# 2.0 LITERATURE REVIEW

## 2.1 *Sargassum*

### 2.1.1 Tropical *Sargassum* species

A total of 110 species of *Sargassum* are widely distributed in the Pacific basin, in tropical and subtropical areas. The tropical regions are situated between 30N and 30S with the seawater temperature of 24-30°C. The regions include:

I) Central Pacific region (Table 1.1):

   I) Northern Hemisphere: known as isolated, are of volcanic origin. This includes the Hawaiian archipelago, Micronesia (Gilbert, Marshall, Caroline, and Mariana islands, and Guam).

   ii) Southern Hemisphere: Indonesia and New Guinea, the Solomon Islands, New Caledonia, Fiji, Samon islands, Society Islands and Australia.

II) Western Pacific (Table 1.2): Mainland of China, Vietnam, Thailand, Cambodia, Malaysia, Singapore and The Philippines.

| Hemisphere | State/Country | Species |
|---|---|---|
| Northern | Hawaii | *Sargassum echinocarpum* |
| | | *S. hawaiiensis* |
| | | *S. obtusifolium* |
| | | *S. polyphyllum* |
| Literature: Doty and Newhouse, 1966; Tsuda, 1966; De Wreede and Jones, 1973; Magruder, 1988; Abbott, 1989; Noro *et al.*, 1994. | | |
| | Micronesia | *S. crassifolium* |
| | | *S. cristaefolium* (=*S. ilicifolium*) |
| | | *S. polycystum* |
| | | *S. tenerrimum* |
| Literature: Tsuda, 1965, 1968, 1972b, 1981, 1988; Tsuda and Belk, 1972; Tsuda and Wray, 1977; Tsuda and Tobias, 1977; Noro *et al.*, 1994. | | |
| Southern | Indonesia | *S. bacciferum* |
| | | *S. baccularia* |
| | | *S. biserrula* |
| | | *S. carpophyllum* |
| | | *S. cristaefolium* |
| | | *S. decaisnei* |
| | | *S. duplicatum* |
| | | *S. echinocarpum* |
| | | *S. gracile* |
| | | *S. granuliferum* |
| | | *S. grevillei* |
| | | *S. hemiphylloides* |
| | | *S. heterocystum* |
| | | *S. ilicifolium* (=*S. sandei*) |
| | | *S. microcystum* |
| | | *S. microphyllum* |
| | | *S. oligocystum* (=*S. binderi*) |
| | | *S. paniculatum* |
| | | *S. plagiophylloides* |
| | | *S. parvifolium* |
| | | *S. polycystum* |
| | | *S. spinifex* |
| | | *S. siliquosum* |
| | | *S. swartzii* |

Table 1.1: Distribution of *Sargassum* in the Central Pacific region

| Hemisphere | State/Country | Species |
|---|---|---|
| | New Guinea | *S. baccularia* |
| | | *S. biserrula* |
| | | *S. granuliferum* |
| | | *S. heterocystum* |
| | | *S. pulchellum* |
| | | *S. siliquosum* |
| | | *S. tenue* |
| | Solomon | *S. cristaefolium* |
| Literature: Reinbold, 1913; Setchell, 1935; Levring, 1960; Womersley and Bailey, 1969. | | |
| | New Caledonia | *S. carpophyllum* |
| | | *S. cinctum* |
| | | *S. coriifolium* |
| | | *S. crassifolium* |
| | | *S. desvauxii* |
| | | *S. duplicatum* |
| | | *S. echinocarpum* |
| | | *S. filifolium* |
| | | *S. fissifolium* |
| | | *S. flavicans* |
| | | *S. ilicifolium* |
| | | *S. lophocarpum* |
| | | *S. myriocystum* |
| | | *S. oligocystum* |
| | | *S. polycanthum* |
| | | *S. spathulaefolium* |
| | | *S. stenophyllum* |
| | | *S. torvum* |
| | | *S. turbinariodes* |
| Literature: Garrigue and Tsuda, 1988. | | |
| | Fiji | *S. cristaefolium* |
| | | *S. horridulum* |
| | | *S. obovatum* |
| | | *S. polycystum* |
| Literature: Garbary *et al.*, 1991; South and Kasahara, 1992. | | |
| | Samoan islands | *S. anapense* |
| | | *S. fonanonense* |
| Literature: Setchell, 1924; Hunter *et al.*, 1993. | | |

Table 1.1: Distribution of *Sargassum* in the Central Pacific region (continued)

| Hemisphere | State/Country | Species |
|---|---|---|
| | Society Islands | *S. boraborense* |
| | | *S. magarevense* |
| | | *S. sociale* |
| | | *S. tahitense* |
| Literature: Setchell and Parks, 1926; Taylor, 1973; Payri and Naim, 1982; Payri, 1987. | | |
| | Australia | *S. distichum* |
| | | *S. filifolium* |
| | | *S. linearifolium* |
| | | *S. peronii* |
| | | *S. podacanthum* |
| | | *S. spinuligerum* |
| Literature: Agardh, 1889; Lucas, 1935; Womersley, 1954, 1959, 1966, 1967, 1987; Shepherd and Womersley, 1971; Clayton and King, 1981. | | |

