# CHAPTER 2: LITERATURE REVIEW

## 2.1 RED ALGAE (RHODOPHYTA)

The red algae are the most diverse in the tropics compared to the green and brown algae. Red algae owe their colour to water-soluble pigments called phycobilins, which are usually red, but sometimes appear light to dark green or even dark brown to black when found in the shallow intertidal zones. The dominant pigment, *r*-phycoerythrin, causes the red colouration, while pigments such as chlorophyll *a* and *d*, carotenes, lutein, zeaxanthin, and *r*-phycocyanin are also present. The Rhodophyta are unicellular, filamentous, parenchymatous (fleshy) or pseudoparenchymatous (rope-like) which some are calcified or calcareous with calcium carbonate or magnesium carbonate (aragonite) (Hilconida *et al.*, 1997). Their cells are eukaryotic and almost all the Rhodophyceae are multicellular. As reported by Lee (1980), there are fewer than ten unicellular genera. Their chloroplasts have thylakoids that occur singly and not in bands as found in the green or brown algae while their cell wall contains little amounts of cellulose and much of gelatinous or amorphous sulphated galactan polymers (agar, carrageenan, funoran, and furcellarin). Floridean starch is their food reserve. The life histories of red algae include the most complicated method of reproduction. Although asexual reproduction is common (cell division and production of non-flagellated spores), most species exhibit three phases – one gamete-producing phase (sexual phase) and two spore-producing phases (asexual

phase) which are carpospore-producing phase (carposporophytic generation) and tetraspore-producing phase (tetrasporophytic generation) (Gavino, 1997). They produce haploid tetraspores, which germinate into gametophytes. The gametophytes produce eggs (carpogonia) which remain attached and spermatia, which are released into the water. When the carpogonia is fertilised, it grows into a carposporophyte, which produces diploid carpospores. The carpospores will germinate into tetrasporophytes. Both spores and gametes are non-flagellated. Vegetative reproduction through fragmentation of the thallus may also occur.

## 2.2 THE GENUS *GRACILARIA*

Under the family *Gracilariaceae*, *Gracilaria* is a large genus from the division of Rhodophyta (Red Algae). However, it has different colour manifestations. It can be black, yellow or red. About 100 species of *Gracilaria* are widely distributed throughout tropical and temperate waters of the world. Xia and Abbott (1987), Abbott *et al.* (1991) and Phang (1994) described several species of *Gracilaria* from Peninsular Malaysia and Singapore. In Malaysia, the eight species of *Gracilaria* reported are *Gracilaria salicornia* (C.Agardh) Dawson, *Gracilaria changii* (Xia et Abbott) Abbott, Zhang et Xia, *Gracilaria edulis* (Gmelin) Silva, *Gracilaria subtilis* (Xia et Abbott) Xia et Abbott, *Gracilaria urvillei* (Montagne) Abbott, Zhang et Xia, *Gracilaria textorii* (Suringar) De Toni, *Gracilaria firma* Chang et Xia and *Gracilaria coronopifolia* J. Agardh.

Amongst the *Gracilaria*, most species show the three phased *Polysiphonia* type of life history. They have morphologically identical diploid tetrasporophyte and haploid gametophyte phases (with equal numbers of male and female plants). *In situ* fertilisation of the female gamete forms the zygote, which then develops into the diploid carposporophyte (the third phase). Each carpospore can develop into a diploid tetrasporophyte, which produces haploid, genetically variable tetraspores (Kain and Destombe, 1995).

Although *Gracilaria* is a very polymorphic genus vegetatively, it has remarkably constant reproductive structures (Dawson, 1949). Dawson was the first to show the taxonomic importance of the shape and origin of spermatangial conceptacles. Yamamoto (1975) divided the genus into three subgenera based on the three different types of male organs: *Gracilariella* – with superficial spermatangia, scattered continuously over the thallus surface; *Textoriella* – with spermatangia in shallowly depressed concave conceptacles in the cortex, with each spermatangial mother cell primordium forming a branch system covering the floor of the conceptacle at maturity; *Gracilaria* – with spermatangia in deep cup-shaped or pot-shaped conceptacles within the cortex with each spermatangial mother cell primordium forming a branch system covering the entire inner surface of the conceptacle at maturity. He also suggested a relationship between type of spermatangial configuration and the presence or absence of tubular cells (nutritive filaments). In 1984, he recognised two additional spermatangial types with no proposed subgeneric names. Then, in 1985, he combined spermatangial and

cystocarpic characters in defining the three subgenera. In 1996, Plastino and Oliveira reported on the consistency of male reproductive structures in the *Gracilaria* species studied, for both natural populations and in the successive generations cultivated *in vitro*. They also observed persistent and reliable characters of the cystocarp anatomy among individuals belonging to the same species in *Gracilaria* and *Gracilariopsis*. However, in assessment of the phenotypic plasticity of red algae using crossability tests, they found that morphotypes with similar morphological characteristics and difficult to be distinguished from one another did not hybridise. Therefore, they concluded that configuration of male reproductive structures and cystocarp anatomy are consistent taxonomic characters but morphological features (gross thallus morphology and branching pattern) are not, in the Gracilariaceae.

## 2.3 ECONOMIC IMPORTANCE OF *GRACILARIA* SPECIES

Of the seaweed phycocolloids, agar has the highest price in the world market and is mostly extracted from red seaweeds like *Gelidium* and *Gracilaria*. Agar has commercial value as the most potent gel-forming agent. The advantage of agar over gelatine is based on agar's higher melting point. Unlike gelatine, which requires refrigeration to set, agar has stronger setting properties and will set at room temperature. Agar extracted from the Gracilariaceae became the first phycocolloid to be used in the human food industry (Armisen and Galatas, 1987; Armisen, 1995). The high price of *Gelidium* and its shortage has caused the

increased use of *Gracilaria* in agar production. To meet the demand, *Gracilaria* is presently cultivated in Chile, China, Taiwan and other countries. Generally, agar from *Gracilaria* may be divided into several groups depending on its gel strength and properties such as charge density, amount of sulphate, pyruvate and methoxyl content (Yaphe, 1984). *Gracilaria* agar has higher degree of sulphation, methoxylation and pyruvylation compared to others (*Gelidium* and *Pterocladia*). Its gelling ability is improved by adapting an alkali pretreatment which converts α-L-galactose 6 sulphate into 3,6-anhydro-α-L-galactose (Murano, 1995). The quality and quantity of *Gracilaria* agar depends on the species used, season or time of collection and their growth environment (Shi *et al.*, 1984; Chirapart and Ohno, 1993). Therefore, strain selection to find a fast growing good agar producer is very important before embarking on mariculture, because variable quality of agar obtained from *Gracilaria* (Hansen, 1984) may present a problem.

