MORPHOLOGICAL CHARACTERIZATION AND DNA FINGERPRINTING OF A PRASINOPHYTE FLAGELLATE ISOLATED FROM KERLA COAST

BY

KANCHAN SHASHIKANT NASARE

DIVISION OF BIOCHEMICAL SCIENCES
NATIONAL CHEMICAL LABORATORY
PUNE-411008, INDIA

2002

MORPHOLOGICAL CHARACTERIZATION AND DNA FINGERPRINTING OF A PRASINOPHYTE FLAGELLATE ISOLATED FROM KERALA COAST

A THESIS SUBMITTED TO THE UNIVERSITY OF PUNE FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (IN BOTANY)

BY

KANCHAN SHASHIKANT NASARE

DIVISION OF BIOCHEMICAL SCIENCES
NATIONAL CHEMICAL LABORATORY
PUNE-411008, INDIA.

October 20002

DEDICATED
TO MY FAMILY

TABLE OF CONTENTS

DECLARATION

Certified that the work incorporated in the thesis entitled **"Morphological Characterization and DNA fingerprinting of a Prasinophyte flagellate isolated from Kerala coast."** submitted by Ms. Kanchan S. Nasare was carried out under my supervision. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

Dr. Aditi Pant
Research Guide

Acknowledgement

I take this opportunity to express my heartfelt gratitude to my Guide Dr. Aditi Pant for her valuable guidance, constant encouragement, and sustained interest during the course of this investigation. She will always be fondly remembered for her infectious enthusiasm and unfailing support.

I express my heartfelt thanks to Dr. Vidya Gupta. It is her suggestions, discussions and constant encouragement that helped me a lot to complete this work. I have to specially thank her for being unconditionally supportive, loving and caring.

I thank the Head, Division of Biochemical Sciences and the Director, National Chemical Laboratory for the infrastructural facilities provided. I would also like to thank the Council of Scientific and Industrial Research for the financial support.

I wish to thank Dr. Meena Lagu for her help during the initial stages of my work. My sincere thanks are also due to Dr. Shama Barnamas, Dr. M. I. Khan, Dr. V. Shankar for timely discussions and help.

I wish to thank Dr. Gokhale, Dr. Khire, Dr. Basatawade, Dr. S. Gaikwad, Dr. S. Deshmukh, Dr. Ameeta, Dr. Prabhune, Dr. Pundale and Dr. M. Sainani, Mrs. Indira, and Satyali for their help and words of encouragement.

I would like to acknowledge Dr. Panicker, and Mrs. Vaishali Kailage, Electron Microscopy Unit, Cancer Research Institute, Mumbai for their help in carrying out electron microscope work.

I am thankful to Dr. Yogesh Shouche and Dayanand, National Institute for Cell Science, Pune and Dr. M. Narsimha Rao for their help in DNA sequencing.

I thank Anil, Atul and Feroz for their co-operation and help during the last phase of my thesis writing and making my stay at hostel comfortable. To Sultha, I owe a special thank for her unconditional caring and cooperation. I would also like to thank Rahul, Rangu, Gauri, Arohi, Monali, Bhushan, Rajesh, Mukund, Swati, Vrunda, Armity, Aditi, and other colleagues at the Division of Biochemical Sciences for all their help.

My labmates, Dr. Vaishali, Shashikala, Suresh and Manoj have always been a great support to me and I enjoyed a very helpful, healthy and unenvious atmosphere in the lab. I am also thankful to Narsimha, Anu, Smita and Sarita for their suggestions and help through out the course my work.

Words cannot suffice to express my feelings towards my mother in-law, Rajubhau, Smita vaihini, Dhanubhau, and Vedangi. It was due to their unconditional support, encouragement and care, I could complete my work. I am also thankful to Vaishutai and Mahendrabhau for their encouragement, emotional support and for being always with me through thick and thin.

The completion would not have been possible without the constant encouragement and support of my parents and grandparents. It is their sacrifice, prayers and blessings, which have made me successfully, complete this work. I express my heartfelt thanks to Anna, Nana, Kaku, Kaki and my brothers and cousins Dinesh, Yogesh, Suvarna, Nilesh, Sachin, Vrushika and Rohan for their unfailing love and caring. I am also thankful to my sister Archana and Daji for being extremely supportive and caring.

My husband Shashi holds a special place in the making of this thesis. The constant love, uncompromising support, and encouragement by him have been the main driving force for me over the whole period.

ABBREVIATIONS

AFLP	amplified fragment length polymorphism
Bp	base pairs
CTAB	hexadecyl-trimethyl-ammonium bromide
^{0}c	degree centrigrade
dATP	deoxyadenisine 5' triphosphate
dCTP	deoxycytidine 5' triphosphate
dGTP	deoxyguaosine5' triphosphate
dNTP	deoxynucleotide5' triphosphate
dTTP	deoxythymidine 5' triphosphate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	dithiothreitol
hrs	hours
ISSR	inter simple sequence repeat
Kb	kilo base pairs
μg	microgram
μl	microlitre
μm	micromolar
ml	mililitre
Mm	milimolar
M	molar
Min	minute
ng	nanogram
PCR	polymerasechain reaction
Pmoles	pico-moles
rDNA	gene coding for ribosomal RNA
RAPD	random amplified polymorphic DNA
RPM	revolutions per minute
RNA	ribonucleic acid
Rnase	ribonuclease
SDS	sodium dodecyl sulphate
Sec	second
SSR	simple sequence repeat
TAE	tris acetate EDTA buffer
TE	tris EDTA buffer
Tris	tris hydroxylmethyl amino methane
U	units of enzyme

ABSTRACT

Oceans and seas cover something like two-thirds of the earth's surface. In them live the photosynthetic plants called algae down to a depth of around 150m, depending on the transparency of the water.

Algae are extremely important not only ecologically, but also phylogenetically. It is thought that all the major groups of animals and plants originated in the sea. Algae play important roles in ecology of aquatic and terrestrial ecosystems and have been used as model protists in physiological and biochemical studies. They have been used to answer many fundamental questions in biology because they are easy to culture and grow fast.

Although these organisms are important, species level identification has remained difficult. The reason for this may be the morphological diversity of the group. There are unicellular, filamentous and macroscopic forms of algae and many of these forms exhibit environmentally induced phenotypic variations.

The aim of present work was to identify a green quadriflagellate isolated from Kochi backwaters to the species level. Using standard protocols of pigment profiles, light microscopy, growth studies, scanning and transmission electron microscopy, followed by DNA fingerprinting, ITS-rDNA nucleotide sequence variation and 18s ribosomal DNA sequences, an attempt has been made to correctly identify the isolate. A detailed discussion has been written on the importance of the data resulting from the use of these techniques in comparison with researches by earlier authors on the genus *Tetraselmis*.

Ten strains of *Tetraselmis*, *Scherffelia dubia*, three species of *Chlamydomonas* and *Pedinomonas minor* are compared with the present isolate. On the basis of results obtained, the organism has been tentatively assigned as *Tetraselmis* species NCIM 7001. (Chlorodendrales, Prasinophyceae, Chlorophyta).

The work presented in this Thesis deals with

1. Culture conditions with respect to nitrate, phosphate and salinity. The HPLC analysis of pigment composition. The morphological and ultrastructural characterization of the isolate.
2. Phylogenetic placement of the species using 18S rDNA sequences.

3. The genetic variability within the genus *Tetraselmis* using ISSR PCR.
4. Species-specific sequences in Internal Transcribed Spacer (ITS) regions of ribosomal DNA of different species.

Chapter 1: General Introduction

In this chapter the characteristics of the prasinophytes have been discussed with respect to their diversity and classification. The ultrastructural details of *Tetraselmis* are noted. A literature survey of the manner in which gene sequences have been used for algal systematics and evolution is also included.

Chapter 2: Morphological characterization of prasinophyte flagellate isolated from Kochi backwaters.

The organism did not grow in fresh water medium and required NaCl for its growth. The minimum concentration of NaCl required for the growth was 0.5% w/v and was able to tolerate 20% w/v of NaCl, with highest growth rate at 2% w/v NaCl. These results indicated that the isolate was euryhaline. Growth conditions studies showed that the organism was able to utilize different nitrogen and phosphorus sources. The lowest cell doubling time of 26.6 hrs was observed, when 2 mM nitrogen supplied as urea and 0.05 mM phosphorus supplied as ammonium dihydrogen phosphate were used in enriched seawater medium.

The light and electron microscopical characters of the isolate and pigment profile suggested that the isolate was member of the genus *Tetraselmis* (Chlorodendrales, Prasinophyceae). The morphological data suggested that the isolate resembled to *T. cordiformis* and *T. contracta*. The ultrastructural characteristics of the Kochi isolate and its marine nature separated it from *T. cordiformis*. The position of pyrenoid and eyespot and the lobing of the chloroplast suggested that the Kochi isolate is not the same species as *T. contracta*. However in absence of ultrastructural data for *T. contracta*, this isolate was deposited in the National Collection of Industrial Microorganisms as *Tetraselmis kochinensis* (?) NCIM 7001.

Chapter 3: Phylogenetic placement of the Kochi isolate among prasinophytes and other green algae using 18s ribosomal DNA sequences.

Small subunit nuclear rDNA (18S) have been widely used for genetic identification of many organisms because they comprise both highly conserved sequences during evolution and low sequence homology regions among species. They show a high degree of functional constancy.

The 18S rDNA sequence of the Kochi isolate showed more than 96 % sequence similarity with other *Tetraselmis* species studied here as well as listed in database which confirmed that the isolate was a *Tetraselmis*. The 18S-rDNA phylogeny showed that all *Tetraselmis* species and *Scherffelia dubia* formed one cluster indicating that the order Chlorodendrales is monophyletic. The *Tetraselmis* cluster separated out from other prasinophytes and grouped with Chlamydomonas and Pedinomonas supporting earlier view that the *Tetraselmis* is advanced genus of the Prasinophyceae and has high affinity with Chlorophyceae.

Chapter 4: DNA fingerprinting of the a prasinophyte flagellate isolated from Kochi backwater and ITS1-5.8S-ITS2 rDNA sequence variation in the genus *Tetraselmis*.

DNA fingerprinting was carried out using ISSR primers. A total of 100 primers were screened. Fifteen dinucleotide and one trinucleotide repeat primers gave clear and reproducible banding patterns. The average band size ranged between 200- 1700 bp.

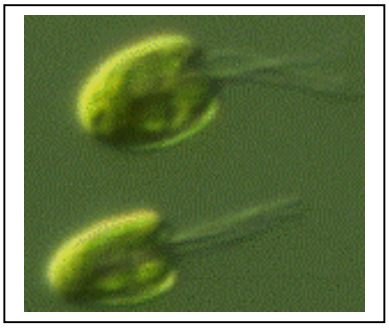
Cluster analysis showed that the five species of *Tetraselmis* separated into three clusters. The Kochi isolate separated out from these five species. The first cluster consisted of the two strains of *T. chui* grouped with *T. gracilis*. The results suggested that *T. chui* CCAP 8/6; CCAP 66/21B and *T. gracilis* CCAP 66/13 might be the same species. The second cluster comprised of two strains *T. striata* and *T. apiculata*. The strains of *T. striata* CCAP 66/5, CCAP 66/16 and *T. apiculata* CCAP 66/15 were extremely closely related with a

bootstrap value of more than 99% and may be the same species. The four strains of *T. verrucosa* formed the third cluster. The species of *Chlamydomonas* and *Pedinomonas minor* grouped into a separate cluster. The Kochi isolate separated at equal distances from both the clusters and formed sister branch with *Scherffelia dubia*.

ITS sequences of *Tetraselmis* were more than 96% similar within the species whereas sequence similarity ranged from 60 to 83% between species. The highest sequence similarity of 83% was observed in ITS1 region of *T. chui-T. gracilis* and *T. striata-T. apiculata*. The sequence similarity between *T. verrucosa* and *T. chui* or *T. striata* ranged between 65-75 % in ITS1 and 60-70 % in ITS2. The Kochi isolate was almost equidistant from *T. chui-T. gracilis*, *T. striata-T. apiculata* and *T. verrucosa* groups. The isolate showed 68-75% sequence similarity in ITS1 and 60-70% sequence similarity in ITS2 with that of other *Tetraselmis* species. ITS sequence results were comparable with ISSR analysis and both the analyses resolved same grouping of species and strains.

Chapter 5: General Discussion

The salient features of the thesis have been discussed with respect to the aim and scope of the present work and future course of investigations have been suggested.



CHAPTER 1

GENERAL INTRODUCTION

The algae comprise a large, heterogeneous and polyphyletic assemblage of relatively simple plants or thallophytes, which lack roots, stems and leaves. Algae occur on shores and coasts, attached to the bottom or live suspended in the water. Freshwaters are also populated by many different species of algae and there are some terrestrial forms, on soils and epiphytic on bryophytes. Altogether, the algae probably account for more than half the total primary production worldwide and all aquatic organisms are dependant on this production. Since oceans and fresh water bodies comprise 75% of the earth's surface, algae are extremely ecologically important.

Algae as we know them are 500-900 million years old and the group comprises a wide variety of taxa (Chapman *et al*., 1999). There are unicellular, filamentous, parenchymatous and macroscopic forms of algae and many of them exhibit environmentally induced morphological variations (Norton *et al*., 1996). The algae include not only the world's largest protists, for example the kelps *Macrocystis* which may be up to 30 m in length, but also many smaller coccoid taxa for example *Chlorella* spp. may be from 1 to 5 μm in size. The great range of diversity of the algae has hampered the definition, characterization and classification of these organisms in any universally acceptable taxonomy. Consequently there now exist as many different systems of classification as there are leading phycologists.

Before the advent of electron microscopy in the 1950s, the algae were classified primarily on the basis of their photosynthetic pigments, storage products in vegetative cells, chemical nature of cell wall and the morphology of motile reproductive cells. As in bryophytes and higher plants, the morphology of the mature vegetative stages did not afford a reliable criterion for distinguishing different classes or phyla of algae. Consequently, several examples became known where morphologically similar algae were placed in different classes or phyla because of their varying physiological and biochemical characters.