Table 1.1: Distribution of *Sargassum* in the Central Pacific region (continued)

| Hemisphere | State/Country | Species |
|---|---|---|
| | China | *S. aemulum* |
| | | *S. angustifolium* |
| | | *S. assimile* |
| | | *S. baccularia* |
| | | *S. cinereum* |
| | | *S. glaucescens* |
| | | *S. graminifolium* |
| | | *S. henslowianum* |
| | | *S. incanum* |
| | | *S. kuetzingii* |
| | | *S. paniculatum* |
| | | *S. polycystum* |
| | | *S. siliquosum* |
| | | *S. tenerrimum* |
| | | *S. vachellianum* |
| Literature: Tseng *et al.*, 1985; Tseng and Lu, 1988, 1992a, 1992b. | | |
| | Vietnam | *S. angustifolium* |
| | | *S. baccularia* |
| | | *S. cinereum* |
| | | *S. crassifolium* |
| | | *S. cymosum* |
| | | *S. duplicatum* |
| | | *S. feldmannii* |
| | | *S. glaucescens* |
| | | *S. gracillimum* |
| | | *S. graminifolium* |
| | | *S. henslowianum* |
| | | *S. incanum* |
| | | *S. paniculatum* |
| | | *S. piluliferum* |
| | | *S. polycystum* |
| | | *S. polyporum* |
| | | *S. siliquosum* |
| | | *S. swartzii* |

Table 1.2: Distribution of *Sargassum* in the Western Pacific region

| Hemisphere | State/Country | Species |
|---|---|---|
| | Vietnam (continued) | *S. tenerrimum* |
| | | *S. vachellianum* |
| | | *S. vietnamense* |
| | | *S. virgatum* |
| Literature: Dawson, 1954; Dinh *et al.*, 1993. | | |
| | Thailand | *S. grevillei* |
| | | *S. polycystum* |
| Literature: Velasquez and Lewmanomont, 1975. | | |
| * | Singapore | *S. asperifolium* |
| | | *S. baccularia* |
| | | *S. cinereum* |
| | | *S. duplicatum* |
| | | *S. glaucescens* |
| | | *S. granuliferum* |
| | | *S. ilicifolium* (=*S. sandei*) |
| | | *S. myriocystum* |
| | | *S. oligocystum* |
| | | *S. polycystum* |
| | | *S. spathulaefolium* |
| | | *S. torvum* |
| Literature: Teo and Wee, 1983; Phang and Wee, 1991. | | |
| | Philippines | *S. aemulum* |
| | | *S. agardhianum* |
| | | *S. baccularia* |
| | | *S. balingasayense* |
| | | *S. bataanense* |
| | | *S. belangeri* |
| | | *S. oligocystum* (=*S. binderi*) |
| | | *S. cinctum* |
| | | *S. crassifolium* |
| | | *S. crispifolium* |
| | | *S. cristaefolium* (=*S. berberfolium*) |
| | | *S. currimaoense* |
| | | *S. cystocarpum* |
| | | *S. cystophyllum* |
| | | *S. duplicatum* |
| | | *S. dotyi* |

* List of Malaysian *Sargassum* are given separately in Table 2.

Table 1.2: Distribution of *Sargassum* in the Western Pacific region (continued)

| Hemisphere | State/Country | Species |
|---|---|---|
| | Philippines (continued) | *S. droserifolium* |
| | | *S. filifolium* |
| | | *S. filiforme* |
| | | *S. feldmannii* |
| | | *S. gaudichaudii* |
| | | *S. giganteifolium* |
| | | *S. gracile* |
| | | *S. granuliferum* |
| | | *S. gracillimum* |
| | | *S. hemiphyllum* |
| | | *S. heterocystum* |
| | | *S. ilicifolium* (=*S. sandei*) |
| | | *S. kushimotense* |
| | | *S. latifolium* |
| | | *S. microcystum* |
| | | *S. myriocystum* |
| | | *S. ohnoi* |
| | | *S. oligocystum* |
| | | *S. oocyste* |
| | | *S. paniculatum* |
| | | *S. parvifolium* |
| | | *S. polyceratum* |
| | | *S. polycystum* |
| | | *S. pteropleron* |
| | | *S. samarense* |
| | | *S. siliquosum* |
| | | *S. spinifex* |
| | | *S. subspathulatum* |
| | | *S. sullivanii* |
| | | *S. tenerrimum* |
| | | *S. turbinarioides* |
| | | *S. umezakii* |
| | | *S. velasquezii* |
| | | *S. vulgare* |
| | | *S. yamadae* |
| | | *S. yoshidae* |

Literature: Silva et al., 1987; Trono, 1992, 1994.

Table 1.2: Distribution of *Sargassum* in the Western Pacific region (continued)

### 2.1.2 Malaysian *Sargassum* species

Malaysia includes both island and mainland regions and has coastlines bounded by four seas, that is, South China Sea, Sulu Sea, Andaman Sea, and Celebes Sea. Due to the geographical proximity of Malaysia and Singapore, they share many algal and endemic species.