Generally, *Gracilaria* agar is sugar reactive and will combine with sugar and still keep its gelling strength. Its non-toxicity and unique rheological properties is appreciated in the food industry where the embedding of preserved cooked fish or meat in a firm jelly prevents breakage during transportation. The contents of the can are also protected from blackening resulting from undesirable reactions. Its ability to bind proteins makes it a good clarifying agent for wines, juices and vinegar. Agar, which contains hydrophilic properties, makes a good moistening additive for bread and pastry. In confectionery, agar is used to allow

clean slicing in marshmallows and candies for example. Agar also plays an important role in various food industries as a stabiliser, thickening and clarifying agent and in the production of skin creams, ointments, lotions, perfumed deodorant sticks and sun creams. In Japan, agar is used to make clear noodles. In pharmaceutical industries, it is widely used for coating pills and capsules and also as a laxative. Agar is used as a suspending agent in the radiological uses of barium sulphate. It also plays a role as antirheumatic agent for prolonged treatment and in the stabilisation of cholesterol solutions. In the field of microbiology, it has been greatly used as a solidifying bacterial media. The market demands for microbiological agar have increased significantly due to new horticultural, entomological and genetic applications (Santelices and Doty, 1989). Agar is the choice medium for cultivation and selection of the transformed microbial hosts in genetic engineering and also in plant callus formation. The high demand for agarose (the neutral fraction of agar, lacking sulphate and other charged groups) will remain to meet the biotechnological and biochemical needs (Renn, 1984). Other biotechnology-oriented applications of agarose are agarose gel electrophoresis (separation and isolation of desired gene DNA fragments & gene mapping); immunology or immunoassays, microorganism & cell culture; chromatography and immobilised systems.

In 1993, Critchley stated the world market value of agar at US $200 million while Hurtado-Ponce and Umezaki (1988) reported that the world's commercial agar comes mostly from gracilaroids. The red seaweed *Gracilaria*

has become an important agar source yielding 60-70% of the world's agar (Schramm, 1991). In addition, it also can be used in removing nutrients from wastewater (Ryther *et al.*, 1979, Bird *et al.*, 1981a), methane production (Bird *et al.*, 1981b), toxicity assessment (Haglund *et al.*, 1996), as beads used in chromatographic columns (Renn, 1984) and served as a food garnish in Japan.

## 2.4 CONTROVERSIES IN THE TAXONOMY OF *GRACILARIA*

According to the definition by Keeton & Gould (1986), taxonomy is the classification of organisms on the basis of their evolutionary relationships. Common approaches used in taxonomic studies are classical evolutionary taxonomy, phenetics (numerical taxonomy), cladistics and molecular taxonomy.

Species delineation in *Gracilaria* is problematic because of the limited distinct morphological characteristics (Papenfuss, 1953, 1967; Bird and MacLachlan, 1982, 1984). There are some controversies in the taxonomic studies among the closely related genera of *Gracilaria, Gracilariopsis* and *Hydropuntia* (*Polycavernosa*).

### 2.4.1 *Polycavernosa*

*Polycavernosa* has been recognised to be a separate taxon from *Gracilaria*. Table 1 shows the morphological differences between *Polycavernosa*

and *Gracilaria*. The main differences that found between these two genera are the appearance and ontogenetic development of the reproductive structures and the mature cystocarp (Chang and Xia, 1963; Federicq and Norris, 1985). In 1982, Bird and McLachlan suggested that the polycavernosa-type spermatangia might be a modification of the verrucosa-type and should be grouped as a subgenus within *Gracilaria*. However, Yamamoto (1984) stated that if these spermatangia were an aggregation of the verrucosa-type, they should be named the Henriquesiana-type or as a separate genus. In 1987, Xia and Abbott placed four new species from the Western Pacific, namely, *Polycavernosa changii* Xia et Abbott from Malaysia and Thailand, *Polycavernosa divergens* Xia et Abbott from the Philippine, *Polycavernosa fisheri* Xia et Abbott from Thailand and *Polycavernosa subtilis* Xia et Abbott from Malaysia into the *Polycavernosa* group based on the clustered spermatangial cavities where the spermatangial mother cells produce internal spermatangia within the inner vortex or outer medulla. However, re-examination of these taxa showed a mixture of spermatangial types, which are used to separate genera in the Gracilariaceae (Abbott, Zhang and Xia, 1991). The simple, pitlike verrucosa-type is seen in the youngest branch portions and gradually changes to the multicavitied *Polycavernosa*-type in older parts. There are some species of *Gracilaria* maintaining these verrucosa-type spermatangia throughout the plants. Hence, the need to transfer the classification from *Polycavernosa* (=*Hydropuntia*) to *Gracilaria*.

