moe, 1996), Spratley Islands (Hodgson *et al*., 2004), Thailand (Hodgson *et al*., 2004), Vietnam (Pham-Hoàng, 1969; Abbott, Fisher and McDermid, 2002; Hodgson *et al*., 2004).

For this species there are 8 varieties or form as follows:

Caulerpa serrulata var. serrulata Weber-van Bosse

Caulerpa serrulata f. *spiralis* (Weber-van Bosse) Gilbert

Caulerpa serrulata var. *(boryana) f. longifolia* Gilbert

Caulerpa serrulata var. *hummii* (Díaz-Piferrer) Farghaly

Caulerpa serrulata var. *["boryana"] f. occidentalis* (Weber-van Bosse) Yamada and Tanaka

Caulerpa serrulata f. *lata* (Weber-van Bosse) C.K.Tseng

Caulerpa serrulata var. *boryana* (J.Agardh) Gilbert

Caulerpa serrulata f. *angusta* (Weber-van Bosse) Eubank

For molecular studies of the *Caulerpa* species different markers have been used by several researchers. Marker *tuf*A was employed by Fama *et al*. (2003), Stam *et al.* (2006), and de Senerpont Domis *et al*. (2003). ITS1 and ITS2 are other markers which have been used by Stam *et al*. (2006), Yeh and Chen (2004). *rbc*L and *ycf*10-*chl*B genes also were reported by de Senerpont Domis *et al*. (2003) and Fama (2002).

2.6 *Caulerpa racemosa* (Forsskål) J. Agardh

Synonyms:

Fucus racemosus Forsskål (1775)

Caulerpa clavifera (Turner) C. Agardh (1817)

Caulerpa uvifera C.Agardh (1817)

Vernacular names:

Seagrapes (En.)

Indonesia – lelato (Lombok), lata (Bangka), lai-lai (South Sulawesi)

Phillipines – ararusip (Ilocano, general and for var. *peltata*), kulinatnat, saluysoy (for var. *peltata*).

C. racemosa is a tropical green alga which is considered as an introduction from the Red Sea and has spread quickly in the south-eastern Mediterranean (Hamel, 1926; Aleem, 1948; Lipkin, 1972; Argyrou *et al*., 1999), recently it has spread also in the western part of the Mediterranean (Piazzi *et al*., 1994, 1997; Gambi and Terlizzi, 1998; Modena *et al*., 2000 Verlaque *et al*., 2000). It was first collected in the Mediterranean Sea in Sousse harbour, Tunisia (Hamel, 1926, 1930, 1931a; Djellouli *et al*., 1998). This species has been found in South-East, and recorded in Myanmar, Thailand, Vietnam, Malaysia, Singapore, Indonesia, the Philippines and Papua New Guinea. In Malaysia, this species can be found particularly in Port Dickson and Pulau Sibu (Ismail, 1995).

C. racemosa is one of the greatest distributed algae of warm-temperate seas and some varieties also occur in subtropical waters. *C. racemosa* is usually found in shallow water with less than 5m depth. For most varieties, favourable environmental conditions consist of calm, relatively clear water with current of weak to medium and loamy or sandy bottom (Prud'homme and Trono, 2001).

This species shows the most taxonomic problems due to its considerable morphological plasticity (Prud'Homme van Reine *et al*., 1996). It can be defined as a specific complex consisting of many varieties or morphological forms. Morphologically, *C. racemosa* is a stoloniferous plant with stolons terete to ovoid in cross-section. Its rhizoid bears decending branches arising at irregular intervals. The fronds are mostly erect, up to 20 cm tall and can be crowded or rather sparse. Branching is simple or irregular. The branchlets basically consist of stalk and head of varied form (club, rounded, ovate or compressed); alternate, opposite, multiseriate or imbricate. When fertile, the thalli are holocarpic (Prud'homme and Trono, 2001).