The advent of electron microscopy allowed a study of algal cell ultrastructure and revolutionised the field of algal taxonomy. One of the

important concepts that came out of these studies was that the vegetative cell morphology forms a poor basis for a natural classification (Mattox and Stewart 1984). The morphological variations in form have led to the mis-classification of species and ecotypes, which contributed to confusion in algal taxonomy. It is now widely recognised that ultrastructure of the motile or reproductive cell particularly the structure of flagella, flagellar roots and basal bodies form a more reliable basis for taxonomic classification because these characters are stable over time. Due to this technique a major revolution has occurred in classification of green algae especially in the members of class Prasinophyceae.

Chadefaud (1941 and 1960) suggested on the basis of light microscopy that those members of volvocales (Chlorophyceae), which were motile and had pits from which flagella emerged should be taxonomically separated from other Chlorophyceae.

The class Prasinophyceae was first established by Christensen (1962) for a group of motile chlorophytes whose bodies and flagella were covered by nonmineralized organic scales. The presence of scales on the surface of these organisms was discovered by Manton and collaborators in their pivotal experiments using the transmission electron microscope (Manton and Parke, 1965; Parke and Manton 1967).

The class was formally described by Moestrup and Throndsen (1988), according to them the class name was descriptive, being named after "prasinos", the Greek word for green, with *Prasinocladus* Kuckuck as the type genus. The name *Prasinocladus* Kuckuck is a synonym of *Tetraselmis* Stein (Norris *et al*., 1980), and the name Prasinophyceae was automatically conserved for the purpose of the class nomenclature. The characteristics of the class have been reviewed by Norris (1980) and Sym and Pienaar (1993).

Electron microscopical investigation of genera traditionally considered belonging to this class show that many possess a covering of scales on the cell body and /or flagella. The flagellar hair scales are arranged in two nearly opposite rows and attached to 4 and 8 axonemal doublets (Moestrup and Throndsen, 1988). The importance of scales in classification of the

Prasinophyceae has been questioned because there are certain "prasinophyte" genera that lack scales, for example *Micromonas* (Manton 1959) and *Pycnococcus* (Guillard *et al*., 1991). Melkonian (1990) in his description of the class, considered the following structural aspects as important to characterize the class: tubular flagellar hairs arranged in two opposite rows along the flagella, a depression or groove at the flagellar insertion, parallel basal bodies and location of golgi bodies close to basal bodies. On the basis of these ultrastructural characteristics Prasinophyceae has been classified into four orders (Melkonian 1990), which are as listed in Table 1.1

Table 1.1: The Classification of Prasinophyceae (chlorophyta)

Order: Mamiellales
- Family: Mamiellaceae
  - Genera: *Mamiella*, *Dolichomastix Mantoniella Bathycoccus*, *Crustomastix*, *Ostreococcus*, *Prasinoderma*
- Family: Micromonadaceae
  - Genus: *Micromonas*
- Family: Pycnococcaceae
  - Genus: *Pycnococcus*, *Prasinococcus*

Order: Pseudoscourfieldiales
- Family: Pseudoscourfieldiaceae
  - Genus: *Pseudoscourfieldia*
- Family: Nephroselmidaceae
  - Genus: *Nephroselmis*

Order: Chlorodendrales
- Family: Chlorodendraceae
  - Genera: *Tetraselmis*, *Scherffelia*

Order: Pyramimonadales
- Family: Pterospermataceae
  - Genera: *Pterosperma, Tasmanites*
- Family: Pyramimonadaceae
  - Genera: *Pyramimonas*, *Halosphaera, Cymbomonas, Prasinopapilla*
- Family: Mesostigmaceae
  - Genus: *Mesostigma*.

The members of Mamiellales are characterized by the presence of a single layer of spider web like scales (Manton 1977) on the cell body and flagella although *Micromonas* (Manton 1959) and *Pycnococcus* (Guillard 1991) are exceptions to this statement. Two genera, *Mantoniella and Mamiella* have an underlayer of smaller, morphologically distinct plate scales (Moestrup 1984, 1990; Marchant *et al*., 1989).

The covering of the cell body in non-mamiellalean prasinophytes comprises two or three layers of scales, which are termed as underlayer, intermediate and outer layers.

Pseudoscourfieldiales have two layers of scales, pentagonal underlayer scales and rod shaped double scales covering the pentagonal scales. *Pseudoscourfieldia* has rod shaped scales, which occur in two layers on the cell body, whereas in *Nephroselmis* there are both rod shaped and stellate scales present in distinct layers (Melkonian, 1990).

The order Pyramimonadales has a complex scaly covering of three layers on the cell body and two layers on the flagella. The scales of the intermediate and outer layers of *Cymbomonas*, *Pterpserma* and *Tasmanites* are similar to the spider web-like plate scales of the Mamiellales. The intermediate layer scales of *Halosphaera* and *Pyramimonas* are like open ended boxes. In *Halosphaera* they are asymmetric, outwardly flanged lips to their side walls (Pennick, 1977) and in *Pyramimonas* there is considerable variation in the ornamentation of the base plate and side walls (McFadden *et al*., 1986; Sym, 1992). The outer layer of body scales of this order consists of large basket or crown shaped scales.

The flagella of Chlorodendrales, (*Tetraselmis* and *Scherffelia*) are covered by three layers of scales: pentagonal, man and rod shaped. Cells are bounded by theca rather than scales. The theca is either single layered as described in *T. tetrathele* and *T. subcordiformis* (Manton and Parke 1965; Stewart *et al*., 1974), or two layered as described in *T. convolutae* (Parke and Manton 1967). The theca is interrupted at the base of flagellar pit by a slit. The theca is usually rigid but can show a remarkable flexibility during division (Manton and Parke 1965; McLachlan and Parke 1967). Scale

structure of the theca is difficult to ascertain as the scales can only be seen in the Golgi vesicles.

The composition of the scales has been investigated only in few genera. Scales of *Tetraselmis*, *Scherffelia* and *Pyramimonas* are unmineralised and are primarily composed of carbohydrate (50-90%), accompanied by traces (3-5%) of protein. The pectinaceous carbohydrate is primarily composed of galacturonic acid and variable amounts of 2-keto-sugar acids. The 2-keto sugar acids have only recently been discovered (Becker *et al*., 1990) and Melkonian *et al*., (1991) suggest that previous records of a predominance of galacturonic acid (Aken and Pienaar 1985) may be due to the 2-keto sugar acids being destroyed by harsh hydrolysis.

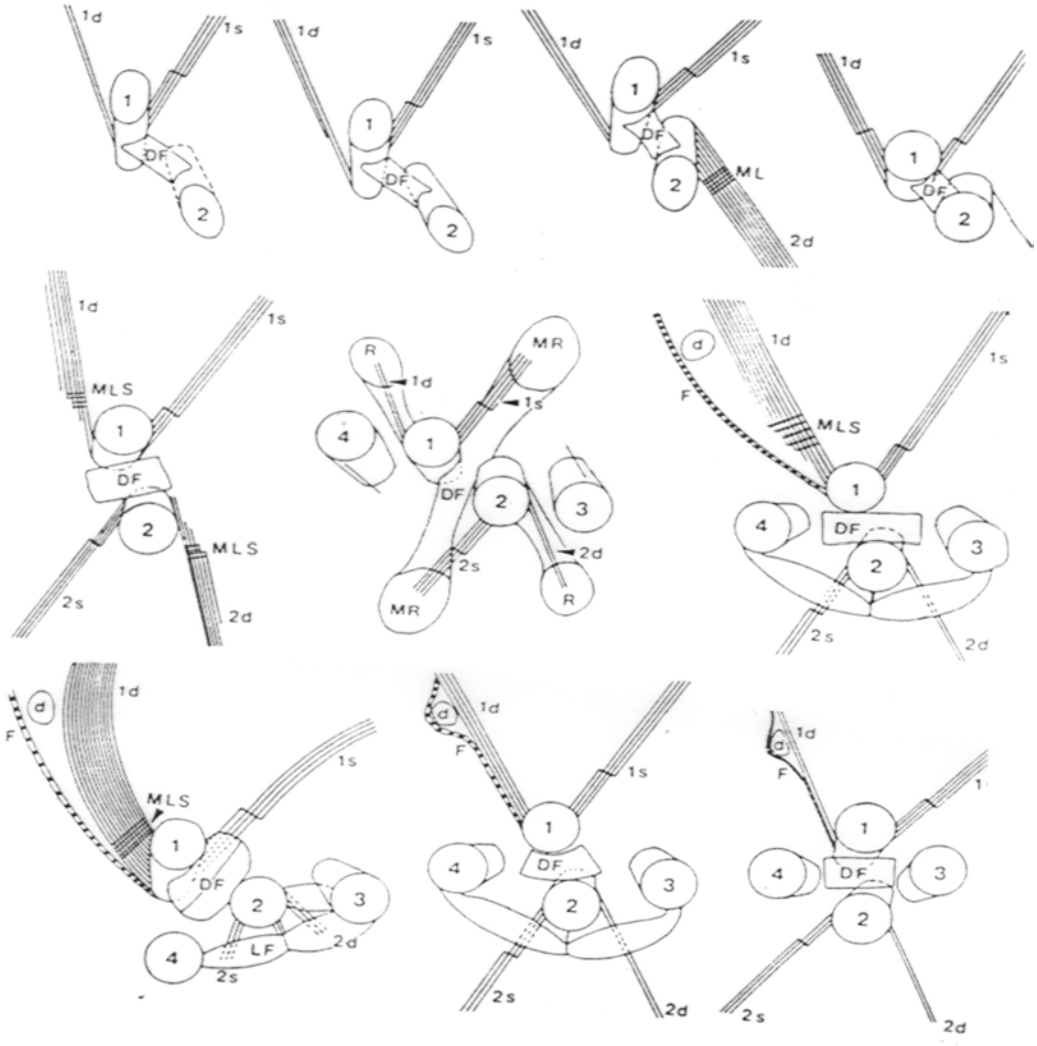
There is great diversity in the architecture of the flagellar apparatus among the members of Prasinophyceae (Fig 1.1).

The basal body of the flagella of prasinophytes is characteristically longer than that found in most other green algae (Moestrup 1982; Melkonian 1984). It is a continuation of the 9 outer axonemal doublets into the cell body with the addition of a C-tubule to each (Andersen *et al*., 1991) thus resulting in triplets. Except for the basal body of *Mantoniella squamata,* the proximal end of all prasinophyte basal bodies has a cartwheel structure. The basal bodies of most prasinophytes are inserted into the cell parallel to the axis of motion and parallel to one another. (Hori *et al*., 1985, Melkonian 1989). Exceptions to this are the basal bodies of *Mantoniella* (Barlow and Cattolico 1980), *Mamiella* (Moestrup 1984), *Dolichomastix* (Moestrup and Ettl 1979), and *Nephroselmis* (Inouye and Pienaar 1984), which are angled relative to one another.

In the quadriflagellate genera, the disposition of basal bodies relative to one another is generally asymmetric, but members of *Pyramimonas* (Sym and Pienaar 1981; O'Kelly 1992), *Tetraselmis* (Salisbury *et al*., 1981) and *Scherffelia* (Melkonian and Preisig 1986) have symmetrically placed basal bodies. The basal bodies are connected to microtubular roots.

Figure 1.1. Diagrammatic representation of the flagellar apparatus of selected members of the Prasinophyceae.

A = *Mantoniella*, B = *Mamiella*, C = *Nephroselmis*, D = *Pseudoscourfieldia*, E = *Mesostigma*, F = *Tetraselmis/ Scherffelia*, G = *Halosphaera*, H= *Pterosperma*, I= *Pyramimonas*. DF= distal fibre, LF= lateral fibre, MLS= multilayered-like structure, 1s, 1d= left and right roots respectively of basal body 1, 2s, 2d= left and right roots respectively of basal body 2.



Microtubular roots also termed "ascending roots" (Manton 1966) are always attached to the oldest two basal bodies of quadriflagellates or to either the oldest or both basal bodies in biflagellates. With the exception of *Pseudoscourfieldia marina* (Moestrup and Throndsen 1988) and species of *Nephroselmis* (Moestrup and Ettl 1979; Inouye and Pienaar 1984) there are always two roots per basal body. Attachment of roots is highly specific and only certain triplets are involved (Melkonian and Preisig 1986). In addition to microtubular roots another type of roots is also present called system II fibres.

System II fibres, also known as deep roots (Manton, 1966) or rhizoplasts (Norris, 1980) are variably striated, contractile and rich in centrin (Salisbury and Floyd 1978; Salisbury *et al*., 1981; Melkonian *et al*., 1988, 1992; Lechtreck and Melkonian 1991). They are present in all prasinophytes, with the exception of *Micromonas pusilla* (Manton 1959). For the most part, system II fibers extend from the basal body around the nucleus and end either near the chloroplast as reported in *Mamiella* (Moestrup 1984) and *Pyramimonas* (Moestrup and Hori 1989) or at the cell membrane as in *Tetraselmis* (Salisbury *et al*., 1981) and *Scherffelia* (Melkonian and Preisig 1986). The system II fibres of *Pterosperma cristatum* differ by terminating on a globose microbody (Inouye *et al*., 1990). *Halosphaera viridis* system II fibers end at the base of the chloroplast, near its eyespot (Hori *et al*., 1985). Extension of the system II fibre beyond the nucleus is a constant feature within the class and is shared by members of the Ulvophyceae (Melkonian *et al*., 1992). System II fibres can vary from thin, insignificant structures as in *Mantoniella* (Barlow and Cattolico 1980; Moestrup 1984) to massive conspicuous structures as in *Tetraselmis* (Melkonian 1979; Salisbury *et al*., 1981).

The system II fibres of *Tetraselmis* and *Scherffelia* are made up of bundles of many fine, parallel filaments (Salisbury *et al*., 1981; Lechtreck and Melkonian 1991; Melkonian *et al*., 1992) interrupted by 9 to 12 cross bars (Salisbury and Floyd 1978; Melkonian and Preisig 1986), but with a variable periodicity (Salisbury *et al*., 1981; Lechtreck and Melkonian 1991).