Table 1 : Morphological Differences between *Polycavernosa* and *Gracilaria*

| *Polycavernosa*<br><br>(Chang and Xia, 1963; Xia and Abbott, 1985; 1987) | Distinguished by<br><br>1. the multi-cavitied spermatangial arrangements that are isolated from each other in surface, cross-sectional views and<br>2. upper absorbing filaments rarely seen,<br>3. the appearance of darkly staining, irregularly shaped, basal absorbing filaments that are large columnar cells with few to many spine like arms found at the lateral bases of, or underneath, the gonimoblast. |
|---|---|
| *Gracilaria*<br><br>(Chang and Xia, 1963; Yamamoto, 1978) | Distinguished by<br><br>1. the simple pitlike verrucosa spermatangial arrangements or so called cup-shaped or pot-shaped spermatangial conceptacles that are isolated from each other in surface, cross-sectional views and<br>2. upper absorbing filaments that are more delicate, elongate and without arms may or may not occur between the carposporophyte mass and the pericarp. |

Gargiulo *et al.* (1992) found the existence of an inner pericarp (a character of both *Gracilariopsis* and *Hydropuntia*) in most of the *Gracilaria* species he studied. These *Gracilaria* species have tubular nutritive cells extending to the outer pericarp (a characteristic of *Gracilaria*). They also observed that the confluent male cavities in *Gracilaria heteroclada* are of *Hydropuntia* type, but the fusion cell of limited extent is of *Gracilariopsis* type while the presence of outwardly directed tubular nutritive cells is of *Gracilaria* type. Ganesan (1993) also observed the formation of spermatangia in multicavitied conceptacles (as *Hydropuntia*) in *Gracilaria damaecornis*, but found that the cystocarp has tubular nutritive cells from the base and top of the cystocarp and not restricted to the floor. Therefore all these lead to the conclusion that it is very difficult to use sexual reproductive studies for differentiating between species of *Hydropuntia*, *Gracilariopsis* and *Gracilaria*.

### 2.4.2 *Hydropuntia*

Xia and Abbott (1987) recognised *Hydropuntia urvillei* Montagne as a species of *Polycavernosa* based on a single young cystocarp with inconspicuous basal absorbing filaments together with a pericarp and synonymised the genus *Hydropuntia* Montagne with *Polycavernosa* Chang et Xia (1963). Based on the priority of the earlier validly published generic name, Wynne (1989) proposed the reinstatement of *Hydropuntia* and the transfer of all taxa recognised under *Polycavernosa* into that genus. Earlier, *Hydropuntia urvillei* was placed in the

genus *Corallopsis* Greville along with all *Gracilaria*-like entities with distinct constrictions at the nodes and in internodal regions. In 1954, Dawson transferred two *Corallopsis* species, including *C. salicornia* into *Gracilaria*, synonymies *Corallopsis* with *Gracilaria.*

### 2.4.3 *Gracilariopsis*

In 1967, Papenfuss compared cystocarpic morphology and concluded that the presence or absence of tubular cells cannot be used to discriminate between the *Gracilariopsis* and *Gracilaria.* Hence, *Gracilariopsis* was placed in synonymy under *Gracilaria* (Yamamoto, 1975, Chang & Xia, 1976). However, the study on spermatangial development and post-fertilisation events in *Gracilariopsis lemaneiformis* (Bory) Dawson by Fredericq and Hommersand (1989) proposed the resurrection of *Gracilariopsis.* Recently, molecular studies strongly support the segregation of *Gracilariopsis* from *Gracilaria* (Bird *et al.*, 1992a, 1992b; Goff *et al.*, 1994). Kapraun and Dutcher (1991) also presented karyological data to support the segregation by comparing the genome sizes using hydroethidine staining of the nuclei and microspectrophotometry. In 1994, Freshwater *et al.* again confirmed the segregation using the *rbc*L gene sequence.

### 2.4.4 Interspecific grouping

There are also some controversies with the interspecific grouping among *Gracilaria* such as distinction between *G. textorii* (sur.) Hariot and *G. sublittoralis* Yamada et Segawa (nom. nud.), which are morphologically very close (Yamamoto, 1994). Problems are also faced in the distinction of *G. crassa* Harvey et J. Agardh, *G. cacalia* (J. Agardh) Dawson, *G. minor* (Sonder) Durairatnam and *G. canaliculata* (Kutzing) Sonder from *G. salicornia* (C.Agardh) Dawson as reported by both Xia (1986) and Meneses & Abbott (1987). The taxonomy of *G. verrucosa* (Hudson) Papenfuss also faces many difficulties (Yamamoto & Sasaki, 1987; Yamamoto & Sasaki, 1988). Bird and McLachlan (1984) has commented that spermatangia based on only three types are not taxonomically useful at the species level for a genus like *Gracilaria*, which consists of more than 100 species. Species found in a single geographic area may show similar morphology but have quite different spermatangia type.

## 2.5 DEOXYRIBONUCLEIC ACID (DNA) ISOLATION AND PURIFICATION

Plant genome analytical tools developed along with the proliferation of mapping, genotyping and diagnostic methodologies, but were inhibited by difficulties faced in obtaining usable DNA from the organisms. Hence, the initial stage of molecular biology is the isolation and purification of sufficient high

quality DNA. Unfortunately, nucleic acid isolation methods applicable to certain plant species may not be so to another species. In addition, the purity of DNA suitable to be used in certain molecular techniques (such as polymerase chain reaction amplification) may not be applicable for others such as restriction fragment-length polymorphisms (Coyer *et al.*, 1995). Modifications to these protocols often lead to time-consuming and complicated methods where large quantity of starting material is needed. Therefore, 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.

Initially, protocols developed for DNA extraction were time and labour intensive, usually incorporating one or two cesium chloride (CsCl) spins in an ultra-centrifuge (Mizukami *et al.*, 1998; Roell and Morse, 1991; Fain *et al*, 1988). However, Lee *et al.* (1997) stated the difficulties in detecting and recovering small quantities of DNA using these gradient techniques besides being expensive and time-consuming. Hence, many techniques have been used to eliminate this step such as the use of sepharose spin columns (Mayes, 1992), a series of purification procedures including CTAB protocols and Qiagen affinity columns (Steane *et al.*, 1991) and gel purification steps (Saunders, 1993).

Unfortunately, extraction of DNA from seaweeds is complicated by the presence of sulfated polysaccharides. Grinding sample using liquid nitrogen normally released these viscous soluble polysaccharides, which attached to the

DNA (Brasch *et al.*, 1981). So, Coyer and Steller (1995) evaluated the necessity of powdering samples in liquid nitrogen and found that the yield obtained by powdering kelp tissue in liquid nitrogen was 64% greater than by grinding tissue in the buffer at room temperature. Therefore, in order to eliminate those problems, several techniques have been developed, such as use of hydroxyapatite binding (Parsons *et al.*, 1990; Villemur, 1990) and cetyltrimethylammonium bromide (CTAB) treatment (Goff and Coleman, 1988; Roell and Morse, 1991). For Phaeophycean taxa, fingerprint-quality DNA has been obtained by submitting liquid nitrogen ground sample to non ionic detergent hexadecyl trimethylammonium bromide (CTAB) and chloroform-isoamyl alcohol (Doyle and Doyle, 1990; Coyer *et al.*, 1994). In 1994, Sinnappah extracted DNA from *Gracilaria* species using several methods and reported that the combination of CTAB and phenol-chloroform was more effective compared to the others. She stated the difficulties in obtaining clean DNA from the *Gracilaria* samples due to high polysaccharide and other contaminants. Ho (1995) reported that CTAB method is effective in obtaining good PCR-grade DNA from *Sargassum*.