According to Verlaque *et al*. (2000), and Durand *et al*. (2002), there are three different co-existent morphological varieties of *C. racemosa.* Two of them, *C. racemosa* var. *turbinata-uvifera* (C. Agardh) J. Agardh and *C. racemosa* var. *lamourouxii* (Turner) Weber-van Bosse f .*requienii* (Montagne) Weber-van Bosse, are typical non-aggressive Lessepsian immigrants. “Invasive variety” is the name given to the third taxon due to its quick and abrupt spread across the Mediterranean Sea. According to Panayotidis and Zuljevic (2001), the dispersal of the invasive *Caulerpa racemosa* is based on sexual production on a huge scale. The majority of *Caulerpa* species are capable of regenerating through fragmentation, based on Belsher and Meinesz (1995); Ceccherelli and Cinelli (1999a) studies. Sexual reproduction is similar to that of other *Caulerpa*, where most of them are considered to be monoecious and holocarpic, releasing their total content in the form of microscopic, mobile, biflagellated presumed anisogametes. These gametes are typically shed from siphonous liberation tubes with length of 1.2-2 mm (Prud’homme and Trono, 2001).

The fronds of *C. racemosa* are eaten fresh as salad or consumed mixed with spices like grated coconut, garlic, shallot, basilleaves, salt and chilli in the Moluccas (eastern Indonesia). On the other hand, the *C. racemosa* side dish is prepared by mixing clean fronds with salt and tomatoes or with tomatoes, onions and pepper in the Cagayan Province of the Philippines. It is also utilized as fish feed and as medicine for human to lower blood pressure and to treat rheumatism (Prud’homme and Trono, 2001). The properties of antibacterial, antibiotic, antifungal and peroxidase activities are also recorded (Prud’homme and Trono, 2001).

In the Moluccas, eastern Indonesia, the fronds of *C. racemosa* are eaten fresh as salad or consumed mixed with spices like grated coconut, garlic, shallot, basil leaves, salt and chilli. In the Cagayan Province of the Philippines, the *C. racemosa* side dish is prepared by mixing clean fronds with salt and tomatoes or with tomatoes, onions and

pepper. It is also utilized as fish feed and as a medicine for humans to lower blood pressure and to treat rheumatism. In addition, antibacterial, antibiotic, antifungal and peroxidase activities are recorded (Prud'homme and Trono, 2001).

2.7 MOLECULAR APPROACHES IN SEAWEED SYSTEMATICS

Molecular biology is a modern branch of biological sciences that deals with biology phenomena and processes at the molecular level, by chemical, physical, and physiochemical methods (King and Stansfied, 1985; Kahl, 1995). It also involves the research of the structure and function of biological macromolecules and the relationship of their functioning to the structure of a cell and its internal components including nuclei, cell membranes and mitochondria (King and Stansfield, 1985; Kahl, 1995).

From the early 1990s onwards, molecular phylogenetic techniques have been playing an increasingly important role in studies of algal taxonomic (Brodie and Lewis, 2007). Molecular systematics is a new sub-dicipline of systematics (Hillis and Moritz, 1990) and is to incorporate molecular data into the phylogenetic relationships studies of organisms.

Proper classification of economically important organisms is important for the cultivation and exploitation activities. Traditionally, the identification of organisms has been based on vegetative morphology, anatomical aspects and reproductive features. Nevertheless, these features are not completely satisfactory. The phenotypic characteristics are subjective and are affected by various environmental factors and depending on the external growing conditions. Due to the limitations of morphological features in identification, genetic markers can play an important role in systematic studies of closely related species.

A number of different molecular techniques can be used to study algal phylogeny; nucleic acid sequencing, RAPD (random amplification of polymorphic DNA), RFLP (restriction fragment length polymorphism) and AFLP (amplified fragment length polymorphism). The application of DNA-based markers is generally determined by the technology that is used to reveal DNA-based polymorphism. The restriction fragment length polymorphism (RFLP) assay (Botstein *et al*., 1980) has been the choice for many species to measure genetic diversity and to construct a genetic linkage map. However, in general, RFLP assay is time consuming and laborious as it detects DNA polymorphism through restriction enzyme digestion, coupled with DNA hybridisation.

2.7.1 Gene Sequencing

The most important technique available to a molecular biologist is probably gene sequencing, by which precise order of nucleotides in a piece of DNA can be determined. This technique has been around for 35 years, but only during the late 1970s, rapid and efficient sequencing has been made possible (Brown *et al*., 2001) and has become one of the most utilized of the molecular approaches for inferring phylogenetic history.