Pigments of Prasinophyceae

All prasinophytes have chlorophyll a and b. Although *Tetraselmis* has been singled out as chlorophyll b-rich (Smith and Alberte 1991) with a proportion of chlorophyll a:b ratio of 0.5, many prasinophytes have a chlorophyll a:b ratio of approximately 1 or less (Ricketts 1970). *Micromonas pusilla, Mantoniella squamata, Mamiella gilva, Nephroselmis pyriformis, Nephroselmis sp. Pyramimonas amylifera, Pachysphaera sp., Pterosperma sp., Pseudoscourfieldia marina* and *Pycnococcus provasolii* all have a marker chlorophyllide called magnesium 2,4 divinyl phaeophyrin a5 monomethyl ester (Mg2, 4D) (Rickets 1970; Jeffrey 1989; Fwaley 1992).

As discussed above there is great diversity in the architecture of cell and flagellar apparatus of prasinophytes. Mitosis and cytokinesis also vary greatly within the class. For example *Pyramimonas* has open mitosis and a persistent telophase spindle. On the other hand *Tetraselmis* has closed mitosis and the telophase spindle disintegrates.

At present there are about 21 genera in the Prasinophyceae (Table 1.1). According to ultrastructural characteristics, the Class Prasinophyceae has been divided into four orders as Mamiellels, Pseudoscourfieldiales, Chlorodendrales and Pyramimonadales. However recent molecular phylogenetic analyses using 18S rDNA indicate that the Class Prasinophyceae is not monophyletic but comprises of five independent lineages: Chlorodendrales, Nephroselmidaceae, Mamiellales, Pyramimonadales and Mesostigmatoophyceae (Melkonian *et al*., 1995; Nakayama *et al*., 1998; Marin and Melkonian 1999).

Order Chlorodendrales is characterised by the presence of theca on cell body (Manton and Parke 1965). The theca is formed by fusion of scales. The flagellar movement is in a breaststroke fashion. The flagellar root system consists of 4-2-4-2 crucially arranged microtubular roots and two massive rhizoplasts. The order Chlorodendrales comprises single family Chlorodendraceae with two genera *Tetraselmis* and *Scherffelia*.

Genus: *Tetraselmis*

The genus *Tetraselmis* species *cordiformis* was first described by Stein (1878), from a fresh water lake. West (1916) described a genus, which he called *Platymonas* from seawater, and considered it very close to *Tetraselmis* Stein. Butcher (1959) suggested that *Platymonas* West was a synonym of *Tetraselmis* Stein and he transferred several *Platymonas* species to *Tetraselmis*. Manton and Parke (Manton and Parke 1965, Parke and Manton 1967) studied the ultrastructure of *Platymonas* West and they rejected the proposal because at the time the ultrastructure of *Tetraselmis* was unknown. Melkonian (1979) studied the ultrastructure of *Tetraselmis* Stein and for the first time showed that the ultrastructure of *Tetraselmis* was identical to that of *Platymonas* (Manton and Parke 1965; Parke and Manton 1967) confirming the thesis by Butcher (1959) that they belong to the same genus.

The life history of *Tetraselmis* has been divided into three phases, a flagellate stage followed by a non-motile vegetative phase and a third stage in which the cells form an aflagellate cyst with a thick wall. Species of *Tetraselmis* usually remain in the motile stage although some species may remain in a non-motile stage for long periods of time under adverse conditions. During this phase new walls develop and old walls may accumulate as concentric rings around the cell. If they are polarized on one side of the cell they may form a pseudo-stalk.

Many species of *Tetraselmis* have compressed cells, although cells with ellipsoidal and cylindrical shape have also been reported. Cells may be slightly to strongly ridged, particularly in middle to posterior regions of the cells. Sometimes the ridges may be twisted in the posterior parts. Most of the species have two or four slight to distinct creases extending much of the length of the cell wall.

The theca closely surrounds the cell membrane. It is smooth with one or two layers. Thecae are formed by deposition of small stellate-like scales. Only one type of scale is deposited in *T. tetrathele* (Manton and Parke 1965) and *T. subcordiformis* (Stewart *et al*., 1974), but two types of fibrilloid

structures are deposited in the formation of thecae in *T. convolutae* (Parke and Manton 1967).

Cells of *Tetraselmis,* are quadriflagellate. Flagella are of equal length and are attached at the bottom of an apical, trough-shaped cell depression. Flagella emerge from the cell in two pairs. They are thick, blunt-ended and covered by cross-striated flagellar hairs and three rows of scales covering the entire flagellar membrane. All these scales are produced in the Golgi apparatus and transported into the scale reservoir before being released on the flagellar surface. At the base of the flagellar pit small thick hairs are generally present although Melkonian (1979) has reported that they are absent in *T. cordiformis*.

The flagellar apparatus of *Tetraselmis* consists of four zig-zag arranged basal bodies associated with microtubular roots and two distinct rhizoplasts (Fig 1.1). Dictyosomes are restricted to the region surrounding the basal body complex

A single large chloroplast is present in each cell. The chloroplast is anteriorly lobed, and the posterior part of the chloroplast may or may not be lobed. A pyrenoid is always present, although in some species the pyrenoid matrix is small. The pyrenoid matrix may be surrounded by starch grains and the matrix may show a cavity on the side adjacent to the nucleus, but some species have no such cavity. The structure of pyrenoid is a useful taxonomic characteristic (Norris *et al*., 1980).

The eyespot is conspicuous, present on the chloroplast and located either in the median or posterior region of the cell. The eyespot is composed of two layers of lipid granules that lie adjacent to the chloroplast envelope.
The nucleus is situated between the two rhizoplasts, adjacent to the pyrenoid.

Species level identification of the genus *Tetraselmis* was difficult (Butcher 1959) until the advent of electron microscope (Norris *et al*., 1980). Identification of subgenera and species in *Tetraselmis* depends on characters that are visible only with the electron microscope. Hori *et al*., (1982, 1983 and 1986) considered pyrenoid ultrastructure as an important criterion for

the separation of species and classified the genus into four subgenera (Table 1.2). The first subgenus *Tetraselmis* (Hori *et al*., 1982) is characterized by the presence of branched cytoplasmic channels in the pyrenoid matrix. These channels contain electron dense material. Species listed under this subgenus are *T. cordiformis, T. ascus, T. convolutae* and *T. astigmatica*.

The second subgenus is *Prasinocladus* (Hori *et al*., 1983), in which the pyrenoid is invaded by a single cavity filled with a lobe of the nucleus. Species included in this subgenus are *T. marina* and *T. verrucosa*.

In the third subgenus *Tetrathele* the pyrenoid cavity is filled with a lobe of cytoplasm connected to several small canaliculi that traverse the pyrenoid matrix. There are no reports on the species listed under subgenus *Tetrathele*.

The fourth subgenus *Parviselmis* (Hori *et al*., 1986) is characterized by the presence of several canaliculi ending blindly in the pyrenoid matrix. The species included under this subgenus are *T. striata, T. levis, T. chui, T. alacris* and *T. suecica.*

Table 1.2: Classification of the genus *Tetraselmis* based on pyrenoid ultrastructure and species listed under each subgenus

Subgenus	Species listed
Tetraselmis	*T. cordiformis*
	T. ascus
	T. convolutae
	T. astigmatica
Prasinocladus	*T. marina*
	T. verrucosa
Tetrathele	--
Parviselmis	*T. striata*
	T. levis
	T. chui
	T. alacris
	T. suecica

(-- Species not reported)

Marin *et al*., (1993) used the ultrastructure of flagellar hairs to distinguish between species. The flagellar hairs of *Tetraselmis* consist of five components: a proximal filament, tubular shaft, a transition zone, distal subunits and the distal filament. On the basis of variation in transition zone and distal filament they grouped flagellar hairs of *Tetraselmis* into four main types and several subtypes (Table 1.3).

Table 1.3: Ultrastructural types of flagellar hairs and species list belonging to each type in the genus *Tetraselmis* (Marin *et al*., 1993)

Type	Subtype	Species
1	a	*T. tetrathele*
		T. convolutae
		T. spec. CCMP 938
	b	*T. chui*
		T. subcordiformis
		T. spec. RG 96
		T. spec. RG 97
		T. spec. CCMP 936
	c	*T. spec*. CCMP 924
		T. spec. CCMP 937
		T. spec. CCMP 952
2		*T. striata*
		T. suecica
		T. levis
		T. spec. CCMP 945
		T. spec. CCMP 956
		T. spec. CCMP 966
		T. spec CCMP 976
		T. spec. RG 79
		T. tetrathele
		T. spec. SAG 161-3
3	a	*T. verrucosa*
		T. verrucosa
		T. spec. CCMP 973
	b	*T. marina*
		T. marina
4	a	*T. striata*
		T. astigmatica
	b	*T. convolutae*
	c	*T. cordiformis*

Type 1 flagellar hairs are characterized by the absence of a transition zone. Species belonging to each flagellar type is given Table 1.3.

Type 2 contain a simple transition zone of only one segment about 30-40 nm in length.

Type 3 has a multiple transition zone composed of 3-6 segments, the segment size decreases in length from the proximal to the distal segment.

Type 4 also is characterized by a multiple transition zone, but the individual segments have approximately the same length.

As shown in Table 1.1 and 1.2, the classification system provided by Hori *et al*., (1982, 1983 and 1986) and Marin *et al*. ,(1993) resolved different relationships for different species. For example Hori *et al*., (1982) placed *T. cordiformis T. convolutae* and *T. astigmatica* to the subgenus *Tetraselmis* (Table 1.2). This is supported by Marin's (Marin *et al*., 1993) results which show that they have similar types of flagellar hairs (Table 1.3).

T. striata and *T. chui* are shown to be closely related by Hori *et al*., (1986), but Marin's (Marin *et al*., 1993) results indicate that these species have different flagellar hair types (Table 1.3). Furthermore Marin *et al*., (1993) have shown that one strain of *T. convolutae* with which they worked has type 1 flagellar hair type whereas the second strain of this species has type 4 flagellar hair type (Table 1.3) and similar case observed in *T. striata* and *T. tetrathele* (Table 1.3). Thus it becomes difficult to place or position new taxa using either of these schemes and the final placement of the new taxa is a subjective decision.

The species level identification has been considerably helped by the use of molecular markers such as 18S rDNA sequences, DNA fingerprinting and ITS sequence polymorphism.

Ribosomal genes are the well characterized, ubiquitous and easily accessible by PCR (White *et al*., 1990). Phylogenetic analysis of living organisms has been revolutionized by comparisons of their ribosomal gene sequences. These studies were first initiated in prokaryotes (Woese, 1987) and have been successfully applied in eukaryotes (Sogin 1991). Nuclear encoded 18S rRNA was first studied in algae by Sogin and colleagues

(Gunderson *et al*., 1987) and subsequently, using partial 18S rRNA sequences from a greater number of taxa, by Chapman and collaborators (Kantz *et al*., 1990; Zechman *et al*., 1990; Buchheim *et al*., 1990, Chapman and Buchheim 1992). More refined analyses have been performed using comparisons of complete nuclear encoded 18S rDNA sequences and taxa from a wide range of algal groups (Huss and Sogin 1990; Lewis *et al*., 1992; Wilcox *et al*., 1992, 1993; Steinkötter *et al*., 1994; Surek *et al*., 1994; Friedl and Zeltner 1994; Friedl 1995; Melkonian and Surek 1995; Nakayama *et al*., 1998; Marin and Melkonian 1999; Diez, *et al*., 2001).

Although most studies within algae have focused upon phylogenetic relationships within organisms from different algal classes, it is also possible to infer reliable relationships between species of single genus. For example, Huss *et al*., (Huss and Sogin 1990 and Huss *et al*., 1999) studied 11 species of *Chlorella* along with other members of Chlorophyta and found that *Chlorella* taxa are dispersed over two Classes of chlorophytes, the Trebouxiophyceae and the Chlorophyceae. Further they proposed that only four species namely *C. vulgaris* Beijerinck, *C. lobophora* Andreyeva, *C. sorokiniana* Shih. st Krauss, and *C. kessleri* Fott et Novakova should kept under the genus *Chlorella* and that other *Chlorella* species belong to different taxa within the Trebouxiophyceae. Recently, Huss *et al*., (2002) have used 18S and ITS1 rDNA sequences to identify taxonomic position of acid tolerant strains such as *Chlorella saccharophila, Chlorella protothecoides var. acidicola* and *Viridiella fridericiana*.

Also based on species specific conserved regions of 18S rDNA molecular probes have been developed for many phytoplankton species. Caron *et al*., (1999) have developed such probes for the identification *Paraphysomonas* (Chrysophyceae).

The 18S sequences are known to be highly conserved within a genus. Therefore sometimes they may not be useful for resolving differences at the species level. DNA fingerprinting has been used for studying between species relationships.

The term DNA fingerprinting was introduced by Jeffreys (Jeffreys *et al*., 1985) to describe a method for the detection of variable DNA loci by hybridization of multilocus probes to electrophoretically separated restriction fragments. DNA typing has rapidly become the primary method for identifying and distinguishing individuals, species and populations. It is also used in forensic science.

One important technique of DNA typing is the Randomly amplified polymorphic DNA (RAPD). This technique has been used to study interspecific variation in three species of *Porphyra* from Western North Atlantic and Gulf of Mexico by Dutcher and Kapraun (1994) who showed that *Porphyra carolinensis* had the greatest heterogeneity, *P. leucosticta* which is widely distributed in North Atlantic consists of a number of discrete populations and that *P. carolinensis* and *P. rosengurttii* which are endemic to the southeastern coast of the United States are genetically more homogeneous than the *P. leucosticta* populations. Coyer *et al*., (1997) compared 24 individuals of *Postelsia palmaeformis* (Phaeophyta) and showed that near-shore populations showed a greater homogeneity than offshore individuals. They attributed this to limited spore dispersal and inbreeding in the near shore populations. Donaldson *et al*., (1998) used Amplified fragment length polymorphism (AFLP) to study three populations of *Chondrus crispus* (Rhodophyta) between latitude 45^0N and 48^0N off the coast of Canada. They showed that plants from the Gulf of St. Lawrence and the Bay of Fundy showed greater similarity than plants sampled off the Atlantic coast of Nova Scotia.