There are a total number of 27 species of *Sargassum* reported in Malaysia (Table 2) (Teo and Wee, 1983; Phang, 1984, Crane, 1988; Ahmad and Hindun, 1991; Phang and Wee, 1991; Ahmad and Go, 1994; Phang and Yoshida, 1997). Among these, 11 species are from the western coasts, 5 species from the eastern coasts, 5 species from Tioman Island (Ahmad and Go, 1994) of Peninsular Malaysia, 6 species from Sava (Borneo) (Phang and Wee, 1991), Recently, a new species was found in Penang Island (Phang and Yoshida, 1997).

| | Species |
|---|---|
| 1 | *Sargassum asperifolium* (Hervey et Mart.) J. Agardh |
| 2 | *S. baccularia* (Mert.) C. Agardh |
| 3 | *S. binderi* (Sonder) J. Agardh |
| 4 | *S. cinereum* J. Agardh |
| 5 | *S. crassifolium* J. Agardh |
| 6 | *S. cristaefolium* J. Agardh |
| 7 | *S. duplicatum* J. Agardh |
| 8 | *S. filipendula* C. Agardh |
| 9 | *S. fluitans* (Borgesen) Borgesen |
| 10 | *S. glaucescens* J. Agardh |
| 11 | *S. granuliferum* C. Agardh |
| 12 | *S. grevellei* J. Agardh |
| 13 | *S. hornschuchii* Agardh |
| 14 | *S. ilicifolium* (Turner) C. Agardh |
| 15 | *S. muticum* (Yendo) Fensholt |
| 16 | *S. myriocystum* J. Agardh |
| 17 | *S. oligocystum* Montagne |
| 18 | *S. polycystum* C. Agardh |
| 19 | *S. sandei* Reinbold |
| 20 | *S. siliquosum* J. Agardh |
| 21 | *S. spathulaefolium* J. Agardh |
| 22 | *S. stolonifolium* Phang et Yoshida |
| 23 | *S. tenerrimum* J. Agardh |
| 24 | *S. torvum* J. Agardh |
| 25 | *S. virgatum* (Mertens) Agardh |
| 26 | *S. vulgare* C. Agardh |
| 27 | *S. wightii* Greville |

Table 2: List of Malaysian *Sargassum* species

### 2.1.3 Problems associated with the taxonomy of *Sargassum*

*Sargassum* is highly differentiated, thus, it is the most anatomically complex genera in the Phaeophyta. The phenotypic expression of its morphology is complex as variations exhibit temporal (as plant morphology may change with its age (Womersley, 1954; Jephson and Gray, 1977; Umezaki, 1986)), intra-individual (variation within and between primary laterals (Umezaki, 1974, 1984)) , inter-individual (dimorphism between sexes (Reinbold, 1913; Collins and Hervey, 1917)), environmental (affecting the morphology established at phenotypic level and the survivorship of progeny at genotypic level) and geographical differences in trait expression between local and broadly distributed populations (Kilar *et al.*, 1992).

Confusions that exist in the classification of *Sargassum* include: Phenotypic plasticity due to different environmental conditions (Taylor, 1960; Soe-Htun and Yoshida, 1986), ontogenetic forms in its life history (Womersley, 1954; Critchley, 1983a, 1983c; Ang and Trono, 1987; Kilar and Hanisak, 1988), low-frequency traits, polymorphisms (Kilar and Hanisak, 1988, 1989), overemphasis on obvious features which is not well understood, hybridization (Taylor, 1960; Paula and Oliveira, 1982), polyploidy, designation of varieties and forms (Grunow, 1915, 1916a, 1916b; Setchell, 1931, 1933, 1935, 1936; Womersley, 1954; Yoshida, 1983), type of specimens (Womersley, 1954; Yoshida, 1983; Hanisak and Kilar, 1990), uncertainty as to which traits are taxonomically important (Parr, 1939; Taylor, 1960), and incomplete

ecological, development and reproductive information (Womersley, 1954; Earle, 1969; Soe-Htun and Yoshida, 1986; Kilar and Hanisak, 1988, 1989).

One of the best examples is the classification of *Sargassum oligocystum* Montagne and *S. binderi* Sonder. The morphological characteristics of these *Sargassum* appear to be similar with only minor differences. They all belong to a group with flattened main branches, leaves which are lanceolate and dentate with small teeth. The only differences to distinguish these *Sargassum* are the vesicles and the receptacles. The vesicles of *S. oligocysumi* are spherical while winged vesicles appear in *S. binderi*. *S. oligocystum* was identified to have spines only at the tips of receptacles whereas *S. binderi* have sharp dentate receptacles. As reported by Womersley and Bailey (1970), *S. binderi* was the synonym of *S. oligocystum*. However, Ajisaka (1998, personal communication) has decided to classify *S. binderi* and *S. oligocystum* separately based on the characteristics of the receptacles.

Approaches to overcome the above mentioned problems include:

i) Seasonal studies where samples in a populations are studied in detail throughout the year.

ii) Geological studies which compare the distribution of a population within the local and widely distributed populations.

iii) Reaction norms where the polyphenism is properly studied.

iv) *In vitro* culture of the plants enable a better understanding of the intra- and inter-specific boundaries.

v) Numerical taxonomy where both the phenotypic and genotypic responses of the plant are considered.

vi) Genetic studies where the molecular data enable the measurement of changes occurring in the genome. Problems associated with phenotypic convergence and plasticity are overcome by using genotypic characters. (Kilar and Hanisak, 1989)

## 2.2 Conventional classification of *Sargassum*

According to the classification system of *Sargassum*, it can be divided into 5 subgenera, namely *Bactrophycus*, *Arthrophycus*, *Phyllotrichia*, *Schizophycus* and *Eusargassum* (= *Sargassum*) (C. A. Agardh, 1820; J. G. Agardh, 1848, 1889; Grunow, 1915, 1916; Setchell, 1931, 1935, 1936) based on morphogenic relations between stem and blade (J. G. Agardh, 1889; Yoshida, 1983). The subgenus of *Eusargassum* was then subdivided by J. G. Agardh (1889) into two groups based on the receptacles, i.e. *Zygocarpicae* and *Cladocarpicae*.