Hong *et al.* (1992) developed a rapid extraction method using lithium chloride (LiCl) to isolate DNA from *Porphyra perfora* J. Agardh with no grinding of tissue, hydroxyapatite binding, CTAB treatments, enzymatic treatments, phenol extractions or CsCl ultracentrifugation. In 1995, Hong *et al.* optimised this method by regulating the composition of the extraction solution, heating time and shaking time. However, Hong *et al.* (1997) reported that this method was not

applicable to a few seaweeds such as *Sargassum fulvellum* (Turner) C. Agardh and *Symphyocladia latiuscula* (Mart.) Yamada as the DNAs isolated were not available as PCR template. *Sargassum* DNA isolated using this LiCl protocol was observed to inhibit PCR reaction (Ho *et al.*, 1995a). Jin *et al.* (1997) also showed that extracts from certain seaweeds inhibit *Taq* DNA polymerase.

In 1995, Shin *et al.* developed a simple conchocelis DNA isolation method eliminating the use of liquid nitrogen and CTAB treatment whose residues affect absorbance at 260nm (Doyle & Doyle, 1990). This method reported also does not require the phenol extraction and the RNase reaction. Liu *et al.* (1995) developed a single-step direct PCR amplification from solid tissue. In the same year, Steiner *et al.* reported a rapid one-tube chemical extraction of genomic DNA (Rose extraction method) which can be used for plant, animal and microbial sources of DNA.

## 2.6 MOLECULAR APPROACHES

Molecular techniques used in molecular systematics studies include protein isozyme electrophoresis, molecular cytogenetics (chromosomes studies) and nucleic acids analysis. The nucleic acid characterisation methods are DNA-DNA hybridisation, the polymerase chain reaction, analysis of fragments and restriction sites sequencing & cloning. In 1985, Jeffreys *et al.* introduced the technique of DNA fingerprinting and since then, it has been used to examine

questions of population variation (Gilbert *et al.*, 1990, Alberte *et al.*, 1994, Reeve *et al.*, 1990, Rogstad *et al.*, 1988). In 1994, Coyer *et al.* demonstrated that DNA fingerprinting could measure genetic diversity within a population and that the majority of DNA bands were stably inherited.

Recently, the development of the polymerase chain reaction (PCR) for the *in vitro* amplification of DNA sequences facilitates gene analyses, recombinant techniques, enables DNA detection from single algal cells (Li *et al.*, 1988), and permits sequence determinations even from extinct organisms. There are quite a few PCR-based techniques for DNA markers identification such as Random Amplified Polymorphic DNA (RAPD) (William *et al.*, 1990); DNA Amplification Fingerprinting (DAF) (Caetano-Anolles *et al.*, 1991) and Single Strand Confirmational Polymorphism (SSCP) (Ortia *et al.*, 1989).

The basic principle of enzymatic reaction was first described by Kleppe *et al.* in 1971. Initially, PCR was introduced as a method using two synthetic oligonucleotide primers that anneal to complementary strands of DNA and flank the region of interest in the target DNA (Saiki *et al.*, 1985). Then, it grew from a theoretical scheme to the detection of mutation in the unique human genes (Gibbs, 1990).

Initially, the Klenow fragment of *Escherichia coli* DNA polymerase I was used to extend the annealed primers, but due to its inactivation by the high

temperature, fresh enzyme addition was required during every cycle. However, the subsequent use of heat stable *Taq* DNA polymerase has allowed the development of simple automated thermal cycling devices for carrying out the amplification reaction in a single tube (Saiki *et al.*, 1988a). Other advantages are the increasing specificity and yield of the amplification reaction because non-specific binding or mispairing rarely happen at high temperatures (Vosberg, 1989).

PCR requires a three step cycling process. The first step is denaturation at 94°C in which the DNA to be amplified is separated into single strands which serve as templates for the annealing of primers. Secondly, the annealing of primers to the different strands of the DNA by lowering the temperature. Lastly, the annealed strands will be extended with the *Taq* polymerase and deoxynucleotide triphosphates. These newly synthesised DNA strands are themselves templates for the PCR primers. A repetitive series of cycles of denaturation, primer annealing and extension lead to exponential accumulation of the desired DNA fragments. The amplification reaction eventually reaches a "plateau" after 20-30 cycles where only little increase in DNA can be measured at this stage. The opposing competition of template-template hybrids and template-primer hybrids or the decreasing activity of the *Taq* polymerase in the reaction may cause this (Rehm *et al.*, 1993).

The standard reaction involves the following components: the template DNA, a pair of oligonucleotides complementary to the 3' ends of the double-stranded template DNA, *Taq* polymerase, deoxynucleotide triphosphates and buffers. A number of factors influence the specificity of *Taq* polymerase mediated amplification: the time of the primer extension step, the amount of enzyme used, concentration of $Mg^{2+}$, the nature of template DNA and primers, and the annealing temperature (Mullis *et al.*, 1986; Saiki *et al.*, 1988b; Wu *et al.*, 1991).

Many short protocols for the DNA purification have been reported (Steiner *et al.*, 1995; Mercier *et al.*, 1990; Winberg, 1991; Yamado *et al.*, 1990). Generally, the success rate for PCR amplification of crude DNA extracts is high when the target fragments were relatively abundant. PCR is highly tolerant to impurities in the DNA sample being amplified. Direct PCR without DNA extraction from solid tissues has been carried out (Panaccio *et al.*, 1993).