DNA polymorphism detected by inter simple sequence repeat (ISSR) offers another potential tool for genome fingerprinting (Zietkiewicz *et al*., 1994). The ISSR technique is similar to the RAPD technique except that ISSR primer sequences are designed from microsatellite regions and the annealing temperatures used are higher than those used for RAPD markers. The banding patterns generated by ISSR primers are more reproducible. Joshi *et al*., (2001) used ISSR markers to study genetic variation among *Oryza* cultivars and their wild progenitors. Their results indicated that the most

divergent species was *O. brachyantha* and that two varieties of cultivated rice: *O. sativa* var. *indica* and *O. sativa* var. *japonica* had a monophyletic origin. In algae these markers have used for studying the genetic diversity among *Batracospermum boryanum* (Rhodophyta) collected at distances of about 5 m, from a water stream (Vis 1999). The result suggested that there was high genetic variation among these individuals, which could be due to outcrossing and recombination through sexual reproduction. Interestingly the author found that the individuals from the most upstream site were closely related to the individuals from the furthest downstream site which might be due either to carpospore establishment or plant fragmentation.

Most of the DNA fingerprinting studies in algae have been done in red and brown algae, and there are fewer reports from the unicellular green algae. The reason for this might be the large amount of DNA required for the analysis. As the present thesis shows it is possible to use ISSR markers to determine similarity indices in unicellular green algae. Further the results are confirmed by studying ITS rDNA sequence polymorphism among these strains.

ITS sequences are widely used in phylogenetic analysis. There are two different strategies, which allow finding out species-specific variation in the ITS region. One by finding restriction fragment length polymorphism in ITS regions of related taxa, the second by cloning and sequencing of these regions. The usefulness of ITS sequences in taxonomic and phylogenetic studies have been well documented (Lee and Taylor 1990; Kooistra *et al*., 1992; Gardes and Bruns 1993; van Oppen *et al*., 1994, Cozzolino *et al*., 1999; Serrão *et al*., 1999).

ITS regions may either exhibit high sequence similarity within a species or show significant variation. In the case of Yeast, Chen *et al*., (2000 and 2001); Fell *et al*., (2000) and Kurtzman (2000) have found that the two strains of one species show more than 99% sequence similarity.

On the other hand Odorico and Miller (1997) showed that four individuals of *Acropora longicyathus* (Scleractinia) had more than 98% similar ITS1 sequences whereas three individuals of *A. valida* had a sequence

similarity of only 71 to 73%. Similarly, when Pillmann *et al*., (1997) compared nine populations of *Caulerpa filiformis* from two biogeographic regions, five from Australia and four from South Africa, their results showed that there was no sequence variation among the Australian populations and only a 4-base variation among the South African populations. On the other hand Fama *et al*., (2000) detected a very high inter and intra individual sequence variation in *C. racemosa*.

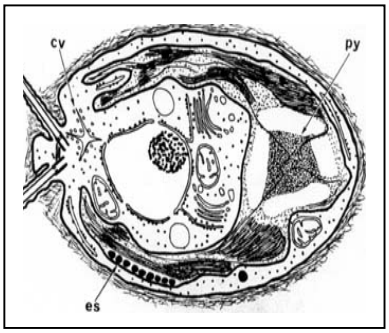
Apart from the evolutionary impact on the ITS sequences the reason for such a kind of sequence variation is that ITS are tandemly repeated and the degree of homogeneity found in tandemly repeated sequences depends on the balance between the rate of homogenization and the rate of new mutations (Ohta and Dover 1983). If the rate of homogenization is low in comparison with the rate of new mutations, one would expect to find multiple ITS variants in a single species as discussed earlier in case of *Acropora*. If in a species the rate of homogenization is high as compared to the rate of mutation then one would expect high sequence similarity as in case of Yeasts, (Chen *et al*., 2000 and 2001; Fell *et al*., 2000 and Kurtzman 2000). We were interested in finding out how much sequence variation occurs in *Tetraselmis* species, which is an advanced member of the Class Prasinophyceae.

In the present thesis, Chapter 2 deals with optimization of culture conditions, pigment analysis, morphological characterization using light and electron microscopy and identification of a prasinophyte flagellate isolated from Kochi backwaters. The organism has been assigned to the genus *Tetraselmis* (Chlorodendrales, Prasinophyceae).

Chapter 3 deals with the confirmation of genus level identification of the Kochi isolate by comparing 18S rDNA sequences with sequences from other *Tetraselmis* and phylogenetic analysis of the genus *Tetraselmis*.

Chapter 4 deals with genetic variability in the 11 strains of genus *Tetraselmis* using ISSR markers. Identification of species-specific regions in the nuclear ITS-rDNA region. Inter and intra-specific relationships inferred from these two sets of data have been discussed.

Chapter 5 deals with general discussion with respect to the aim and scope of the present work and future possible course of investigations.



CHAPTER 2

MORPH0L0GICAL CHARACTERIZATION OF A PRASINOPHYTE FLAGELLATE ISOLATED FROM THE KOCHI (KERALA) BACKWATER

1 ABSTRACT

The organism did not grow in fresh water medium and required NaCl for its growth. The minimum concentration of NaCl required for the growth was 0.5% w/v and was able to tolerate 20% w/v of NaCl, with highest growth rate at 2% w/v NaCl. These results indicated that the isolate was euryhaline. Growth conditions studies showed that the organism was able to utilize different nitrogen and phosphorus sources. The lowest cell doubling time of 26.6 hrs was observed, when 2 mM nitrogen supplied as urea and 0.05 mM phosphorus supplied as ammonium dihydrogen phosphate were used in enriched seawater medium.

The light and electron microscopical characters of the isolate and pigment profile suggested that the isolate was member of the genus *Tetraselmis* (Chlorodendrales, Prasinophyceae). The morphological data suggested that the isolate resembled to *T. cordiformis* and *T. contracta*. The ultrastructural characteristics of the Kochi isolate and its marine nature separated it from *T. cordiformis*. The position of pyrenoid and eyespot and the lobing of the chloroplast suggested that the Kochi isolate is not the same species as *T. contracta*. However in absence of ultrastructural data for *T. contracta*, this isolate was deposited in the National Collection of Industrial Microorganisms as *Tetraselmis kochinensis* (?) NCIM 7001.

2 Introduction

The Backwaters of Kochi are situated between lat $9^0$28' and 10^0N and long $76^0$13'E and $76^0$31'E. Surface water salinity ranges from 1.40 to $33.51^0/_{00}$ and temperature ranges from 28 to 31^0C during the course of the year. The euphotic zone is about 4-5 m deep and the area is highly productive. There is spatial heterogeneity in the composition of phytoplankton occurring in different regions of the Backwaters. Gopinathan (1972) has reported 120 different species of phytoplankton occurring in this region. The present *Tetraselmis* sp. (Kochi Isolate) was isolated from this region by Dr. K. J. Joseph.

Tetraselmis cells are usually found as solitary, free-swimming, thecate cells with four flagella or as solitary or colonial, stalked or unstalked, aflagellate sessile cells. Most species descriptions are based on the flagellate stage.

Within the genus *Tetraselmis* cell sizes are highly variable. Cells may range from 5 to 25 micrometers in length (Butcher 1959, Norris *et al*., 1980) and individual species may have characteristic size ranges within these limits. The cells are ellipsoidal to cylindrical in lateral view, and more or less flattened in end view. The four flagella are slightly shorter than the cell and emerge in two pairs. Cells rotate on their axis while swimming and may abruptly change direction, *T. convolutae* for example repeatedly changes the direction (Manton and Parke 1965).

There is a single chloroplast which occupies most of the volume of the cell and a single central or posterior pyrenoid. The chloroplast may or may not be dissected into lobes and strands of various sizes. The plastid color is usually green, but in a few species it may be reddish due to the accumulation of one or more of the carotenoids or xanthophylls pigments. A single nucleus is located near the anterior end of the cell directly beneath the flagellar bases.

Cells divide in the non-motile stage into two daughter cells, although in few species daughter cells may undergo one more division before being liberated from the parent theca thus giving four cells. Sometimes one of the daughter cells inverts within the parent theca so that the two cells lie in a reversed position although *T. roscoffensis*, cells do not invert after division (Norris *et al*., 1980).

In the genus *Tetraselmis* subgenera and species level identification has always been difficult (Butcher 1959, Norris *et al*., 1980). Identification of subgenera and species in *Tetraselmis* depends on characters that are visible only with the electron microscope. Hori *et al*., (1982, 1983 and 1986) considered pyrenoid ultrastructure as an important criterion for the separation of the species. On the basis of pyrenoid ultrastructure they have classified the genus into four subgenera (Table 1.2, Chapter 1). Marin *et al*.,

(1993) used the ultrastructure of flagellar hairs to separate the species. He found that the flagellar hairs of *Tetraselmis* consist of five components: a proximal filament, tubular shaft, a transition zone, distal subunits and distal filament. On the basis of variation in transition zone and distal filament they have grouped flagellar hairs of *Tetraselmis* into four main types and several subtypes (Table 1.2, Chapter 1).

This chapter presents culture conditions studies, and identification of the Kochi isolate on the basis of pigment analysis, morphological and ultrastructural characteristics.

3 MATERIALS AND METHODS

Materials

HPLC grade Acetone, Acetonitrile, Ethyl acetate and Methanol were purchased from Merck India Ltd. Glutaraldehyde, Osmium tetraoxide, Sodium cacodylate, Uranyl acetate Araldite and DMP-40, were purchased from Pelaco chemicals (Canada). Ultramarine Synthetica Sea salt was obtained from Water Life Research LTD. UK. All salts, solvents and chemicals used were analytical grade.

Algal culture

Unialgal culture was kindly gifted by Dr. K. J. Joseph, which was isolated from Kochi Backwaters and was tentatively identified as *Tetraselmis* by its isolator. Culture was made axenic by repeated sub-culturing and with antibiotic treatment. Other cultures listed in Table No. 2.1 were purchased from Culture Collection of Algae and Protozoa (CCAP) UK and Sammlung von Algenkulturen at Universität Göttingen (SAG) Germany.

Methods

Growth Medium

Marine algae were grown in enriched seawater medium of Guillard (Guillard *et al*., 1962) which contained Na_2EDTA 4.36, $FeCl_3.{}_6H_2O$ 3.15, $CuSO_4$ $5H_20$ 0.01, $ZnSO_4$ $7H_2O$ 0.022, $CoCl_2$ $6H_2O$ 0.01, $MnCl_2$ $4H_2O$ 0.18,

Na_2MoO_4 $2H_2O$ 0.006, $NaNO_3$ 75, NaH_2PO_4 5.65 all salts in mg in 1 liter of sea water pH 8.0. Seawater was prepared by dissolving 20 gms of Ultramarine Sea salt in 1 liter double distilled water and pH was adjusted to 8.0.

Table 2.1: List of cultures used in our study

Sr. N0	Culture	CCAP Cat. Number	Origin
1	*Tetraselmis chui* Butcher	8/6	Isle of Cumbrae, Scotland
2	*Tetraselmis chui* Butcher	66/21B	Yorkshire, England
3	*Tetraselmis striata* Butcher	66/5	Gwynedd, Wales
4	*Tetraselmis striata* Butcher	66/16	Caramarthen, wales
5	*Tetraselmis apiculata* Butcher	66/15	Licolnshire, England
6	*Tetraselmis verrucosa* Butcher	163/3	Essex, England
7	*Tetraselmis verrucosa fo rubens**	66/18B	Isle of wight, England
8	*Tetraselmis verrucosa fo rubens**	66/6	Norfolk, England
9	*Tetraselmis verrucosa fo rubens**	66/46	Qingdao, China
10	*Tetraselmis gracilis* Kylin	66/13	Northumberland, England
11	*Kochi Isolate*	-	India.
12	*Scherffelia dubia* (Perty) Pascher	SAG17/26	-
13	*Pedinomonas minor* Korschikov	1965/3B	River Danube, Slovak Replublic
14	*Chlamydomonas moewusii* Gerloff	11/11	Rice field, Allahabad, India
15	*Chlamydomonas proteus* Pringsheim	11/21	Sand, Hirschberg, Czechoslovakia
16	*Chlamydomonas plethora* Butcher	11/86B	Brakosh, Butley River Suffolk, England

* (Butcher) Hori, Chihara and Norris 1983

Fresh water algae were grown in Jaworski medium (Beaks 1988) which contained $Ca(NO)_3.4H_2O$ 20, KH_2PO_4 12.4, $MgSO_4.7H_2O$ 50, $NaHCO_3$ 15, EDTAFeNa 2.2, $EDTANa_2$ 2.2, H_3BO_3 2.4, $MnCl_2.4H_20$ 1.4, Vit. B_{12} 0.008, $NaNO_3$ 80 Na_2HPO_4 36 all salts in mg per liter and pH was adjusted to 8.0.

MiEB12 medium for *Scherffelia dubia* KNO_3 100 mg, $(NH_4)_2HPO_4$ 10mg, $MgSO_4.7H_2O$ 10mg, $CaSO_4$ saturated solution 10 ml, soil extract 30 ml, micronutrient solution 5 ml, volume was adjusted to 1 lit. with deionised water. Vitamin B_{12} 5 μg/l was added in sterile solution after cooling.

Optimization of Culture Conditions

Initial inoculum in all optimization experiments was 40 cells/ml.

Effect of various Nitrogen Sources on growth

The effect of various nitrogen sources on growth of alga was studied using enrichment with $NaN0_3$, $NaNO_2$, $(NH_4)_2SO_4$, NH_4Cl, and Urea at a concentration of 0.85 mM nitrogen in seawater medium. Seawater medium without added nitrogen served as a control. The concentration of the best nitrogen source was varied from 0.5 to 6.0 mM.

Effect of various Phosphorus Sources on growth

The effect of various phosphorus sources on growth of alga was studied using enrichment with various phosphorus sources Na_2HPO_4, NaH_2PO_4, K_2HPO_4, KH_2PO_4, $(NH_4)_2HPO_4$, $NH_4H_2PO_4$ at a concentration 0.036mM phosphorus in sea water medium. The concentration of the best phosphorus source was varied from 0.01 to 1.0 mM.

Effect of NaCl concentration on growth

The effect of NaCl on growth was studied by increasing NaCl concentration from 0.5 to 25% NaCl in artificial seawater medium.