Plant genome analytical tools developed along with the proliferation of mapping, genotyping and diagnostic methodologies, but were inhibited by difficulties faced in obtaining usable pure DNA from organisms. The success of genetic studies can be determined by the sufficient quantity and good quality of DNA. Therefore, the initial stage of molecular biology involves the isolation and purification of sufficient high quality DNA. Unfortunately, nucleic acid isolation methods applicable to certain species may not be so to different species. The modifications to these protocols often lead to

time consuming and complicated methods where large quantity of starting material is needed. Hence, the development of algal molecular genetics will be enhanced by the development of nucleic acid isolation methods that are quick, convenient and applicable to diverse species.

Gene sequencing refers to the determination of the actual nucleotide sequence in genomic DNA. Thousands to millions of nucleotides make up DNAs. It is essential to reduce the complexity of larger DNAs to much smaller fragments as a single sequencing experiment normally delivers the order of only a few hundred bases. Two different techniques were developed almost simultaneously; they are the chain termination method by Sanger and Coulson (1977), and the chemical degradation method by Maxam and Gilbert (1977). The two techniques are radically different but equally valuable. The methods of Maxam and Gilbert and of Sanger and Coulson have incessantly undergone numerous incremental improvements in technology, but have always relied on the nested set approach. A nested set is known as a collection of single stranded sub-fragments of a DNA molecule (Volckaert *et al.*, 2008). DNA sequencing has become a significant technique for inferring phylogenetic history between different organisms. This is because sequencing can provide us with the actual nucleotide sequence of a particular organism. DNA sequence data are the most informative tool for molecular systematics, and comparative analysis of DNA sequences is becoming increasingly important in plant systematics.

In systematic studies, nucleic acid sequencing is employed in three major aspects: (i) the evolution of genes, including studies of the processes that produce sequence-level variation, researches of the origin of new alleles or new loci and investigations of convergence and selection; (ii) the intraspecific or populational studies including the tracing of organismal and alleic genealogies within species and studies of geographic variation, gene flow, hybridization and conservation studies; (iii) the

interspecific studies such as construction of species phylogenies to evaluate macroevolutionary patterns and processes.

There are three common approaches in preparing DNA templates for sequencing in molecular systematics. Direct PCR amplification of the whole genomic DNA or cDNA is the most widely used method. This is because as of now, the specific primers for PCR amplification and sequencing for most of the organisms are available. This method is fast and cost effective. Secondly is cloning, performed by using the recombinant technology. However, it is very hardly ever used by taxonomists these days as it is labour intensive and very expensive. Thirdly is the direct sequencing on nuclear-encoded ribosomal RNAs or messenger RNAs that are particularly abundant in certain tissues (Hillis *et al*., 1996).

For algae, the advantage of nucleic acid sequencing methods is that large numbers of independently evolving characters can be used to reconstruct phylogenies as compared to the much smaller numbers of morphology, biochemical, and other characters which are present. Nucleic acid sequencing is one of the solutions used to overcome problems associated with the phenotypic plasticity of algae such as *Caulerpa racemosa*. Thus, some unique genotypes of algae regions have been widely used for the taxonomy of algae.

2.7.2 Random Amplified Polymorphic DNA (RAPD)

Random Amplification of Polymorphic DNA (RAPD) or Arbitrary Primed PCR (AP-PCR) uses a single short random primer in a PCR reaction, mostly decamers, to amplify the DNA between the primers (Welsh and McClelland, 1990; Williams *et al*., 1990). RAPD involves the use of a single DNA primer to direct amplification under PCR based amplifications of random sequences. This method was first accounted, almost simultaneously, by two research groups (Welsh and McClelland, 1990; Williams *et al*., 1990).

Application of RAPD-PCR in constructing genetic maps, assessing genetic stability, tagging chromosomes, assessing pedigree, determining parentage, identifying DNA markers linked to disease resistance genes, detecting genetic variation and generating genetic fingerprints, studying populations and determining taxonomic identity has been discussed in a recent review by Hardy *et al.* (1992). RAPD is popular because of its quickness, simplicity and automation in operation.