PCR product detection and identification can be accomplished by size-dependent means where ethidium bromide-stained gel electrophoresis is sufficiently sensitive to provide size-dependent detection (Maniatis *et al.*, 1989). Acrylamide gels give greater resolution than agarose gels and silver staining can increase the sensitivity. In 1989, Allen *et al.* reported that by using discontinuous buffer system, the resolution of acrylamide could be increased. High Performance Liquid Chromatography (HPLC) and capillary

electrophoresis may also be used. The desired PCR product can be purified for subsequent use (Warren *et al.*, 1991; Kalnoski *et al.* 1991). Recently, there have been many reviews on the applications of PCR in forensic analysis, genetic diseases, microbiology, environmental studies, evolutionary biology, developmental diseases (Vosberg, 1989; White *et al.*, 1989; Grainger and Madden, 1993). In the field of seaweed molecular biology, PCR was used to study the heterosis in *Gelidium* (Patwary & Van der Meer, 1994), characterisation of *Porphyra* (Dutcher & Kapraun, 1994) and *Sargassum* (Ho *et al.*, 1995b). PCR amplification of nuclear and plastid genes from algal herbarium specimens and algal species was successfully done by Goff and Moon in 1993.

### 2.6.1 Random Amplified Polymorphic DNA (RAPD)

In 1990, Williams *et al.* developed a new technique to detect genetic polymorphisms in a relatively simple way using a technique known as RAPD (Random Amplified Polymorphic DNA). The AP-PCR (arbitrarily primed PCR) technique was introduced in 1990 by Welsh and McClelland. The genome contains several priming sites close to one another that are in an inverted orientation. The random primer (around 10 bases long with a GC content of 50% or higher) anneals randomly to individual strands of denatured DNA through standard A-T, G-C base pairing. Based on the amplification of DNA with these random primers, RAPD needs neither prior knowledge of DNA sequences nor specific DNA probes. It has other advantages of being technically simple; quick

to perform, requires only small amounts of DNA and does not involve the use of radioisotopes. In RAPD reactions, competition between potential priming sites in the template rather than the total number of priming sites available determines the composition of the amplification products (Rafalski *et al.*, 1991).

RAPD assays were used to access the genetic diversity within and between species of *Allium* (Wilkie *et al.*, 1993), *Amaranthus* (Chan and Sun, 1997), cotton varieties (Iqbal *et al.*, 1997), *Eucalyptus* (Baril *et al.*, 1997), sugar beet (Lorenz *et al.*, 1997) and *Theobroma cacao* (Lerceteau *et al.*, 1997). It is also widely used in detecting genetic markers linked to a gene for a particular trait such as resistance to certain disease and pest (Besnard *et al.*, 1997, Dweikat *et al.*, 1997, Hu *et al.*, 1997, Scholten *et al.*, 1997). Genetic markers representing unique DNA sequences can be used as starting points for chromosome walking in gene mapping (Haring *et al.*, 1996). Such genotypes are valuable in breeding programmes and plant improvement. Other applications of RAPD are in the study of mating systems (Gaiotto *et al.*, 1997), selection of apomictic seedlings from hybrids (Ur-Rahman *et al.*, 1997) and in detecting recombinants (King *et al.*, 1993). In addition, RAPD was used by Chen *et al.* (1997) as tool to detect genomic alterations during plant development or when subjected to certain stress environments.

Among seaweeds, RAPD has been used to study genetic variability of *Porphyra* (Dutcher and Kapraun, 1994), *Gracilaria* spp. (Sinnappah, 1994),

*Sargassum* spp. (Ho *et al.*, 1995a), *Postelsia palmaeformis* (Coyer *et al.*, 1997) and *Gelidium sesquipedale* (Alberto *et al.*, 1997). Patwary *et al.* (1993) revealed the usefulness of RAPD to discriminate algal species and determine relationships within species. They also indicated the possibilities of establishing unique fingerprints for individual plants based on the combined results of several primers. In 1994, Patwary and Van der Meer generated RAPD markers for the detection of heterosis or hybrid plant in *Gelidium vagum*.

### 2.6.2 Amplified Fragment Polymorphism (AFLP)

AFLP analysis is a technique involving the amplification of selected restriction fragments from the digestion of total plant DNA by the polymerase chain reaction (Vos *et al.*, 1995) which produces DNA fingerprints with a large number of genetic markers. The multiplex ratio (the number of information points analysed per experiment) is much higher for AFLP than for other types of markers such as RFLP (Restriction Fragment Length Polymorphism), RAPD or SSRP (Simple Sequence Repeat Polymorphism) (Powell *et al.*, 1996).

AFLP is easy to perform and can identify significant polymorphisms. The genomic DNA is digested by restriction endonucleases such as *Eco*R 1 and *Mse* 1, ligated to *Eco*R 1 and *Mse* 1 adapters, and amplified by PCR using primers that contain the common sequences of the adapters and one to three arbitrary nucleotides as selective sequences. The numbers of selective nucleotides in the

*Eco*R 1 primers and *Mse* 1 primers are critical to AFLP analysis where the number of different amplified DNAs decreased as the number of selective nucleotides of the *Mse* 1 primers increased (Lin *et al.*, 1997). Hence, the number of selective nucleotides in the PCR primers and the complexity of the genome determine the polymorphism.

AFLP is a reliable, reproducible molecular marker assay and has more polymorphisms per reaction compared to RFLP or RAPD. Unlike microsatellite marker assays, the predetermination of the genomic DNA sequences is not needed in AFLP application. Moreover, AFLP has the potential to become a universal molecular marker test as polymorphisms are found with both prokaryotic and eukaryotic organisms.

In 1996, Janssen *et al.* proved the applicability of AFLP in taxonomic studies by comparing the obtained data with results obtained by genotypic and chemotaxonomic methods such as DNA-DNA hybridisation and cellular fatty acid analysis. The discriminative power of AFLP towards the differentiation of highly related strains of the same species or strain characterisation at the intra-subspecific level highlights its potential in epidemiological and evolutionary studies (O'Neill *et al.*, 1997). Diversity and genetic differentiation using AFLP were also studied (Paul *et al.*, 1997; Roa *et al.*, 1997; Schut *et al.*, 1997; Hongtrakul *et al.*, 1997).