*Sargassum* is one of seven genera in the family Sargassaceae (Kuetzing), in the order of Fucales. As might be imagined with more than 400 species (Yoshida, 1983), *Sargassum* taxonomy is complex. The classification and identification of *Sargassum* subgenus *Sargassum* is based on the descriptions of the morphological features. These include the leaf-like blade which range from linear to lanceolate to ovate with entire, serrate, or highly dentate margins (Kilar *et al.*, 1992). Also, blades can be flat, recurved, undulate or inflated. The arrangement and size of crypstomata on the blades is also important for taxonomic purposes. The number of laterals arising from the main stem and the degree of branching (Womersley, 1954) are also characters that are used for the description. Stems (axes) can be terete or triangular in cross section, smooth or verrucose, with or without acute spines. Vesicle numbers and features which included winged/alated or non-winged, spherical to rarely elliptical, terminated by a

sharp tip or not are used. (Critchley, 1983a; Yoshida, 1983; Tseng and Chang, 1984; Kilar and Hanisak, 1988). Fruiting branches which contain sexual structures in the receptacles can be terete or angular, smooth or armed with teeth, in racemes, panicles or cymes. There are two kinds of reproductive bodies, i.e. antheridia (small and motile sperms) and oogonia (large and non-motile eggs). The presence of antheridia, or oogonia, or both, within the conceptacle of the receptacle is also used for classification. The plant may be dioecious, monoecious, androgynous or hermaphrodite.

Due to the highly differentiated morphology and anatomically complex genera, the recognition and description of *Sargassum* is difficult. Also, systematic confusion exists due to the variations in morphological features in form, size, and numbers (frequencies) (Kilar and Hanisak, 1989). As a result, errors or uncertainty of classification originating from morphological characteristics will affect the creation of major taxonomic groups (Morris, 1973).

Therefore, studies that include both morphological and molecular data will endow much better descriptions and assessment of biological diversity than those that concentrate on just one approach (Moritz and Hillis, 1990).

## 2.3 DNA extraction

DNA characterisation has enabled plant scientists to further understand the plant genome, especially through the rapid proliferation of mapping, genotyping, and diagnostic methodologies. For all these procedures, high quality DNA is required as a starting material. However, plants are particularly notorious for their intrastablility with many isolation procedures. Using published procedures for purifying plant genomic DNA of one plant group will often fail miserably with others, an outcome that is not unexpected given the diversity of plants and their secondary compounds.

A major contamination problem to deal with in plants, is the presence of polysaccharides and phenolic compounds that exist in the cell walls and intercellular matrix of plants, which always happen to coextract with nuclear DNA. Polysaccharides have been shown to inhibit the activity of a wide range of DNA-modifying enzymes such as restriction enzymes, polymerases and ligases (Aoki and Koshihara, 1972; Furukawa and Bhavadna, 1983; Richards, 1988; Shioda and Muofushi, 1987). Besides polysaccharides and phenolic compounds, some secondary metabolites such as tannins will also interfere with the extraction strategy.

The difficulty of using DNA isolation methods is also one of the common problems in the purification of plant DNA. Many existing methods employ technically demanding techniques such as CsCl ultracentrifugation (Goff and Coleman, 1988; Roell and Morse, 1991). Secondly, the speed to obtain the DNA is also considered. Some existing methods vary from several hours to days. For example, samples are ultracentrifuged for over 15 hours in a CsCl density gradient in order to remove the contaminating polysaccharides. Also, this method is very costly, time-consuming and requires a large amount of starting material.

As a result, a popular method with lower cost is used as an alternative, that is the hexadecyltrimethylammonium bromide (CTAB) method (Richards, 1987). The CTAB method is widely used by plant geneticists to separate contaminating polysaccharides from nucleic acids based on their different solubilities in the presence of CTAB. Another simple, rapid method of DNA purification involves high concentrations of salts and detergents such as sodium dodecyl sulfate (SDS) or potassium acetate. Proteins and polysaccharides form insoluble complexes with SDS or potassium acetate and are thereby separated from the DNA. Using lithium chloride (LiCl) to purify the plant DNA inexpensively is also one of the choices among plant researchers. By the LiCl treatment, grinding of tissues, protoplast release, or other expensive tools are not required as reported by Hong *et al.* (1992) who successfully extracted the DNA from *Porphyra perforata* (Rhodophyta) as a template for PCR amplification.

DNA, defined nucleic acid probes, laborious hybridization procedures, difficulty in standardization, the requirement of a great deal of operator time and effort, the relatively small numbers of polymorphisms generated, and the extrapolation of genetic relationships based on variation of small, discrete regions of the chromosomes.