Pigment Analysis

About 20 ml of an exponentially growing culture (about 10^6 cells/ml) was harvested by centrifugation at 4000 rpm for 5 minutes. Cell pellet was washed twice with saline water. Pigments were extracted in 1 ml HPLC grade acetone by sonication at 4^0C in the dark. Cell debris was removed by centrifugation at 10,000 rpm followed by filtration through 0.22 µm Millipore filter. Pigments were separated on C-18 column with a solvent gradient as given in Table 2.2. Absorbance was measured on UV-visible spectrophotometer detector on Waters® HPLC system. The pigments were identified by comparing their retention time with standard pigments and also by comparing with retention time given by Wright and Jeffrey (1997)

Table 2.2: HPLC solvent system program

Time (min)	Flow rate (ml/min)	% A[1]	% B[2]	%C[3]
0	1.0	100	0	0
4	1.0	0	100	0
18	1.0	0	20	80
21	1.0	0	100	0
24	1.0	100	0	0
29	1.0	100	0	0

1. 80:20 methanol: 0.5 mM ammonium acetate (pH 7.2, v/v)
2. 90:10 acetonitrile :water (v/v)
3. Ethyl acetate

Light and Electron microscopy

For light microscopy cells were fixed with Lugol's Iodine and observed under Inverted Photo Zoom microscope (Cambridge Instruments).

For scanning electron microscopy cells were fixed in 0.2% glutaraldehyde made up in seawater and dehydrated using increasing

concentration of acetone (20 to 100%). Samples were air dried, coated with silver and observed with Leica Stereoscan 440 microscope.

For transmission electron microscopy cells were first concentrated by low speed centrifugation and then fixed in 2% glutaraldehyde made up in 0.1 M sodium cacodylate buffer (pH 7.0) for 30 min. at 4^0C and then washed with 0.2% sucrose for 10 min at room temp. Excess glutaraldehyde and sucrose were removed by washing twice with 0.1 M sodium cacodylate buffer. Postfixation was done in 2% osmium tetraoxide for 1 hr. at 4^0C in dark. Then washed thoroughly with 0.1 M sodium cacodylate pH 7.0. Dehydration was performed in series of alcohol grade (20-95%) at room temperature. Infiltration was done with alcohol and Araldite A (1:1) for 1 hr. at 60^0C, followed by only Araldite A at 60^0C for 1 hr and then at room temp. Cells were embedded in freshly prepared Araldite B (Araldite A 23 ml and 0.4 ml DMP-40). Blocks were polymerized at 60^0C for 48 hrs.

The blocks formed after polymerization were carefully trimmed to expose the underlying cells in the form of pyramid like shape to get serial sections. 600-700 A^0 thick sections were cut with glass knife on a LKB Bromma 2088 ultratome V. Sections were then picked up on Formvar coated copper grids and stained for 10 min in 10% alcoholic solution of uranyl acetate in the dark and then washed thoroughly with deionised water followed by lead citrate for 10 min. and viewed with Ziess EM 109 microscope.

4 RESULTS AND DISCUSSION:

Culture conditions

Effect of Nitrogen Sources on Growth

The organism grew well in all the nitrogen sources tested (Table 2.3). Urea was the best nitrogen source giving the lowest cell doubling time of 37.9 hrs. The cell doubling time decreased, when the nitrogen concentration was increased from 0.5 to 2.0 mM as urea and thereafter the growth rate decreased (Table 2.4) possibly due to either the limitations of other nutrients or toxicity by nitrogen.

Table 2.3 Effect of different nitrogen sources on growth

Nitrogen source (0.85mM Nitrogen)	Cell doubling time (hrs)
$NaNO_3$	44.6
$NaNO_2$	48.0
NH4Cl	42.5
Urea	37.9

*Results are average of two independent data sets.

Table 2.4 Effect of variation of Urea concentration on growth

Urea Concentration (mM)	Cell doubling time (hrs)
0.5	38.5
1.0	36.1
2.0	28.9
4.0	40.1
6.0	42.5

*Results are average of two independent data sets.

Effect of different Phosphorus sources on Growth

Using 2 mM nitrogen as urea the phosphate source was varied. The best phosphate source was ammonium di-hydrogen phosphate (Table 2.5) and at a concentration of 0.05 mM of phosphorus as ammonium di-hydrogen phosphate, cell doubling decreased to 26.6 hrs (Table 2.6). Thus the best growth conditions for this alga were 2mM nitrogen as urea and 0.05 mM phosphorus as ammonium dihydrogen phosphate supplied in enriched seawater medium of Guillard at 20ºC under constant light intensity of 2.5 W m-2.

Table 2.5 Effect of Phosphorus on Growth

Phosphorus Source (0.036 mM Phosphorus)	**Cell Doubling Time (hrs)**
NaH_2PO_4	40.1
Na_2HPO4	40.1
KH_2PO_4	36.1
K_2HPO_4	37.9
$NH_4H_2PO_4$	34.2
$(NH_4)_2HPO_4$	34.6

***** Results are average of two independent data sets.

Table 2.6 Effect of different concentration of $NH_4H_2PO_4$

Different $NH_4H_2PO_4$ Concentration (mM)	**Cell Doubling time (hrs)**
0.01	39.0
0.03	28.9
0.05	26.6
0.07	31.4
0.09	36.12
0.10	42.8

* Results are average of two independent data sets.

Effect of NaCl on growth of the organism

The alga did not grow in fresh water and the minimum requirement of NaCl for the growth of the organism was 0.5% w/v. The cell doubling time decreased exponentially with increase in NaCl concentration upto 2.0% and growth decreased with further increase in NaCl concentration (Table 2.7). Although the cell doubling time increased to 72 hrs, the alga was able to tolerate upto 20% NaCl. At NaCl concentrations higher than 20% the alga did not grow (Table 2.7). An extended lag phase was observed after 8% w/v NaCl and cells lost their flagella. In 15% and 20% NaCl lag phase was extended to 192 hrs and cell doubling time was also increased (Table 2.7). This indicated that the isolate was highly salt tolerant. Butcher (1959) has pointed out that the species of the genus *Tetraselmis* are highly euryhaline and can tolerate wide range of salt concentrations. Carter (1937) isolated *Platymonas* (*Tetraselmis*) *contracta* from Isle of Wight where salinity ranges from 1 to 4%. This is the first report of *Tetraselmis* tolerating such a high salt concentration.

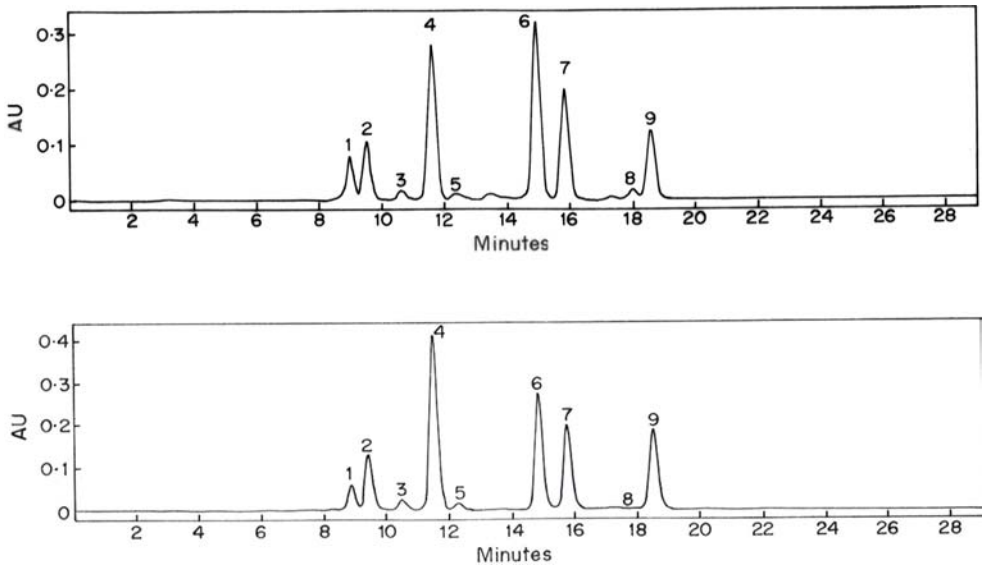
Table 2.7: Effect of NaCl concentration on growth of the Kochi isolate

NaCl Concentrations % w/v	Generation Time (hrs)
0.5	37.9
1.0	36.1
2.0	28.9
4.0	34.1
6.0	38.8
8.0	41.2
10.0	44.0
15.0	52.2
20.0	71.8
25.0	No growth
30.0	No growth

Pigment Analysis

The pigment profile of the Kochi isolate (Fig 2.1b) was similar to that of *Tetraselmis striata* CCAP 66/5 (Fig 2.1a). The chlorophyll a:b ratio was less than 1, which is the reported data for most of the *Tetraselmis* species (Ricketts 1970). Magnesium -2 DVP and Prasinoxanthin were absent. Ricketts (1970) has pointed out that the genus *Tetraselmis* though a member of Prasinophyceae, contains pigments similar to those of the Chlorophyceae.

Figure 2.1: HPLC pigment profile of the pigment extract from a) *T. striata* CCAP 66/5 and b) Kochi Isolate Absorbance recorded at 445 nm. Pigments are as 1-Siphonaxanthin, 2-Neoxanthin, 3- Pheophorbide a, 4-Pheophorbide a-like, 5- Lutein, 6-Chlorophyll b, 7- Chlorophyll a, 8-β-carotene.



Light Microscopy

Light microscope observations showed that the green cells were 16-25 μm in length and 12-18 μm in width with 58% of cells above 20 μm in length and 42% of cells less than 20 μm in length. They were quadriflagellate and ellipsoidal in shape with a 4 lobed apex (Fig. 2.2 a). The four flagella emerged from the base of an apical depression and were blunt ended (Fig. 2.2 b). The organisms swam with rapid anterior movement of the flagella and while swimming cell body always twisted around its vertical axis. Sometimes while swimming cells abruptly stopped and changed the direction. When cells stopped flagella continued beating.

Inside the cell a single large cup shaped chloroplast was present. The pyrenoid was central in position and located immediately beneath the nucleus. A prominent orange eyespot was located posteriorly (Fig 2.2 c).

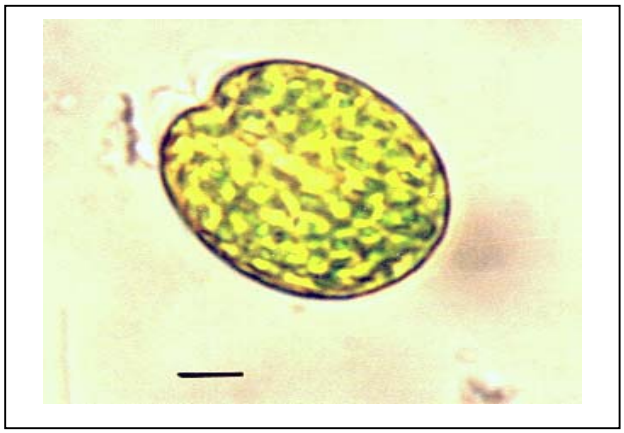
Cells divided in the non-motile stage and the flagella detached at the onset of division. Longitudinal division resulted in two daughter cells (Fig 2.2 c) which sometimes underwent one more division before the daughter cells were liberated from the parent theca to give four daughter cells (Fig 2.2 d). The daughter cells were completely flagellated before release. Sometimes one or two of the daughter cells turned through 180^0 immediately after division so that they lay reversed relative to each other within the parental theca (Fig 2.2 e), sometimes the daughter cells did not turn so and remained parallel to each other (fig 2.2 f). The dormant phase consisted of thick walled cysts and occasionally a papilla like structure was seen on the wall of cyst. An apical depression was never observed on the cyst wall.

The light microscopical characters such as four lobed apex, four flagella inserted in apical depression, granular chromatophores and large red colored eyespot suggest that this organism might belong to the genus *Tetraselmis* Class Prasinophyceae, Chlorophyta. The species level identification of the genus depends on ultrastructural characteristics.

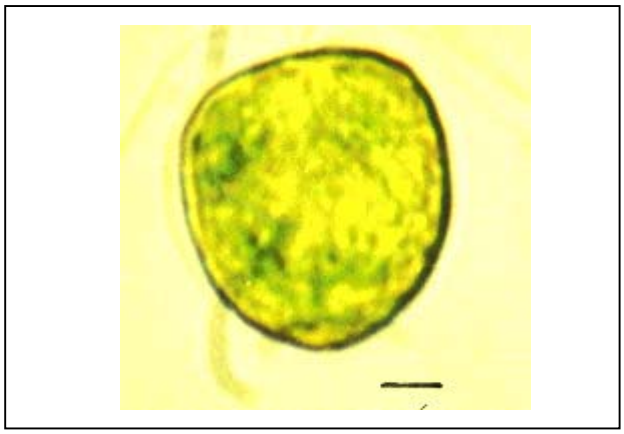
Figure 2.2 Light Micrographs of cells of Kochi Isolate

a) Apical view of cell showing four lobed apex b) A cell showing four flagella arranged in two pairs. c) Two daughter cells showing orange colored eyespot. Scale Bar-10 µm.

a.



b.



c.