The main advantages of RAPD are that it requires no prior knowledge about any particular gene in a target taxon. The standard RAPD technology (Williams *et al*., 1990) utilizes short synthetic oligonucleotides (ten bases long) of random sequences as primers to amplify nanogram amounts of total genomic DNA under low annealing temperatures by PCR. These oligonucleotides serve as both forward and reverse primer and usually are able to amplify fragments from three to ten genomic sites simultaneously.

Also, RAPD has an edge over restricted fragment length polymorphism (RFLP). This is because the RAPD method can be used to detect higher levels of polymorphism (Williams *et al*., 1990). RAPD can be used for fingerprinting individuals of small

localized populations as it can reveal diversity between individuals even of a biological species, especially a geographically widespread species (Coleman and Goff, 1991; van Oppen *et al*., 1994).

RAPD proved to be a useful tool in the taxonomy and classification of seaweeds at the genus and species level: *Gracilaria* species (González *et al*., 1996), *Gelidium* species (Patwary *et al*., 1993), *Porphyra* (Dutcher and Kapraun, 1994), *Sargassum* species (Ho *et al*., 1995a; Ho *et al*., 1995b), *Hizikia fusiformis* (Park *et al*., 1998), and *Furcellaria lumbricalis* (Valatka *et al*., 2000). However, RAPD is only preferred when resources and time are limited and no previous information about the species under investigation is known.

2.7.3 Restriction Fragment Length Polymorphism (RFLP)

Restriction fragment length polymorphism (RFLP) is a technique in which used to estimate DNA sequence divergence by analyzing DNA fragment variation patterns caused by changes in the bases that are either due to deletions, substitutions or insertions of nucleotide across the entire genome through the use of restriction endonuclease. The enzymes recognize and cleave specific sites in the DNA consisting of four to eight nucleotides in a specific sequence. Separation of DNA fragments cleaved by enzymes according to size is performed by using electrophoresis on agarose gel. The detection of the fragments of interest can be done by several means. Electrophoretic fragment patterns can be observed and compared directly after being stained with ethidium bromide and for greater sensitivity; the DNA can be transferred to nitrocellulose membrane and hybridized with a specific probe.

RFLPs have been extensively applied in the studies of phylogenetic relationships of algae at the population, genus and species level. This method is used in *Gracilaria* (Goff and Coleman, 1988; Rice and Bird, 1990), *Pandorina* (Moore and Coleman, 1987) and within *Laminaria* species (Bhattacharya and Druehl, 1990).

Advantages of RFLPs include the relatively low cost and simple methods associated with both their first identification and subsequent analysis, the capability to use gene-containing DNA segments (cDNA or genomic) as RFLP-detecting probes, and the generation (as a by-product) of single-copy probes that are useful for various other purposes (Biren *et al.*, 1997).

However, the separation of plastid DNA from nuclear DNA is a time-intensive and expensive process that will often include contaminating mitochondrial or plasmid DNA. Besides, although RFLP analysis is informative and reproducible, it is very laborious and time-consuming as it may require the testing of all different kinds of restriction enzyme / probe combinations to find polymorphic bands and also needs large quantities of high-quality DNA for digestion and blottin (Karp *et al*., 1996). This approach is not a suitable technique for the study of species from higher taxa (Goff and Coleman, 1988).

2.7.4 Amplified Fragment Length Polymorphism (AFLP)

Amplified Fragment Length Polymorphism (AFLP) is a new molecular approach which combines the advantages of RAPD and RFLP method into a powerful tool to produces information that appears useful for analyses from large biogeographic scales to smaller population-level investigations. This DNA fingerprinting technique is based on selective PCR amplification of restriction fragments from a total digest of

genomic DNA (Vos *et al.*, 1995), and it has been found to be an efficient method for studies of organisms in which insufficient variation is detected through sequence analysis

AFLP involves the restriction of genomic DNA, followed by ligation of adaptors complimentary to the restriction sites and selective PCR amplification of a subset of subset of the adapted restriction fragments. These fragments are visualized on denaturing polyacrylamide gels either through autoradiographic or fluorescence methodologies. AFLP technique requires isolation of DNA with high purity for restriction digestion. The availability of many different restriction enzymes and corresponding primer combinations provides a great deal of flexibility, enabling the direct manipulation of AFLP fragment generation for defined applications (e.g. polymorphism screening, QTL analysis, genetic mapping).