### 2.4.2 Random Amplified Polymorphism DNA (RAPD)

The Random Amplified Polymorphism DNA (RAPD) (Williams *et al.*, 1990) is a technique based on the polymerase chain reaction (PCR) (*Saiki et al.*, 1988; Erlich *et al.*, 1991; Hardys *et al.* , 1992) to differentially amplify anonymous regions of genomic DNA fragments using oligonucleotide primers and low annealing temperatures (Williams *et al.*, 1990; Welsh & McClelland, 1990; Caetano-Anollés *et al.*, 1991a). This procedure is also generally referred to as arbitrary primed PCR (Welsh & McClelland, 1990), or DNA amplification fingerprinting (Caetano-Anollés *et al.*, 1991a,).

RAPD technique is very easy to perform and requires no prior investigation of the genomes. The advantages of this technique resulted in widespread acceptance of its use to generate genetic markers for mapping studies (Klein-Lankhorst *et al.*, 1990; Williams *et al.*, 1990; Chaparro *et al.*, 1994), identifying both inter- and intra-specific somatic hybrids in crop plants (Williams *et al.*, 1990; Waara *et al.*, 1991; Bird *et al.*, 1992), taxonomic sorting tools (Adams and Demeke, 1993; Smith *et al.*, 1994), molecular ecological investigations (Hardys *et al.*, 1992), biogeographic studies (van Heusden and Bachmann, 1992; Thorpe *et al.*, 1994; van Oppen *et al.*, 1994), strain identification and genomic fingerprinting (Welsh and McClelland, 1990; Wilde *et al.*, 1992), comparing polymorphisms in anonymous (arbitrary) nucleotide sequences for *Gelidium vagum* Okamura (Patwary *et al.*, 1993), population genetic studies

(Chalmers *et al.*, 1992; Dawson *et al.*, 1993), and paternity analysis (Hadrys *et al.*, 1993). Previously, this method was also used in the recognition of animal species (Fong *et al.*, 1995; Pfenninger *et al.*, 1995) as well as variety and hybrid distinction in commercial plants and crops (Welsh *et al.*, 1991; Novy *et al.*, 1994). RAPD enables construction of linkage maps of higher plants (Giese *et al.*, 1994) and insects (Hunt and Page, 1995), and detection of polymorphisms in inbred strains of mice (Woodward *et al.*, 1992). For marine algae, where relatively little is known about population structure at the genetic level, RAPD is a good approach. This technique was used to characterise *Sargassum polycystum* and *S. siliquosum* (Phaeophyta) by Ho *et al.*, 1995.

Compared to other DNA techniques, the use of RAPD markers is relatively fast, easy and inexpensive. The technique only requires a very small amount of target DNA in each reaction and many reactions can be amplified simultaneously in commercially available thermal cyclers In addition, the reaction products can be resolved and documented quickly using gel electrophoresis and photography. The study of RAPD in comparison with allozyme analysis (Aagaard *et al.*, 1995) shows that RAPD emphasises greater differentiation among populations since its variation is likely to result from noncoding DNA subjected to more rapid changes than the functional genes encoding allozymes which will be also less variable due to the action of natural selection. The advantage of RAPD is further enhanced as the technique can also be applied when only small amounts of material are available or when it is required not to

affect the viability of the sample under study, for example, an endangered species (Pfenninger *et al.*, 1995). RAPD can also be used as genetic markers in unmutagenised plant lines due to its simplicity, rapidity, and ability to reveal naturally existing DNA polymorphisms.

However, the shortcoming of this technique is its primer. As the primer is short, the probability of the genome containing several priming sites close to one another that are in an inverted orientation is high. Another disadvantage of this technique is the preferential amplification of DNA fragments. As a result of the preferential amplification of DNA fragments, 'false' or 'masked' relationships between taxa or populations can occur. This technique may have reproducibility problems between laboratories (Wilkerson *et al.*, 1993).

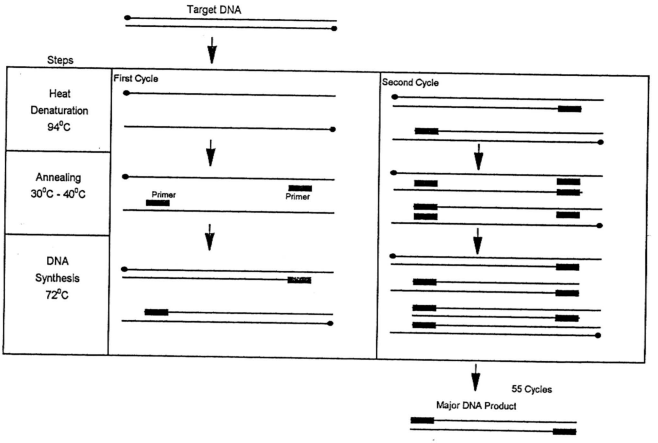


Figure 1: Steps for RAPD-PCR

### 2.4.3 Molecular data / DNA Sequence data

Molecular data are the most appropriate data for addressing phylogenetic questions (Avise, 1994). For example, recent analysis of DNA sequences provided an independent set of data to address brown algal phlyogenetic issues at various taxonomic levels: from generic to ordinal relationships (Saunders and Druehl 1992; Tan and Druehl, 1993, 1994; Saunders and Kraft, 1995).