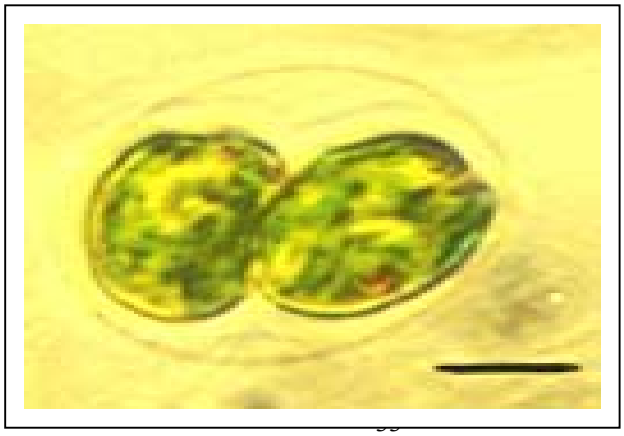
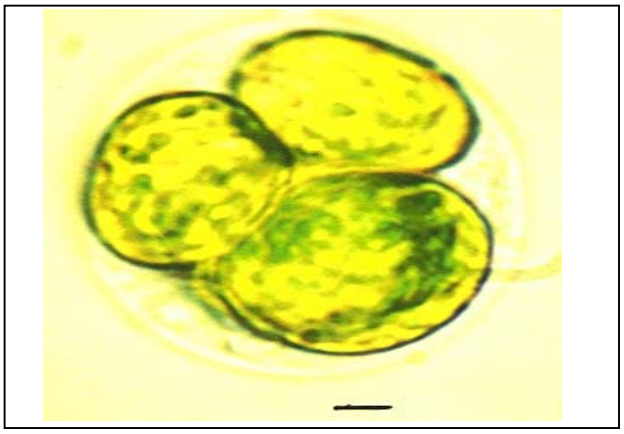
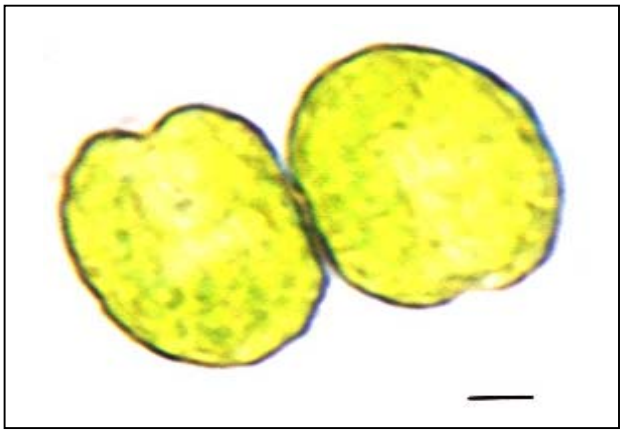


Figure 2.2 continued d) A cell divided into four daughter cells. e) Two daughter cells in antiparallel position. f) Two daughter cells in parallel position. Scale Bar-10 µm.

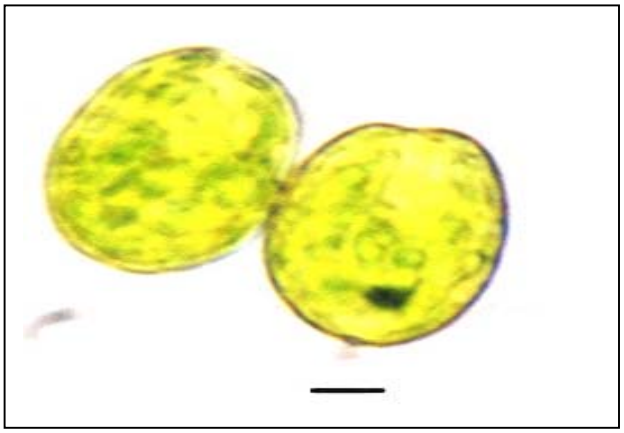
d.



e.



f.

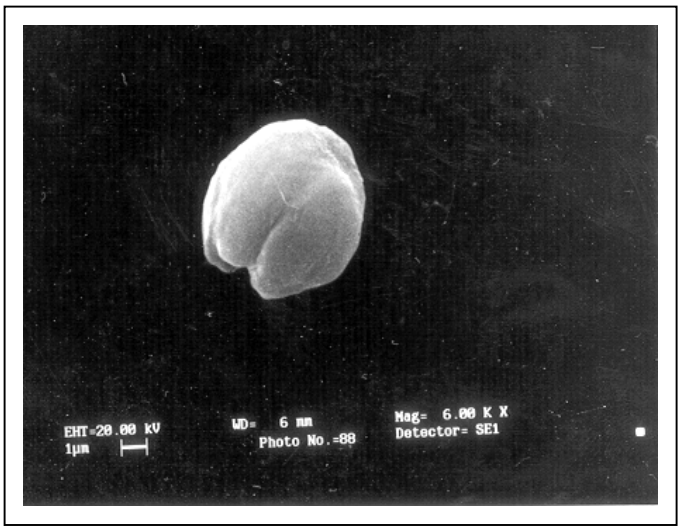


Scanning Electron Microscopy

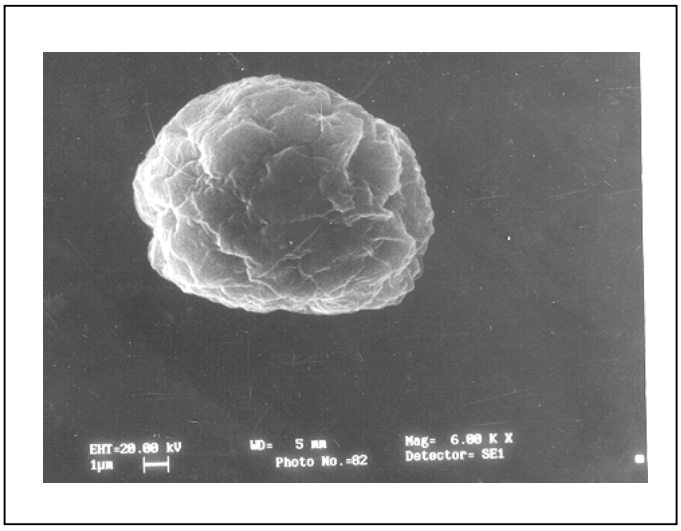
Scanning electron micrograph of Kochi isolate was compared with that of *T. chui* CCAP, *T. striata* CCAP 66/5, *T. gracilis* CCAP 66/13 *T. verrucosa* 66/18B and *T. apiculata* CCAP 66/15. The results indicated that the outer surface of the theca of the Kochi isolate was somewhat rough whereas the thecal surface of other species checked showed smooth surface This suggest that that the outer surface of theca is different in present isolate than the other species of *Tetraselmis*. Figure 2.3 shows the rough thecal surface of the smooth thecal surface of *T. gracilis* CCAP 66/13 and (A) Kochi isolate (B).

Figure 2.3. Scanning Electron Micrograph

A. *T. gracilis* (CCAP 16/13) showing smooth outer surface.



B. Kochi Isolate showing rough outer surface of theca.



Transmission Electron Microscopy

The detailed intracellular structure was best seen in longitudinal sections. (Fig. 2.4). The cell body was covered with fused layer of scales known as theca and it covered whole cell body except for the flagellar groove.

The four flagella were equal in length, thick and blunt ended. The flagella were covered with layers of hairs and scales. Each flagellum was covered with two rows of tubular hair scales on both sides (Fig 2.5). In addition to hair scales, three types of flagellar scales were reported to be present in the genus *Tetraselmis*, Pentagonal or star shaped scales, Man or rod shaped scales and Knotted scales (Manton and Parke 1965; Parke and Manton 1965; Becker *et al*., 1990). The pentagonal (star) and man (rod) shaped flagellar scales are seen in figure 2.6 The flagellar pit hairs were absent.

The basal bodies were long, parallel and arranged in zigzag row (Fig 2.7). They were interconnected by different sets of connecting fibers. It is suggested that the connecting transfibers of *Tetraselmis* may be involved in co-ordinating opposite pair of flagella while flagella of each outer pair linked by hydrodynamic coupling (Salisbury *et al*., 1981). Several dictyosomes lay surrounding the basal bodies (Fig.2.8).

The four anchoring sites of the four basal bodies at the plasma membrane represent laminated oval disks (Fig 2.7), which had earlier been recognised by Manton and Parke (1965) and termed half desmosomes by Schnepf and Maiwald (1970). Salisbury *et al*., (1981) proposed to replace the term by rhizanchora which was again modified to rhizankyra (Melkonian and Preisig 1986).

The flagellar root system consisted of 4 roots, and in addition to this two system II fibrous roots were present. Presence of two massive fibrous, rhizoplasts is a character typical of *Tetraselmis*, very well seen in most of the sections. The rhizoplasts passed on either side of the nucleus and terminated adjacent to the plasma membrane. The striated rhizoplasts are contractile

organelles and play a role in initiating the flagellar movement (Salisbury *et al*., 1984). The flagellar apparatus and the rhizoplasts of *Tetraselmis* have been well studied. The isolated rhizoplasts from *T. striata* consisted of 60-65 percent of a single protein called centrin which is a contractile protein based on supercoiling of these filaments. Near the flagellar apparatus, the distal portion of rhizoplast branched into unequal arms (Fig 2.9). The branching of rhizoplast was reported in case of *T. cordiformis* (Salisbury *et al*., 1978) and *Scherffelia dubia* (Melkonian and Preisig 1996).

The nucleus was central to anterior in position and was ellipsoidal in shape and contained darkly stained nucleolus. The nuclear envelope was continuous in outline towards anterior side whereas at its posterior side that is near the pyrenoid, small projection of nuclear envelope invaded the pyrenoid matrix.

There was single massive chloroplast, which was anteriorly lobed while posterior region was not lobed. Pyrenoid was central in position located very close to nucleus (Fig 2.4). Starch plates or grains were absent surrounding the pyrenoid matrix, while lens shaped starch grains occurred in the chloroplast (Fig 2.10). Pyrenoid was invaded by many cytoplasmic channels from the nuclear side. These channels were filled with electron dense materials.

The eyespot in this species of *Tetraselmis* was located posteriorly (Fig 2.4) however its position may vary from central to posterior. The eyespot consisted of 2 rows of eyespot lipid globules separated by a single thylakoid layer, which was connected, to both globule layers. The eyespot globules were homogenous in electron contrast and size.

We found that the position of the major organelles was fixed and stable in this isolate and the configuration was similar to that of other prasinophyte flagellates. The arrangement of basal bodies was also constant.

The morphological features of this isolate that is the presence of hair scales and long basal bodies show that this alga is a member of the Prasinophyceae. Furthermore presence of theca, striated flagellar hairs and two massive rhizoplast places this genus under the order chlorodendrales.

The order chlorodendrales comprises two genera *Scherffelia* and *Tetraselmis*. In *Scherffelia* pyrenoid is absent (Melkonian and Preisig 1986). Therefore presence of the pyrenoid places this genus under *Tetraselmis*.

The morphological data show that the cell size of the present isolate is larger than any previously reported species in the genus *Tetraselmis*. The nearest in size were *T. contracta* (Carter 1937, Butcher 1959) and *T. cordiformis* (Stein 1878, Melkonian 1979).

Melkonian (1979) has shown that *T. cordiformis* Stein has a central pyrenoid away from the nucleus, the pyrenoid matrix is surrounded by starch plates and the matrix is invaded by cytoplasmic channels from all sides which contain microbodies filled with electron dense material. The chloroplast is highly reticulate. In contrast the Kochi isolate had a pyrenoid which was placed immediately below the nucleus and pyrenoid matrix was not surrounded by starch plates. The pyrenoid matrix was invaded by cytoplasmic channels only from the nuclear side and contain electron dense material which were not surrounded by membrane. The chloroplast was smoothly cup shaped with almost equal anterior lobes. These differences in the ultrastructure between *T. cordiformis* and the present isolate lead to the conclusion that the present isolate is not *T. cordiformis*.

As described by Carter (1937) and Butcher (1959) cells of *T. contracta* Carter are compressed, ellipsoidal with acute posterior end. The apical lobes are unequal. The pyrenoid is basal and eyespot is central to anterior in position. Cysts are thick walled with apical depression. The ultrastructural data for *T. contracta* is not available.

In the Kochi isolate, four apical lobes were equal in size. The pyrenoid was central in position and its position was highly stable under different culture conditions. Also cysts did not have any apical depression, however few cysts showed papilla-like structure. From these points it is clear that the Kochi isolate is not *T. contracta*. The isolate has been deposited in National Collection of Industrial Microorganism as *Tetraselmis kochinensis* (?), NCIM 7001.

Figure 2.4: Longitudinal section of a cell showing elliptical shape and distribution of cell organelles. Magnification Scale Bar: 1 μm.

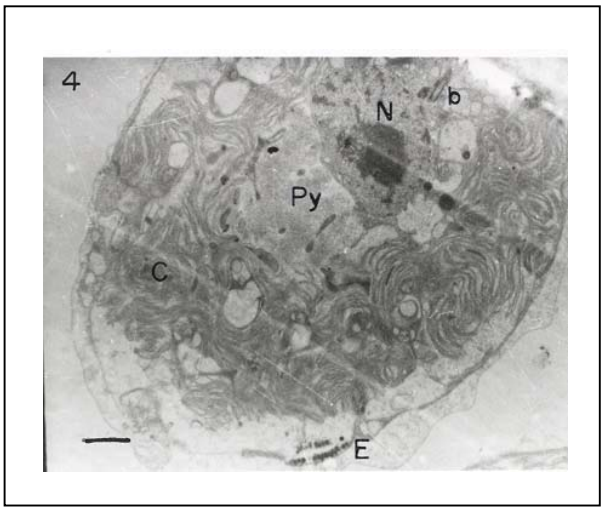


Figure 2.5: Whole mount preparation of flagella showing hair scales on both sides. F=flagellum, H=hair scales. Scale Bar: 0.5 μm.

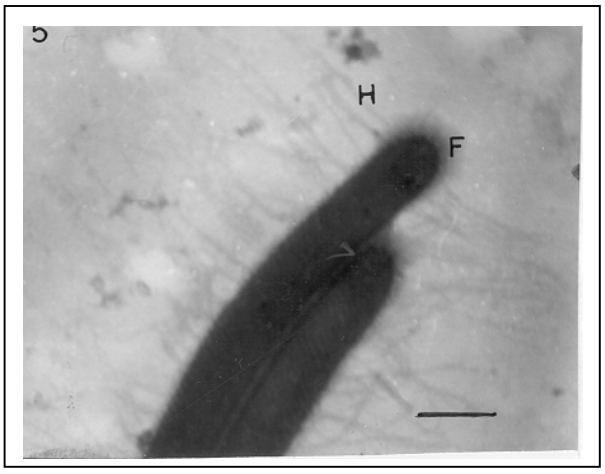


Figure 2.6: Section passing through apical pit and flagella showing pentagonal scales shown by arrow head and rod shaped scales shown by arrow b= basal bodies. Bar: 0.5 μm.