The major advantage of this technique is AFLP provides equal or greatly enhanced performance in terms of reproducibility, resolution, and time efficiency. Most likely the single greatest advantage of the AFLP technology is its sensitivity to polymorphism detection at the total-genome level. The large number of bands it produces, giving a very good chance of finding a large number of polymorphic bands among them. The polymorphisms detected by this method come from the same sources as in RFLPs, insertions, deletions and point mutations leading to the presence or absence of restriction sites, but compared to RFLPs, AFLPs are normally scored only as dominant markers, even when some researchers gave possible methods for using them co-dominantly.

Nevertheless, AFLP methods are technically demanding, sensitive to the quantity and purity of DNA to be digested, need some experience to be performed and data analysis of the hundreds of amplified bands must be done by computer analysis.

2.8 MOLECULAR APPROACHES IN UNRAVELLING THE TAXONOMY OF *Caulerpa*

Taxonomy can be defined as the theory and practice of describing, naming and classifying objects, particularly organisms. Taxonomy has evolved, and will continue to progress, through interaction of three processes: (i) progressive addition, modification, and abandonment of criteria suggested by information obtained by means of continually improving observational and analytical instruments and techniques; (ii) discovery of organisms with combinations of characteristics that do not fit existing definitions of taxonomic units; and (iii) changing philosophical concepts.

Early workers continued to classify additional species, notably Kützing (1849), who listed 41 species. *Caulerpa* were then organized into 13 tribes by Jacob Agardh (1872). In 1898, Weber-van Bosse monographed the genus, bringing together descriptions and drawings for the entire array of name species and varieties. Svedelius (1906) discussed the ecology and biogeography of *Caulerpa* in his report on the genus in Ceylon. He described and illustrated 21 species and many varieties. In 1913, Børgesen listed 11 species of *Caulerpa* from the Danish West Indies; the (1940) described 5 species from Mauritius. *Caulerpa* variants from Java and the Philippines are reported by Gilbert (1942) and then in Meñez and Calumpong (1982). In 1940 and 1944, Yamada worked on *Caulerpa* from the northern Marshall Isalnds (1950) and the Andaman Sea (1965), as well as the Indian Ocean (1967).

Coppejans and colleagues provide useful keys, illustrations and descriptions on Caulerpales from the Southwestern Pasific in a series of papers (Coppejans, 1992; Coppejans and Beeckman, 1989, 1990; Coppejans and Meinesz, 1988; Coppejans and Prud'homme van Reine, 1992). In their papers, the term "ecad" instead of "variety" was

used, which does not have taxonomic standing. Their term may, nevertheless, more accurately reflect the ecologically variable forms of *Caulerpa*.

Caulerpa species are infamously variable, which has led to the description of a great number of varieties, subspecies, forms and ecads, many of which intergrate. More than one "variety" growing from a single stolon has been produced from cultural studies; they have also shown significant changes in morphology under different environmental conditions such as light, temperature, salinity or turbulence (Ohba *et al*., 1992; Carruthers *et al*., 1993). As a result, the status of such varieties is unclear, and this probably will remain so until genetic analyses are carried out.

Molecular techniques in comparison with conventional methods, measure the changes in the genome more than the phenotype and the usage of genotypic characters can overcome the problems related to phenotypic convergence plasticity (Donoghue and Sanderson, 1992).

In 2002, Famà *et al*. inferred molecular phylogeny from chloroplast *tuf*A sequences of 23 taxa for better understanding of the evolutionary history of the genus *Caulerpa*. They included a sequence of *Caulerpella ambigua* as a potential outgroup. The results reveal that the latter taxon is, in fact, sister to all ingroup. From their studies, high bootstrap values support *monophyly of C. mexicana, C. sertularioides, C. taxifolia, C. webbiana,* and *C.* prolifera, whereas most other *Caulerpa* species show para- or polyphyly. The *tuf*A phylogenetic results reveal that *Caulerpa* itself consists of a series of relatively ancient and species-poor lineages and a relatively modern and rapidly diversifying clade containing most of the morphological and species diversity.