Ribosomal DNA (rDNA) and ribosomal RNA (rRNA) sequences are often employed to study phylogenetic relationships between either closely or distantly related species (Brown, 1990; Barnes, 1991; Penny and O'Kelly, 1991). In eukaryotes, the rRNA genes are cotranscribed, producing a single transcript consisting of an external transcribed spacer (ETS), the small-subunit rRNA gene (SSU), the first internal transcribed spacer (ITS1), the 5.8S rRNA gene, the second internal transcribed region (ITS2), and the large-subunit rRNA gene (LSU) (Appels & Honeycutt, 1986). In many lower eukaryotes and most higher eukaryotes examined, the 5S RNA genes are found at a different locus than that of larger rDNAs on the nuclear genome (Gerbi 1985, Appels and Honeycutt, 1986). The SSU, 5.8S gene, 5' region of LSU and SSU rRNAs (18S rDNAs) have been employed in eukaryote phylogenies (Sogin *et al.*, 1989; Baroin *et al.*, 1988; Yokota *et al.*, 1989, Cedergren *et al.*, 1988; Field *et al.*, 1988; Lake, 1990; Ariztia *et al.*, 1991; Barta *et al.*, 1991; Chapman & Buchheim, 1991). These sequences are characterised by conserved regions common to all

eukaryotes, and variable regions that reflect finer details of phylogenetic descent. However, the 5.8S gene is considerably smaller than the SSU or LSU and its usefulness in taxonomic investigations for close and distantly related organism has been questioned (McCarrol *et al.*, 1983; Sogin *et al.*, 1986). Several such sequences have been reported for members of the Rhodophyta (Bhattacharya *et al.*, 1990; Bird *et al.*, 1990; Hendriks *et al.*, 1991) as well as the Phaeophyta (Saunders and Druehl, 1992) and Chlorophyta (Kantz *et al.*, 1990; Zechman *et al.*, 1990; Chapman and Buchheim, 1991). Such a variety of rRNA gene arrangements reflects the evolutionary changes of rRNA genes in various phylogenetic groups and is therefore considered to have phylogenetic implications at intrafamilial and intrageneric levels (Appels and Honeycutt, 1986).

Nuclear ribosomal DNA sequences have been extensively used over the past decade to infer phylogenetic relationships among taxa at different hierarchical ranks (Hillis & Davis, 1986; Woese, 1987; Cedergren *et al.*, 1988; Zimmer *et al.*, 1989; Huss & Sogin, 1990; Mindell & Honeycutt, 1990). In algae, rDNA sequences have been used to assess phylogenetic relationships among species and genera (Perasso *et al.*, 1989; Rausch *et al.*, 1989; Druehl, 1990; Huss & Sogin, 1990; Zechman *et al.*, 1990). For studies at and below the species level, however, rDNA coding regions reach their limit of resolution (Jŋgensen and Cluster, 1988; Hillis and Moritz, 1990) and, for this reason, nuclear rDNA internal transcibed spacer (ITS) sequences offer the best resolution potential (Baldwin 1992; Kooistra *et al.*, 1992, 1993; Pleyte *et al.*, 1992;

Soltis & Kuzoff, 1993; Bakker *et al.*, 1995) as compared with slowly evolving chloroplast DNA sequences or the structurally variable mitochondrial genomes (Clegg & Zurawski, 1992; Palmer, 1992). Located between the three genes within the nuclear rDNA cistron (i.e. 5'-18S-ITS1-5.8S-ITS2-26S-3'), the ITS regions are part of the initial transcript but is spliced out later during maturaton of pre-rRNA (Gerbi, 1985). The 5.8S rDNA occurs in the middle of the rDNA cistron and is flanked by two internal transcribed spacers (ITSs): ITS 1 and ITS 2. ITS sequences are believed to have few evolutionary constraints, thus they are free to evolve at a faster rate, as reflected in the number of substitutions and well-documented length variation (Yokota *et al.*, 1989; Gonzales *et al.*, 1990; Gardes *et al.*, 1991; Steane *et al.*, 1991; Venkateswarlu & Nazar, 1991), despite the fact that the secondary structural elements in both ITS regions are known to play an important role in the processing of the pre-rRNA molecule (Mattaj *et al.*, 1993; van Nues *et al.*, 1994). These characteristics allow their usage for biogeographic and population studies. Taken together, the genes and spacers of the nuclear ribosomal DNA cistron provide a combination of conserved and variable regions making them suitable for molecular evolutionary studies (Jorgensen & Cluster, 1988). In particular, the internal transcribed spacers (ITS1 and ITS2) are fast evolving and have been found to provide suitable levels of variation among populations of a single species, between species and occassionally between closely related genera. Within and among species of marine algae, variation of ITS sequences ranging from 0 to 20% have been reported (Bakker *et al.*, 1992; Kooistra *et al.*, 1992; van Oppen *et al.*, 1993). Some other groups in which ITS sequence

variation has been widely applied include the fungi, where they show a range of variation (Gardes *et al.*, 1991; Lee & Taylor, 1992), the flowering plant group Madiinae within the Compositae (Baldwin, 1992), and among species of salmonid fishes (Phillips and Pleyte, 1991).