### 2.6.3 DNA Sequencing

DNA sequencing is the unravelling of the genetic information contained in DNA (in linear order of nucleotide bases) which uses techniques such as dideoxy sequencing (Sanger *et al.*, 1977) and partial chemical degradation (Maxam and Gilbert, 1977, 1980). The separation method in DNA sequencing must be able to distinguish long oligomers, which differ by only one single nucleotide in length.

## 2.7 MOLECULAR APPROACHES IN UNRAVELLING THE TAXONOMY OF ALGAE

Bhattacharya *et al.* (1990) pointed out that morphological and organelle data alone are not reliable in determining the phylogenetic relationships. The limitations faced with traditional morphology systematic study bring forth the development of additional features. Approaches such as crossability test (Plastino & Oliveira, 1996), chromosome numbers (Bird & Rice, 1990), various chemical substances (Brown & McLachlan, 1982; Craigie *et al.*, 1984) and molecular characteristics (Dutcher *et al.*, 1990; Bird *et al.*, 1992a, 1994; Goff *et al.*, 1994) are widely used. However, data collected from one approach alone will not provide reliable species concept. Therefore, different approaches have to be combined to provide a solution to the identification of any species.

Generally, molecular systematic can be applied to the study of population structure (including geographic variation, mating systems, heterozygosity and individual relatedness), identification of species boundaries (hybridisation) and estimation of phylogenies. In the identification of locally polymorphic markers, samples were collected from various populations representing from closely spaced to geographically distant locations. In addition, the distribution of variation "within" versus "among" populations can also be determined. Characterisation of red algal genomes using this technique provides information for the classification and evolutionary study of Rhodophytes (Li and Cattolico, 1987; Palmer, 1985).

### 2.7.1 Algae

The ribosomal ribonucleic acid (rRNA) coding regions have been amplified using polymerase chain reaction by reverse transcriptase in the presence of deoxynucleotide triphosphates and chain-terminating dideoxynucleotide triphosphates (Hamby *et al.*, 1988). Sequences comparison of this region and their genes provide sufficient evolutionary information for phylogenetic relationship studies of various algal species. In 1991, Buchheim and Chapman used sequences comparison to provide an independent source of data for the inference of phylogenetic relationships among the green algae and embryophytes. In 1992, they reported a cladistic analysis of partial sequences of nuclear-encoded ribosomal RNA (rRNA) genes for both the small (18S) subunit (SSU) and large (26S) subunits (LSU) of both the green algae and land plants (Chapman and

Buchheim, 1992). Sequences of the ribosomal internal transcribed spacer region (ITS) were also used in species delineation of algae. ITS sequences are very similar within species, but provide enough divergence between closely related species. It has been employed in distinguishing biogeographic groups of marine Cladophorales (Bakker *et al.*, 1992; Kooistra *et al.*, 1992, Marks and Cummings, 1996). It has also been used in delineating species of red algal agarophytes (Goff *et al.*, 1994), providing genetic markers for *Ulva* species (Coat *et al.*, 1998) and in inter-and intraspecific studies of genetic variation in *Caulerpa* (Chlorophyta) (Pillmann *et al.*, 1997).

Restriction fragment length polymorphism (RFLP) analysis of nuclear DNA was used by Bhattacharya and Druehl (1990) to produce taxonomic markers for *Laminaria* species. RFLP assays of plastid DNA from *Gracilaria, Smithora, Porphyra, Plocamium, Lomentaria, Polysiphonia, Polyneura* (Goff and Coleman, 1988); *Gymnogongrus*, Phyllophoraceae (Parsons *et al.*, 1990), organellar DNA of *Gracilaria* (Rice and Bird, 1990, Bird *et al.*, 1990b); 18S rDNA of *Gracilaria, Gracilariopsis* (Scholfield *et al.*, 1991) have been carried out.

The identification of algal species by direct comparison of genomes is another alternative of molecular genetics which gives a measure of relatedness that is independent of environmental conditions (Kapraun *et al.*, 1996b). In 1988, Goff and Coleman compared the electrophoretic band patterns of endonuclease-digested plastid DNA to delineate red algal species and populations in order to

establish clearly the proper identification of one *Gracilaria* population of previously uncertain taxonomic affinity. Parsons *et al.* (1990) also used a similar analysis to link the heteromorphic phases of a *Gymnogongrus* population with an uncoupled life history.

### *2.7.2 Gracilaria*

Various molecular approaches have been used in the phylogenetic studies of Gracilariaceae, especially the species of *Gracilaria*. Yabu and Yamamoto (1988,1989) reported on the chromosome counts of *Gracilaria chorda, Gracilaria vermiculophylla* and *Gracilaria verrucosa* from Hokkaido to have a value of n=24. Bird *et al.* (1990b) reported the conspecificity of the two species from opposite sides of the southern Pacific Ocean, *Gracilaria chilensis* Bird, McLachlan et Oliveira from Chile and *G. sordida* Nelson from New Zealand which have similarities in reproductive anatomy, chromosome number, interfertility and organellar DNA restriction profiles. Recently, the nuclear genome profiles have been developed for some species of *Gracilaria* providing information on nuclear genome size, chromosome numbers, karyotype symmetry, genome organisation and also complexity (Kapraun & Dutcher, 1991, Kapraun, 1993, Kapraun *et al.*, 1993, Lopez-Bautista & Kapraun, 1995, Kapraun *et al.*, 1996a, 1996b). In 1993, Kapraun reported that species of *Gracilaria* and *Gracilariopsis* have nearly identical genome sizes of 0.41±0.04 pg (0.37-0.45 pg) despite their distinct chromosome complements of n=24 and n=32, respectively. They observed no significant variation in genome size at both interspecific and

intraspecific levels. However, they found that repeated sequences varied from 13% to 95%, even among taxa of nearly identical genome sizes, hence, the association of speciation with processes such as translocations and fusion/fission which change genome composition but not genome size. In 1995, Lopez-Bautista and Kapraun determined nuclear genome organisation and complexity of *Gracilaria* species from the Mexican Gulf Coast using DNA reassociation kinetics and found the presence of three second order components corresponding to fast/highly repetitive sequences, intermediate/mid repetitive sequences and slow/single copy sequences. They reported that the percentage of unique sequences to repetitive sequences showed wide variations among species of *Gracilaria* and speciation may have been associated with this ratio. In 1996b, Kapraun *et al.* reported similar observations with *Gracilaria* from the Philippines. In estimating the nuclear DNA base composition by the determination of thermal denaturation temperatures (Tm), they reported that at the intraspecific level, Tm profiles indicated maximum similarity values of 79-90% in *Gracilaria tikvahiae* populations, but a range of 46-69% and 73-81% at the interspecific level for *Gracilaria* species and *Gracilariopsis* species, respectively