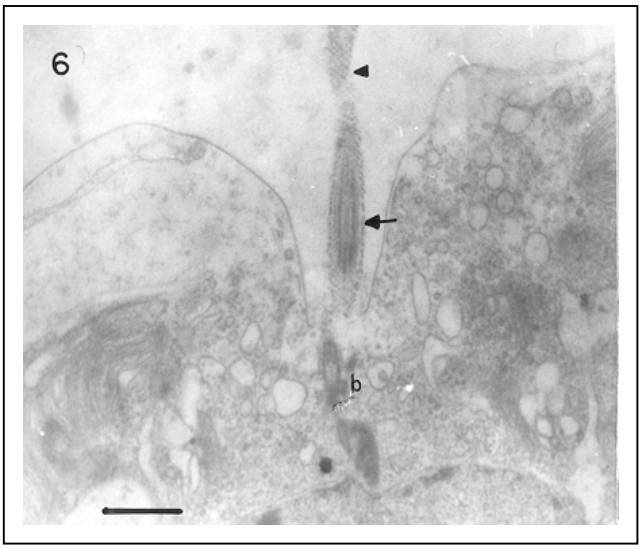


Figure 2.7: Cross section showing zig-zag arrangement of basal bodies (b) and their attachment points, rhizankyra (Ra). Bar: 0.5 μm.

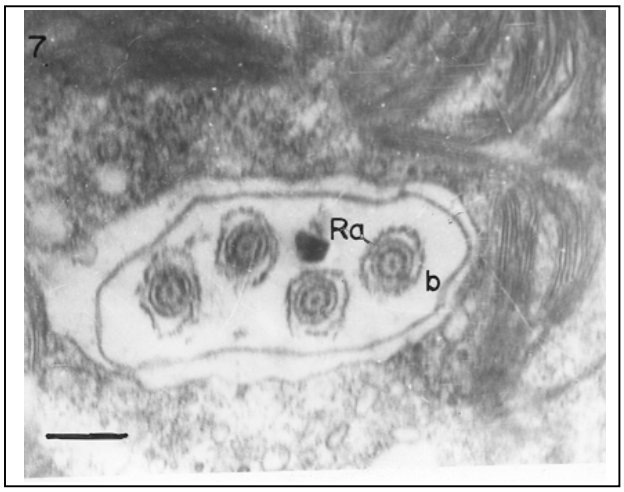


Figure 2.8: Cross section of cell showing interconnected basal bodies and surrounding dictyosomes. Arrow= basal bodies, Arrow head= connecting fibers, Rh= rhizoplast. Bar: 0.5 μm.

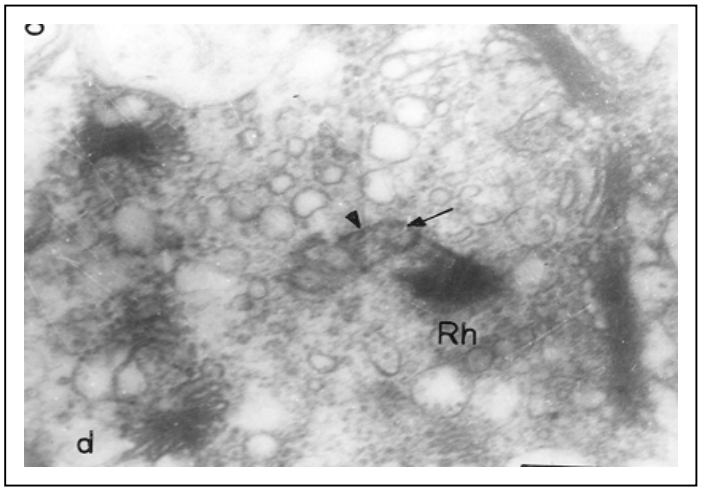


Figure 2.9 Cross section showing flagellar bases (Fb) attached to branched rhizoplast (Rh). Bar: 0.5 μm.

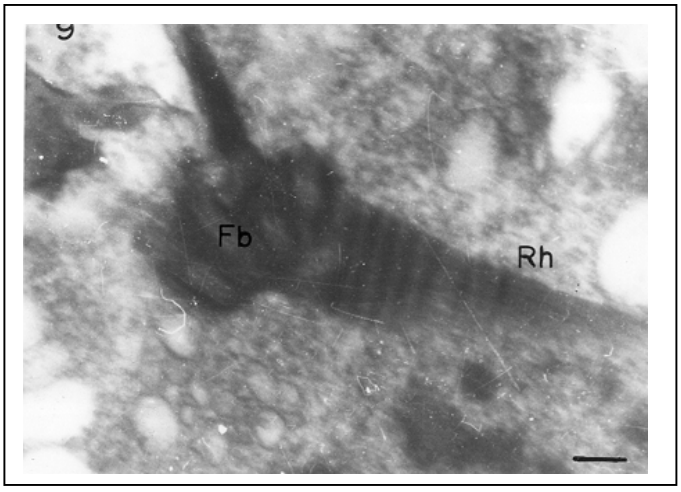
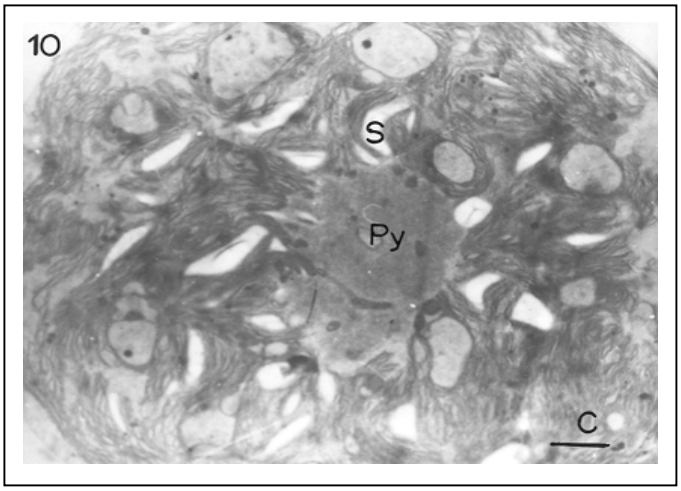


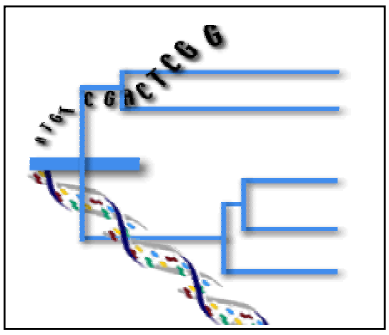
Figure 2.10 Cross section of cell passing through pyrenoid (Py) showing lens shaped starch grains (S) in chloroplast (C). Bar: 1 μm.



Description of *Tetraselmis kochinensis* (?)

Motile cells are 16-25 μm in length and 12-18 μm in width. Cells are ellipsoidal in shape with four apical lobes. Four flagella are slightly shorter than cell length. Flagellar pit hairs are absent. Thecal surface is rough. The chloroplast is cup shaped and lobed at the anterior end. The pyrenoid is central in position, located immediately below the nucleus. The pyrenoid matrix is not surrounded by starch plates, and the matrix is invaded by cytoplasmic channels filled with electron dense material. The orange colored eyespot is mostly located in the posterior region of the cell. Cysts are spherical and may show a papilla like structure.

The isolate is marine and tolerate wide range of salt concentration ranging from 0.5 to 20% w/v with highest growth rate at 2.0%. The strain has been deposited in the National Collection of Industrial Microorganisms (India).



CHAPTER 3

PHYLOGENETIC PLACEMENT OF THE KOCHI ISOLATE AMONG PRASINOPHYTES AND OTHER GREEN ALGAE USING 18S RIBOSOMAL DNA SEQUENCES

1. ABSTRACT

The small subunit nuclear rDNA (18S) have been widely used for genetic identification of many organisms because they comprise both highly conserved sequences during evolution and low sequence homology regions among species. They show a high degree of functional constancy.

The 18S rDNA sequence of the Kochi isolate showed more than 96 % sequence similarity with other *Tetraselmis* species studied here as well as listed in database which confirmed that the isolate was a *Tetraselmis*. The 18S-rDNA phylogeny showed that all *Tetraselmis* species and *Scherffelia dubia* formed one cluster indicating that the order Chlorodendrales is monophyletic. The *Tetraselmis* cluster separated out from other prasinophytes and grouped with Chlamydomonas and Pedinomonas supporting earlier view that the *Tetraselmis* is advanced genus of the Prasinophyceae and has high affinity with Chlorophyceae.

2. INTRODUCTION

Ribosomal RNA or DNA sequences are at present most useful and most used for the phylogenetic analysis of organisms. They occur in all organisms and show a high degree of functional constancy. The different nucleotide positions in their sequences change at very different rates, allowing most phylogenetic relationships to be measured (Woese, 1987).

Phylogenetic analysis using ribosomal RNA sequences was first initiated in prokaryotes (Woese, 1987) and have been successfully applied to eukaryotes (1991). The sequences have been widely used by phycologists to address the question of evolution of different algal classes. Molecular phylogenetic studies in algae began with sequence comparisons of 5S rRNA (Hori et al 1985) which have been completely superseded by sequence comparisons using 18S rRNA molecule. (Sogin 1991; Gunderson *et al*., 1987; Kantz *et al*., 1990; Zechman *et al*., 1990; Buchheim *et al*., 1990; Chapman and Buchheim 1992; Huss and Sogin 1990; Lewis *et al*., 1992; Wilcox *et al*., 1992, 1993; Steinkötter *et al*., 1994; Surek *et al*., 1994; Friedl and Zeltner

1994; Friedl 1995; Melkonian and Surek 1995; Nakayama *et al*., 1998; Marin and Melkonian 1999; Diez, *et al*., 2001).

Although most studies within algae have focused upon phylogenetic relationships within organisms from different algal classes, it is also possible to infer reliable relationships between species of single genus. For example, Huss *et al*., (Huss and Sogin 1990 and Huss *et al*., 1999) studied 11 species of *Chlorella* along with other members of Chlorophyta and found that *Chlorella* taxa are dispersed over two Classes of chlorophytes, the Trebouxiophyceae and the Chlorophyceae. Further they proposed that only four species namely *C. vulgaris* Beijerinck, *C. lobophora* Andreyeva, *C. sorokiniana* Shih. st Krauss, and *C. kessleri* Fott et Novakova should kept under the genus *Chlorella* and that other *Chlorella* species belong to different taxa within the Trebouxiophyceae. Recently, Huss *et al*., (2002) have used 18S and ITS1 rDNA sequences to identify taxonomic position of acid tolerant strains such as *Chlorella saccharophila, Chlorella protothecoides var. acidicola* and *Viridiella fridericiana*.

Based on species specific conserved regions of 18S rDNA molecular probes have been developed for many phytoplankton species. Caron *et al*., (1999) have developed such probes for the identification of *Paraphysomonas* (Chrysophyceae).

In 18S rDNA phylogenetic tree, the *Tetraselmis* resolved near the base of green algal class Chlorophyceae and Ulvophyceae indicating that it is a advanced member of the Class Prasinophyceae having closer relationship with Chlorophyeae (Steinkötter *et al*., 1994; Nakayama *et al*., 1998).

In the present chapter we used 18S rDNA sequences to place the Kochi isolate in the genus *Tetraselmis*. An attempt has been made to study phylogeny of *Tetraselmis* species and three representative of Chlorophyceae listed in Table 2.1 (Chapter 2).

3. MATERIALS AND METHODS

Materials

List of algae used in these studies is given in chapter 2, Table 2.1. Primers were purchased from Bangalore Genie (India). Taq Polymerase and Big Dye terminator sequencing Kit was from Perkin Elmer (USA). dNTPs were from Promega (USA). DNase free RNase A, CTAB and PVP were Sigma (USA) products. Other chemicals used were of Molecular Biology Grade.

Methods

DNA Isolation

Algal cultures were grown in 1 lit. of medium in 2 liter flasks as detailed in chapter 2 and harvested in late log phase of growth by centrifugation at 4000 rpm for 5 min at room temperature. Cell pellet was washed with saline solution and rapidly frozen using liquid N_2. It was stored at -80^0C till DNA required.

DNA was extracted using the CTAB method of Rogers and Bendich (1988) with a few modifications. Frozen 1 gm of cell pellet was ground into fine powder using a glass rod in liquid N_2 and suspended in extraction buffer (2% CTAB, 100mM TrisCl pH 8.0, 20 mM EDTA pH 8.0, 1.4 M NaCl, 1% PVP and 0.5% DTT). Extraction buffer 10-15 ml preheated at 60^0C was added to per gram of cell powder and incubated at 60^0C for 90 min and then allowed to cool down at room temperature. To this an equal volume of chloroform: isoamyl alcohol (24:1) mixture was added. The two phases were separated by centrifugation at 6000 rpm for 10 min. at room temperature. The aqueous layer was taken into another tube and to this an equal volume of CTAB precipitation buffer (1% CTAB, 50 mM TrisCl pH 8.0, 20 mM EDTA pH 8.0) was added. The DNA was allowed to precipitate at room temperature for 30 min and then centrifuged at 10,000 rpm for 10 min at room temperature. The supernatant was decanted and pellet was allowed to dry at room temp. The pellet was then re-dissolved in high salt TE buffer (1 M NaCl, 10 mM TrisCl pH 8.0, 1 mM EDTA pH 8.0). The DNA was re-precipitated with two volumes of

chilled ethanol, washed with 70% ethanol and dissolved in TE buffer (10 mM TrisCl and 1 mm EDTA). This DNA solution was given an RNase treatment (with DNase free RNase A) at 37^0C for 1 hr. and RNase was removed by extracting with 24:1 chloroform: isoamyl alcohol. The DNA was re-precipitated and dissolved in minimum volume of TE buffer. The quality of DNA was checked on 0.8% agarose gel electrophoresed in 0.5X TAE buffer pH 8.0 and visualised by ethidium bromide staining. The DNA was quantified spectrophotometrically by measuring the absorbance at 260 nm on a Shimadzu UV-Visible spectrophotometer (Model UV-1601PC).