The *psa*B gene codes for one of the two similar $P_{700}$ chlorophyll a apoproteins of photosystem I. The composition of the photosystem I complex from green plants and cyanobacteria has been extensively studied (Thornber, 1986; Wollman, 1986), but this is not true for chromophyte and red algae. Photosystem I fractions have been isolated from brown algae and diatoms and are now being characterised (Berkaloff *et al.*, 1990). The largest core polypeptides of the photosystem I complex are two related transmembranes with approximate molecular weights of 83 kDa, which probably evolved from a duplication of a preexisting gene (Fish *et al.*, 1985). These polypeptides are coded on the chloroplast genome by the *psa*A and *psa*B genes.

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39) is used during the primary step of $CO_2$ fixation in the Calvin cycle. The holoenzyme consists of eight large and eight small subunits (Miziorka & Lorimer, 1983). In chlorophytes (land plants and green algae), the small subunit is encoded on the nuclear genome and the large subunit on the plastid genome (Palmer, 1985). In contrast, in the chromophyte *Olishodiscus luteus* (Reith & Cattolico, 1986) and cryptophyte *Cryptomonas Φ* (Douglas & Durnford, 1989), and the rhodophytes *Porphyridium*

*aerugineum* (Valentin & Zetsche, 1989) and *Cyanidium caldarium* (Valentin & Zetsche, 1989), the small and the large subunits are both encoded on the plastid genome and are separated by a short spacer. Sequence of *rbc*L and *rbc*S genes in *Pleurochrysis carterae* (Fujuwara *et al.*, 1993) demonstrates that these genes constitute an operon on the plastid genome as in other chlorophyll *c*-containing algae and the Rhodophyta.

The algae genes (*rbc*L, *rbc*S, rubisco, ITS1, ITS2, ITS, 5.8S rRNA, ITS SSU rRNA, 18S rRNA, rRNA, srRNA, SSU rRNA, 18S rDNA, 5S rDNA, 5.8S rDNA, *psa*B, *rps*12, F*cp*, *atp*B and *atp*E) from Prymnesiophyta, Chromophyta, Cryptophyta, Chlorophyta, Rhodophyta and Phaeophyta that have been sequenced from 1990 to 1997 are shown in Table 3.

| Sequenced gene | Species/ Genus/ Family | Taxon | References |
|---|---|---|---|
| *rbc* L | Isochrysidales, Coccosphaerales, Prymnesiales, Pavlovales | Prymnesiophyta | Fujiwara *et al.*, 1994 |
| *rbc* L | Pyramimonas (Prasinophyceae) | Chlorophyta | Daugbjerg *et al.*, 1994 |
| *rbc* L | Gelidiales | Rhodophyta | Freshwater *et al.*, 1995 |
| *rbc* L | Gelidiales | Rhodophyta | Wilson *et al.*, 1992 |
| *rbc* L and *rbc* S | *Ectocarpus siliculosus*<br>*Cryptomonas* $\phi$ | Chromophyta<br>Cryptophyta | Valentin and Zetsche, 1990 |
| Rubisco | *Gracilaria verrucosa* | Rhodophyta | Destombe and Douglas, 1991 |
| Rubisco | *Gymnogongrus devoniensis* | Rhodophyta | Maggs *et al.*, 1992 |
| ITS1 and ITS2 | *Cladophora albida* | Chlorophyta | Bakker *et al.*, 1992 |
| ITS1 and ITS2 | Desmarestiaceae | Phaeophyta | Peters *et al.*, 1997 |
| ITS1 and ITS2 | *Cladophoropsis membranacea* | Chlorophyta | Kooistra *et al.*, 1992 |
| ITS | *Cladophora vagabunda* | Chlorophyta | Bakker *et al.*, 1995 |

Table 3: Algae genes that have been sequenced from year 1990-1997

| Sequenced gene | Species / Genus/ Family | Taxon | References |
|---|---|---|---|
| ITS 2 | Nizymeniaceae (Gigartinales) | Rhodophyta | Chiovitti *et al.*, 1995 |
| ITS and 5.8S rRNA | Dictyotaceae | Phaeophyta | Lee and King, 1996 |
| ITS SSU rRNA | *Gracilaria lemaneiformis* | Rhodophyta | Bhattacharya *et al.*, 1990 |
| 18S rRNA | Gracilariales | Rhodophyta | Bird *et al.*, 1992 |
| 18S rRNA | Chlamydomonas | Chlorophyta | Buchheim *et al.*, 1990 |
| 18S rRNA | *Dictyochloropsis reticulata*<br>*Myrmecia astigmatica*<br>*M. bisecta* | Chlorophyta | Friedl, 1995 |
| 18S rRNA | Chordariales, Desmarestiales, Dictyosiphonales, Dictyotales, Ectocarpales, Fucales, Scytosiphonales, Syringodermatales | Phaeophyta | Tan and Druehl, 1993 |
| rRNA | Ulvophyceae | Chlorophyta | Zechman *et al.*, 1990 |
| rRNA | Sphaerococcacean, Phacelocarpaceae Nizymeniaceae | | Saunder and Kraft, 1993 |