Bhattacharya *et al.* (1990) studied the phylogeny of *Gracilaria lemaneiformis* Bory Weber-Van Bosse based on nucleotide sequences of the ITS small subunit ribosomal RNA. Comparison of the *Gracilaria lemaneiformis* small subunit rRNA to homologous genes of diverse eukaryotes showed that the red algae diverged simultaneously with the separation of plants, fungi, animals

and other protist lineages. Algae were found to be of polyphyletic origin and there is no relation between red algae and higher fungi. Bird *et al.* (1990a) sequenced the 18S rRNA of *Gracilaria tikvahiae* McLachlan, *Gracilaria verrucosa* (Hudson) Papenfuss and *Gracilariopsis* species. Later (1992b), they compared the 18S rDNA sequences to determine the phylogenetic relationships in the Gracilariales and reported that there are differences in 45 or more nucleotide positions between genera, while subgenera differed by 12-24 nucleotides. They further suggested that *Hydropuntia* may be a subgenus of *Gracilaria*, and *Gracilaria chilensis* Bird, Mclachlan et Oliveira should represent another subgenus and not belong to the subgenus *Textoriella*. Then, in 1994, they used sequence comparison to determine the relationships of unidentified species among the Gracilariaceae.

RFLP was also applied as a molecular tool in the study of genetic variation among *Gracilaria* species. In 1996, Gonzalez *et al.* used RFLP to conduct DNA analyses of morphotypes of *Gracilaria* collected from different geographical locations and observed identical DNA banding patterns from each enzyme digest irrespective of morphotypes and locations. Biogeographic variation among *G. verrucosa* (Hudson) Papenfuss was also studied by Rice and Bird (1990) using this technique. They reported that no two gracilariod strains were identical in all restriction enzyme digests. Their studies revealed a major group composed of strains from Wales, Norway, France, Argentina and Japan, which showed the possibilities of transoceanic distribution via shipping. However, the similarity of

this group was observed to be more pronounced with *Eco*R1 and less with other enzymes. The Japanese strain was found dissociated from the group by *Bam* HI. They also observed that both English strains were found to be appreciably different from each other in all digests even though they are from the same site. Although in one grouping, Brazil and North Carolina strains were reported as different species as the difference in their restriction profiles were more pronounced that within the other identifiable groups.

Another technique was reported by Intasuwan *et al.* (1993) who used starch-gel electrophoresis to assess the allozyme variation among New Zealand populations of *Gracilaria chilensis* Bird, McLachlan and Oliviera. They reported that within populations of *Gracilaria chilensis*, the level of allozyme variation detected was very low and the genetic distances between populations showed a wide range (D=0.00-0.43). Small genetic distances correlated with small geographical distances except in the North Island where the largest genetic distance (0.43) occurred within this area rather than at a maximum geographic distance (between northwest population and the populations in the south and southeast of North Island). Sosa *et al.* (1996) also reported low level of allozyme variation within populations of gametophytes of *Gracilaria cervicornis* (Turner) J. Agardh from Canary Islands along with the observation of vegetative fragmentation. Significant genetic differentiation was detected between the Maspalomas population with the other two populations at two loci, which are

suspected to link to micro-environmentally controlled selection as no significant differences in salinity and temperature of these three locations were observed.

Single locus microsatellites were also used in the taxonomic study of *Gracilaria*. In 1997, Wattier *et al.* reported that two (GvIAAG and GvIAAC) out of four locus microsatellites in *Gracilaria gracilis* were polymorphic. They also observed high polymorphism variation within population and the combination of these two highly polymorphic loci was powerful in identifying individuals where 93% was found to have a unique genotype.

In 1994, Sinnappah showed that the amplification of the 18S rRNA gene of *Gracilaria* species was achieved only when the annealing temperature was increased from 37°C to 55°C as the DNA was unstable at 60°C. All the *Gracilaria* species she studied showed 1800bp product except for *Gracilaria salicornia,* which showed two amplified products of 1900bp and 2100bp. RFLP fragments from the digestion of 18S rRNA by *Msp* I showed *Gracilaria changii* samples and *Gracilaria edulis* samples have similar patterns. She also found difficulties in amplifying the ITS1/5.8S/ITS2 gene probably caused by the presence of inhibitors or polysaccharides. However, she reported that with RAPD technique, individual fingerprints for individual specimens of *Gracilaria* was successfully generated. With one of the primer tested, OPA11 (CAATCGCCGT), she observed that *Gracilaria changii* (Morib, Malaysia) was totally dissimilar to *Gracilaria edulis* (Morib, Malaysia) and *Gracilaria salicornia* (Cape Rachado,

Malaysia) but there was 40% similarity between *Gracilaria salicornia* and *Gracilaria edulis*. Two bands (possible species markers) were observed in *Gracilaria salicornia* with OPA1 (CAGGCCCTTC). Two possible markers were also reported with OPA11 for both *G. edulis* and *G. salicornia*, which show high similarity to each other. OPA7 (GAAACGGGTG) and OPA9 (GGGTAACGCC) were reported to be good in separating individual samples as no common band was detected. OPA 1 was reported to be able to separate *G. salicornia* samples collected from different habitat. From her observation, different primers used give different value of similarity for two different samples depending on either a conserved or variable region been amplified.