Biochemical study of the pyrenoid indicates it to consist of mainly ribulose-1, 5-bisphosphate carboxylase/oxygenase (RUBISCO) and some minor proteins (Holdsworth, 1971; Kerby and Evans, 1978; Salisbury and Floyd, 1978; Satoh *et al*.,

1984; Kuchitsu *et al*., 1988; Okabe and Okada 1988; Okada *et al*., 1991). In 1989 and 1991, Miyamura and Hori have demonstrated localization of most of the chloroplast DNA (ct-DNA) in the pyrenoid core of several siphonous green algae namely *Caulerpa okamurae, C. lentillifera, C. fergusonii* (Murray), and in a siphonous xanthophyte alga, *Pseudodichotomosiphon constrictus* (Yamada) using fluorescence microscopy after staining it with the DNA-specific fluorochrome 4',6-diamidino-2-phenylindole (DAPI). Five years later, Miyamura *et al*. (1996) employed immunofluorescence microscopy to ensure the presence of RUBISCO in the pyrenoid cores of *C. lentillifera* and *C. okamurae*.

The use of nuclear rDNA ITS sequences (Pillmann *et al*.,1997) had proven to be useful in resolving the inter- and intraspecific genetic variation in five species of *Caulerpa,* including nine populations of *C. filiformis* from two biogeographic regions; five from Australia and four from South Africa. Species relationships were well resolved by internal transcribed spacer (ITS) sequences and supported by high bootstrap values as follows: (*C. geminata* (*C. simpliciuscula* (*C. trifaria* (*C. scalpelliformis*(*C. filiformis*-Australia, *C. filiformis*-South Africa).

In 1997, Benzie and colleagues examined the allozyme variation in seven species and four varieties of *Caulerpa* sampled from the Great Barrier Reef region. In their study, the population genetic analysis of polymorphisms, which occurred in some taxa, demonstrated strong spatial differentiation among populations of *C. cuppressoides, C. racemosa* vars *laetevirens* and *racemosa, C. serrulata,* and *C. taxifolia* and noteworthy but the variable degrees of clonality and/or inbreeding within these populations. Allozyme demonstrated to be a useful tool for defining species boundaries and investigating population structure in *Caulerpa,* but not for determining phylogenetic relationships within the genus.

A number of approaches were undertaken by Lisette *et al.* in 2003 to assess the genetic diversity of *Caulerpa* species in the Bolinao reef system. They analysed partial sequences of chloroplast *rbc*L and *tuf*A genes and a noncoding region flanked by the chloroplast *ycf*10 and *chl*B genes of intraspecific taxa of *C. racemosa, C. sertularioides*, *C. serrulata*, and *C. cupressoides*. In all phylogenetic analyses, *C. sertularioides*, *C. serrulata*, *C. cupressoides* and the three ecads of *C. racemosa* appeared as distinct genetic units.

Verlaque *et al*. (2003), recently had a study on the invasive variety by molecular analyses (rDNA ITS1 and ITS2) which demonstrated that this variety is closer to the warm-temperate Australian *C. racemosa* var. *laetevirens* f. *cylindracea* (Sonder) Weber-van Bosse, than the tropical varieties, but it does not imply that the species was introduced from Australia. Nuclear rDNA ITS sequences has been employed for the study of investigating the root of the invasive species of both *C. racemosa* (Durand *et al.*, 2002; Verlaque *et al*., 2003; Verlaque *et al.*, 2004; Nuber *et al*., 2007) and *C. taxifolia* (Meusnier *et al.*, 2001). In addition to this, the combination of ITS regions and *rbc*L gene has also been used to investigate the invasive species of *Caulerpa* to Europe (Fama *et al*., 2002).

The chloroplast gene *tuf*A encodes for elongation factor TU, a molecule that mediates the entry of an amino-acyl-tRNA into the acceptor site of a ribosome during elongation of the nascent polypeptide chain in protein synthesis (Lewin, 1997). This gene is encoded by the chloroplast genome of photosynthetic algae but is nuclear encoded in some Charophyceae and in land plants (Baldauf *et al*., 1990; Bonny and Stutz, 1993). The *tuf*A gene is a good candidate for phylogenetic studies above the species level because of its conserved nature across a wide range of organisms. Until recently, *tuf*A sequences have been used only to address phylogenetic questions at suprageneric levels (Ludwig *et al*., 1990; Delwiche *et al*., 1995; Baldauf *et al*., 1996).