Table 3 : Algae genes that have been sequenced from year 1990-1997 (continued )

| Sequenced gene | Species / Genus/ Family | Taxon | References |
|---|---|---|---|
| rRNA | *Lessoniopsis* | Phaeophyta | Saunder and Druehl, 1993 |
| srRNA | *Porphyra umbilicalis*<br>*Leucosporidium scottii* | Rhodophyta<br>Basidiomycete | Hendriks *et al.*, 1991 |
| SSU rRNA | *Chlamydomonas parkeae* | Chlorophyta | Kim *et al.*, 1994 |
| SSU rRNA | *Chondrus crispus* (Gigartinales) | Rhodophyta | Leblanc *et al.*, 1995 |
| SSU rRNA | Laminariales | Phaeophyta | Saunders and Druehl, 1992 |
| 18S rDNA | Gracilariales | Rhodophyta | Bird *et al.*, 1992 |
| 18S rDNA | Sporochnales, Desmarestiales and Laminariales | Phaeophyta | Tan and Druehl, 1996 |
| 18S rDNA | Acrichaetiales-Palmariales | Rhodophyta | Saunders *et al.*, 1995 |
| 18S rDNA | Chlorococcalean | Chlorophyta | Wilcox *et al.*, 1992 |
| 5S rDNA | *Scytosiphon lomentaria* | Phaeophyta | Kawai *et al.*, 1995 |
| 5.8S rDNA | Gigartinalean species | Rhodophyta | Steane *et al.*, 1991 |

Table 3 : Algae genes that have been sequenced from year 1990-1997 (continued )

| Sequenced gene | Species / Genus/ Family | Taxon | References |
|---|---|---|---|
| *psa* B | *Pylaiella littoralis* | Phaeophyta | Assali and Goer, 1992 |
| *rps* 12 | *Spirogyra maxima* | Chlorophyta | Lew and Manhart, 1993 |
| F*cp* | *Macrocytis pyrifera* | Phaeophyta | Apt *et al.*, 1993 |
| *atp* B and *atp* E | *Pylaiella littoralis* | Phaeophyta | Martin *et al.*, 1993 |

Table 3 : Algae genes that have been sequenced from year 1990-1997 (continued )

### 2.4.4 Amplified Restriction Fragment Polymorphism (AFLP)

Amplified Restriction Fragment Polymorphism (AFLP) is a powerful DNA fingerprinting technique used to visualise DNA polymorphisms between samples, establish genetic linkage or mapping for genetic loci, identifying or generating molecular markers (*Vos et. al.*, 1995) and to access the levels of genomic variations among species and isolates. This technique allows numerical analysis for characterisation (typing) and identification purposes.

AFLP combines both Restriction Fragment Length Polymorphisms (RFLP) and Random Amplified Polymorphic DNA (RAPD) strategies. The AFLP technique consists of three steps:

I) Restriction endonuclease digestion of genomic DNA followed by ligation of adapters;

ii) PCR amplification of the restriction fragments; and

iii) Analysis of the amplified fragments on polyacrylamide gels

It is based on the selective amplification of a subset of genomic restriction fragments using PCR. For amplification, DNA is digested with restriction endonucleases and double-stranded DNA adapters are ligated to the ends of the DNA fragments to generate template. The sequence of the adapters and the adjacent restriction site thus serve as primer binding sites for subsequent amplification of the restriction fragments

by PCR. Selective nucleotides extending into the restriction fragments are added to the 3' ends of the PCR primers such that only a subset of the restriction fragments are recognised. Only restriction fragments in which the nucleotides flanking the restriction site match the selective nucleotides will be amplified. The subset of amplified fragments are then analysed by denaturing polyacrylamide gel electrophoresis to generate the fingerprint.

When the DNA fingerprints of related samples are compared, common bands, as well as different bands will be observed. These differences, referred to as DNA polymorphisms, are observed in an otherwise identical fingerprint. Polymorphisms detected in DNA fingerprints obtained by restriction cleavage can result from alterations in the DNA sequence including mutations abolishing or creating a restriction site, and insertions, deletions, or inversions between two restriction sites. The DNA polymorphisms identified using AFLP are typically inherited in Mendelian fashion.

This technique has been used with DNA from plants, bacteria, nematodes and fungi (Vos *et al.*, 1995; Lin and Kuo, 1995; Folkertsma *et al.*, 1996; Lin *et al.*, 1996; and Mueller *et al.*, 1996). O'Neill *et al.* (1997) successfully asessed the levels of genomic variations among species and isolates of the genus *Colletotrichum*, pathogens of alfalfa, using this technique. As a new tool in bacterial taxonomy, Janssen *et al.* (1996) reported that this technique showed differentiation of highly related bacterial

strains that belong to the same species or even biovar. As a result, this method is suitable for epidemiological and evolutionary studies. The polymorphisms detected between different *Escherichia coli* strains or *Agrobacterium tumefaciens* strains indicated that AFLP is able to resolve differences in F' episomal DNA (Lin *et al.*, 1996).

In contrast to RAPD techniques, AFLP uses sequence-specific primers, which eliminates the variability associated with nonspecific primers. This method is powerful because it generates numerous DNA fragments from nanogram quantities of DNA, and reaction conditions are stringent, which improves reproducibility. AFLP DNA mapping was more useful than RFLP and RAPD techniques in identifying molecular markers in soybean (Lin *et al.*, 1996).