Meneses (1996) have carried out the assessment of RAPD techniques as a tool in phylogenetic studies of *Gracilaria*. In her study on the gametophytic samples of *Gracilaria chilensis* collected from different locations, Meneses reported that RAPD was able to distinguish populations although variability in DNA fingerprints was present within populations. She attributed the cause of this intrapopulation variation to the constant mixing of material through massive transplants (cultivation), spore coalescence and mitotic recombination (especially vegetative propagation). Besides sequence differences, differences in banding patterns may be caused by other factors such as the ploidy of the samples tested where bands present in diploids may not be found in haploids. She also observed that RAPDs analysis was able to separate *Gracilaria chilensis* and *Gracilaria tenuistipitata*, which were shown to have close relationship by nucleotide

sequence of both rDNA and Rubisco spacer. Meneses *et al.* (1998a) observed intraclonal variability in growth rates of *G. chilensis* when fragments of the same individual were cultured under the same conditions. RAPD analysis revealed genetically-based variability in the clones which changes in growing thalli and seemed to be related to temperature where higher levels of polymorphism was observed at 18°C than at 14°C. They linked this variation to mitotic recombination as these fragments are actively growing apical fragments. In 1998(b), Meneses *et al.* observed that intraclonal variability in *G. chilensis* increases with increasing growth rates under laboratory conditions. They reported that genetic differences were detected with time where after a certain period of growth, genetic variability starts being expressed. They also observed that genetic variability is also induced by the different concentration of copper in the medium.

## 2.8 BIOTECHNOLOGY AND PLANT BREEDING

Biotechnology is the integrated application of microbiology, biochemistry and engineering towards the use of microorganisms, cell and tissue cultures to manufacture useful products. "New biotechnology" which involves genetic engineering and cell fusion to produce organisms capable of making useful products is predicted to dominate the biotechnology industry in the future (Crueger and Crueger, 1989).

In any breeding or genetic conservation programme, knowledge about relationships between genotypes and genetic diversity is very important for its success. Conventional methods of plant breeding have been successful in generating improved varieties of many of the major world crops (Batchvarov, 1993; Chiang *et al.*, 1993; Crisp & Tapsell, 1993). This has mostly been in the improvement of yield and selection of lines resistant to pest and diseases, which can tolerate stressful environments and also improvements in the intrinsic quality of the crop. Furthermore, the creation of new plant varieties with the incorporation of value-added traits using recombinant DNA techniques is the long range objective of biotechnology. In China, genetic and plant breeding has been applied in the cultivation industry of *Laminaria japonica*. The new varieties obtained yield 8-40% more biomass with 20-58% higher iodine content than the control plants (Wu and Lin, 1987).

The non-recombinant approaches to plant improvement are the exploitation of spontaneous or induced variation in cultured plant cells or tissues and the use of intra- and inter-specific protoplast fusion to mediate genetic exchange. Somaclonal variation technology takes advantage of the naturally occurring genetic variation that appears in the plants regenerated from somatic cells grown in tissue culture (Scowcroff and Larkin, 1988). The genetic diversity of plants emerging from disorganised callus tissues provides the breeder with a means of introducing variability into established cultivars without the use of sexual crosses. Unfortunately, callus formation is rather rare with seaweed,

occurring on 0.5-2.7% of sectioned tissues. Calluses of seaweeds are slow growing, require low light levels (Polne-Fuller and Gibor, 1987) and is influenced by the culture medium (Liu and Gordon, 1987).

However, it is more desirable to cross two related species that are sexually incompatible and this can be achieved by fusing the relevant plant protoplasts. Protoplasts are cells that have had their walls removed by enzymatic or detergent treatment and are protected against lysis due to osmotic shock by suspension in a buffer containing a high solute concentration. Isolated protoplasts can be induced to fuse with each other even when derived from different species using certain chemicals (as fusogens) such as polyethylene glycol, dextran and poly-L-ornithine or electrofusion. Protoplast fusion is a useful technique for achieving gene transfer and genetic recombination in organisms (Fujita and Migita, 1987; Mizukami *et al.*, 1995; Lee and Tan, 1988). Protoplast isolation and fusion in *Porphyra* (Bangiales, Rhodophyta) produced chimeral thalli which has a mixture of chromosome numbers and pigmentation. In addition, Fujimura and Kajiwara (1990) have successfully produced bioflavor by immobilised cells regenerated from protoplasts of *Ulva pertusa* (Ulvales, Chlorophyta).

Recently, molecular biology has experienced a revolutionary change with the development of genetic engineering. Its important feature is the ability to cross natural species barriers and place genes from any organism in an unrelated host organism resulting in the propagation of a defined and relatively small piece

of DNA in the host organism. This gives way to other molecular biological opportunities including nucleotide sequence determination, site-directed mutagenesis and manipulation of gene sequences to ensure very high level expression of an encoded polypeptide in a host organism. Therefore, genetic engineering has the advantage of introducing genes into a plant, which do not exist in any member of the same plant family, or even in any plant and in altering the key cellular proteins which changes are unlikely to happen spontaneously through protein engineering. However, two basic capabilities must be considered. Firstly, DNA sequencing where the knowledge of the sequence of a cloned DNA fragment is a prerequisite for planning any substantial manipulation of the DNA and secondly, gene synthesis. In short, if genetically engineered plants are to be used commercially, the following requirements must be met (Primrose, 1987):

1. introduction of the gene(s) of interest to all plant cells
2. stable maintenance of the new genetic information
3. transmission of the new gene to subsequent generations
4. expression of the cloned genes in the correct cells at the correct time

Several studies have been carried out. In 1995, Matsunaga and Takeyama studied different gene transfer methods and characterised a plasmid from the marine cyanobacteria, which was used in developing a stable gene expression system. Chauvat *et al.* (1988) emphasised the importance of having a cloning vector that allows a conditional expression of foreign genes to be cloned. Lluisma and Ragan (1998a, 1998b, and 1999) have cloned and characterised the nuclear

gene encoding a starch-branching enzyme, a galactose-1-phosphate uridylyltransferase gene and a UDP-glucose pyrophosphorylase gene from *Gracilaria gracilis*. Patwary and Van der Meer (1992) have looked into the genetics of *Gracilaria* for developing fast growing strains. Shen and Wu (1995) generated chloramphenicol and cadmium resistant mutant in *G. tenuistipitata* through a mutagenesis and selection procedure using ultraviolet treatment and DNA insertion, respectively. In 1997, Stevens and Purton reported on the progress and prospects of genetic engineering of eukaryotic algae and its role in the advancement of algal biotechnology based industries.