Amplification of 18S rDNA

The 18S rDNA was amplified from genomic DNA by polymerase chain reaction using oligo-nucleotide primers (5'-ACCTGGTTGATCCTGCCAG -3' and 5'- TGATCCTTCYGCAGGTTCAC -3' (Staay *et al*., 2001). The PCR reaction contained 10 mM TrisCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.15 mM dNTPs, 1 unit of Taq DNA polymerase, 10 picomoles of primer, 60 ng of template DNA in 25μl volume for 35 cycles. PCR reactions were performed in Perkin Elmer 9700 for 35 cycles. After initial denaturation for 2 min at 94ºC, each cycle comprised of 1 min denaturation at 94ºC, 1 min annealing at 55^0C, 2 min extension at 72^0C with final extension for 10 min at 72ºC at the end of 35 cycles. The amplified PCR products were electrophoresed on 2% agarose gel in 0.5X TAE buffer, pH 8.0 and visualised by ethidium bromide staining. The PCR product was approximately 1700 bp in size.

Sequencing of 18S rDNA

The PCR product was purified by PEG-NaCl precipitation. The PCR products was mixed with 0.6 volumes PEG-NaCl solution (20% PEG 6000, 2.5 M NaCl) and incubated for 10 min at 37^0C. The precipitate was collected by centrifugation for 30 min at 13,500 rpm. The pellets were washed twice with 70% ethanol and dried under vacuum which was then re-suspended in nuclease free glass distilled water at concentration of >0.1 pmol/μl.

The purified product was directly sequenced using Big Dye terminator Kit (ABI-Perkin Elmer USA). The sequencing reactions were run on ABI-PRISM

310 automated sequencer. To obtain the complete sequence of 18S rDNA, in addition to above primers internal primers SR1/SR3 5'-AGGCTCCCTGTCCGGAATC-3', SR2/SR5 5'-CATTCAAATTTCTGCCCTATC-3 / 5'-ACTACGAGCTTTTTAACTGC-3', SR4/SR7 5'-AGCCGCGGTAATTCCAGCT-3'/ 5'-TCCTTGGCAAATGCTTTCGC-3', SR6/SR9 5'-GTCAGAGGTGAAATTCTTGG-3'/5'-AACTAAGAACGGCCATGCAC-3', SR8/SR11 5'-GGATTGACAGATTGAGAGCT-3'/5'-CGCTTACTAGGAATTC-CTCG-3', SR 10/SR12 5'-AGGTCTGTGATGCC-CTTAGA-3' were used (Nakayama *et al*., 1998). Due to some experimental problem sequence of *C. moewusii* could not be obtained and for *T. cordiformis* only 500 bp sequence was obtained.

Sequence Analysis

The resulting sequences were aligned using CLUSTAL W (Thompson *et al*., 1994) program at www.ebi.ac.uk\clustalw. The phylogenetic placement of the Kochi isolate was done at the Ribosomal Database Project (RDP-II, release 7.1, Maidak *et al*., 2000).

A data set of 81 taxa of Viridiplantae was used in Neighbor-Joining method to position the organisms listed in Table 2.1. A smaller data set of 21 prasinophyte taxa was used to find out the relationships of *Tetraselmis* species with other Prasinophyceae. The genetic distances were calculated according to the two-parameter model of Kimura (1980) using Mega 2 software (Kumar *et al*., 2001).

The following references were used in the phylogenetic analysis: *Acrosiphonia* sp. (U03757), *Ankistrodesmus stipitatus* (X56100), *Anthoceros agrestis* (X80984), *Asteromonas gracilis* (M95614), *Chaetosphaeridium globosum* (AJ250110), *Chara foetida* (X70704), *Characium hindakii* (M63000), *Characium saccattum* (M84319), *Chlamydomonas reinhardtii* (M32703), *Chlorella vulgaris* (X13688), *Chlorokybus atmophyticus* (M95612), *Coleochaete orbicularis* (M95611), *Coleochaete scutata* (X68825), *Cosmarium botrytis* (X79498), *Cyanophora paradoxa* (X68483), *Cyanoptyche gloeocystis* (AJ007275), *Cymbomonas tetramitiformis* (AB017126), *Dunaliella salina* (M84320), *Fossombronia pusilla* (X78341), *Friedmannia israeliensis*

(M62995), *Funaria hygrometrica* (X74114), *Fusochloris perforata* (M62999), *Ginkgo biloba* (D16448), *Glaucocystis nostochinearum* (X70803), *Gloeotilopsis planctonica* (Z28970), *Halosphaera sp.* (AB017125), *Hydrodictyon reticulatum* (M74497) *Hylochomium splendens* (X95477), *Klebsormidium flaccidum* (M95613), *Klebsormidium nitens* (AJ250112), *Lychnothamnus barbatus* (U81272), *Mamiella sp.* (AB017129), *Mantoniella squamata* (X73999), *Marchantia polymorpha* (X75521), *Mesostigma viride* (AJ250109), *Mesostigma viride* (AJ250108), *Mesotaenium caldariorum* (X75763), *Micromonas pusilla* (AJ010408), *Microthamnion kuetzingianum* (Z28974), *Mnium hornum* (X80985), *Myrmecia biatorellae* (Z28971), *Nanochlorum eukaryotum* (X06245), *Neochloris aquatica* (M62861), *Nephroselmis olivacea* (X74754), *Nitella flexilis* (U05261), *Oltmannsiellopsis viridis* (D86495), *Ostreococcus tauri* (Y15814), *Pellia epiphylla* (X80210), *Pediastrum duplex* (M62997), *Pinus luchuensis* (D38246), *Prototheca wickerhamii* (X56099), *Pseudoscourfieldia marina* (X75565), *Pseudoscourfieldia marina* (AJ132619), *Pterosperma cristatum* (AJ010407), *Pycnococcus provasolii* (X91264), *Pyramimonas australis* (AJ404886), *Pyramimonas parkeae* (AB017124), *Pyramimonas disomata* (AB017121), *Scenedesmus abundans* (X73995), *Scenedesmus obliquus* (X56103), *Scherffelia dubia* (X68484), *Sphagnum palustre* (Y11370), *Staurastrum* sp. (X74752), *Tetraselmis convolutae* (U05039) *Tetraselmis striata* (X70802), *T.* Sp. RG-07 (U41900), *Trebouxia arboricola* (Z68705), *Trebouxia asymmetrica* (Z21553), *Trebouxia impressa* (Z21551), *Ulothrix zonata* (Z47999), *Volvox carteri f. nagariensis* (X53904).

4. Results and Discussion

The 18S sequence of the Kochi isolate was 1701 bp and that of other *Tetraselmis* species listed in Table 2.1 were 1691 to 1704 bp. The sequence similarity program run at RDP II site for taxonomical placement of the Kochi Isolate and the BLAST search program also showed that the 18S rDNA sequence of the isolate was significantly similar with that of a Prasinophyte symbiont of protozoan *Spongodrymus* (AF166381) and *Tetraselmis*

convolutae (Fig. 3.1 and Fig. 3.2). The prasinophyte symbiont has been shown to be closely related to the *Tetraselmis convolutae* on the basis of its 18S rDNA sequence (Gast *et al*., 2000). Thus these results confirm the earlier ultrastructural results that the Kochi isolate is a *Tetraselmis*.

The sequence comparison of the Kochi isolate with other *Tetraselmis* listed here and three other *Tetraselmis* sequences retrieved from the databases is given Figure 3.3 in the form of CLUSTAL W multiple alignment. The results showed that 18S rDNA sequences of *Tetraselmis* species were more than 96% similar. The genetic distance calculated for pairwise species comparison, is given in Table 3.1.

The sequence comparisons showed that 18S rDNA sequences of *T. striata,* CCAP 66/15 and *T. striata* (X70802) were 100% similar with that of *T. apiculata* CCAP 66/15 (Fig 3.3 and Table 3.1) suggesting that this *T. apiculata* CCAP 66/15 might be a strain of *T. striata*. Very similar conclusions may be drawn from ISSR fingerprinting and ITS sequence comparisons. These results are discussed in Chapter 4.

Similarly *T. gracilis* CCAP 66/13 showed 99.5% similarity with *T. chui* CCAP 8/6 and 99.1% similarity with *T. chui* CCAP 66/21B. Between the two *T. chui* strains CCAP 8/6 and CCAP 6/21B, there was 99.5% similarity. Thus suggesting that these *T. gracilis* CCAP 66/13 and *T. chui* CCAP 8/6 and CCAP 6/21B might be the same species. Again, very similar conclusions may be drawn from ISSR profiles discussed Chapter 4.

The phylogenetic tree drawn using Neighbor-Joining method is given in figure 3.4. All the *Tetraselmis* species separated out from other prasinophycean taxa. They were more closely related to *Chlamydomonas proteus*, *Chlamydomonas plethora* and *Pedinomonas minor* than they are to other prasinophyte taxa. The phylogenetic closeness of *Tetraselmis* with members of Chlorophyceae has already been shown by Steinkötter *et al*., (1994) and Nakayama *et al*., (1998). In the present analysis also grouping of *Tetraselmis* with *Chlamydomonas* taxa was well supported by 100% bootstrap value in all analyses.

All *Tetraselmis* species and *S. dubia* separate from other prasinophyte taxa and grouped together with high bootstrap value of 100% suggesting that the order Chlorodendrales is monophyletic.

Although the Kochi isolate showed high sequence similarity with *T. convolutae* (Fig. 3.2) phylogenetic analysis using Neighbor-joining method with other seven *Tetraselmis* species separates the kochi isolate from *T. convolutae*. Morphologically, *T. contracta* Carter and *T. cordiformis* Stein were the nearest relatives of the Kochi isolate. Both ultrastructural studies and the salt tolerance of the Kochi isolate presented in chapter 2 clearly showed that it is not *T. cordiformis*. Further more 500 bp sequence of 18S rDNA of T. cordiformis showed only 92% sequence similarity with that of the Kochi isolate confirmed that the isolate is not *T. cordiformis.* On the other hand it is difficult to ascertain whether the isolate is *T. contracta*, since no information on ultrastructure or 18S rDNA is available. The isolate has been deposited in the National Collection of Industrial Microorganism as *Tetraselmis kochinensis* (?) NCIM 7001.

Scherffelia dubia, has grouped with *T. verrucosa* and the Kochi isolate suggest that *S. dubia* is very closely related to the *Tetraselmis*. The morphological closeness of these two genera was first shown by Melkonian and Preisig (1986). The difference between *Scherffelia* and *Tetraselmis* is the presence of pyrenoid *Tetraselmis* (Melkonian and Preisig 1986). In the present analysis *Scherffelia* grouped with other *Tetraselmis* species suggesting a close relationship (Fig 3.4).

Figure 3.5 clearly demonstrates the polyphyletic nature of the prasinophytes. The polyphyletic nature of the Prasinophyceae has been extensively discussed by Steinkötter *et al*., (1994); Nakayama *et al*., (1998) and Marin and Melkonian (1999). Within the Chlorophyta the Prasinophytes formed five independent lineages namely Chlorodendrales, Pyramimonadales, Mamiellales, Nephroselmidaceae and Mesostigmatophyceae (Fig 3.5).

Marin and Melkonian (1993) have shown that the order Chlorodendrales is heterogeneous with respect to flagellar hair types. However the 18S rDNA phylogenetic analyses of 18 taxa of Chlorodendrales

representing two genera *Tetraselmis* and *Scherffelia* showed that, the order Chlorodendrales is monophyletic (Fig 3.4). The grouping was strongly supported by 100 % bootstrap value even with different tree drawing methods. From the results it can be stated that the observed morphological heterogeneity in the genus *Tetraselmis* and or the order Chlorodendrales might be due to environmental conditions. The characters that are routinely used for the separation of *Tetraselmis* species such as shape and size of cell, position of eyespot, ultrastructure of pyrenoid and flagellar hairs appear to vary depending on the environmental conditions and age of the culture. Hence molecular tools are a necessary adjunctive to ultrastructure for delimiting the *Tetraselmis* species.

Table3.1: Genetic distances calculated for pairwise species comparison using Kimura two parameter model

1- *T. verrucosa* CCAP163/3, 2- *T. verrucosa* CCAP 66/6, 3- *T. verrucosa* CCAP 66/46, 4- *T. verrucosa* CCAP 66/18B, 5- *T. chui* CCAP 66/21B, 6- *T. gracilis* CCAP 66/13, 7- *T chui* CCAP 8/6, 8- *T. striata* CCAP 66/5, 9- *T. convolutae* (U05039), 10- *T*. sp. RG 07 (U41900), 11- *T.striata* (X70802), 12- *T. apiculata* CCAP 66/15, 13- *T. striata* CCAP 66/16, 14- *S. dubia,* 15- *S. dubia* SAG 17.26, 16- Kochi Isolate.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
[2]	0.002														
[3]	0.008	0.008													
[4]	0.004	0.004	0.004												
[5]	0.031	0.030	0.032	0.030											
[6]	0.029	0.028	0.032	0.028	0.009										
[7]	0.026	0.025	0.028	0.024	0.005	0.005									
[8]	0.028	0.028	0.030	0.026	0.019	0.017	0.014								
[9]	0.027	0.026	0.029	0.025	0.019	0.016	0.014	0.007							
[10]	0.025	0.025	0.027	0.023	0.017	0.014	0.012	0.005	0.003						
[11]	0.025	0.024	0.026	0.022	0.017	0.014	0.011	0.004	0.002	0.001					
[12]	0.025	0.024	0.026	0.022	0.017	0.014	0.011	0.004	0.002	0.001	0.000				
[13]	0.025	0.024	0.027	0.022	0.017	0.014	0.011	0.004	0.002	0.001	0.000	0.000			
[14]	0.028	0.028	0.028	0.023	0.035	0.033	0.031	0.029	0.029	0.027	0.026	0.026	0.026		
[15]	0.028	0.028	0.028	0.023	0.035	0.033	0.031	0.029	0.029	0.027	0.026	0.026	0.026	0.000	
[16]	0.028	0.026	0.026	0.024	0.034	0.034	0.031	0.033	0.033	0.031	0.031	0.031	0.031	0.031	0.031

Figure 3.1: Taxonomical Placement of the Kochi Isolate according to Ribosomal Database Project

SEQUENCE_MATCH version 2.7
written by Niels Larsen.
Comments
: A minimum of 100 unique oligos required
: A total of 166 sequences were excluded
: 34362 sequences were included in the search
: The screening was based on 7-base oligomers

EUKARYOTES

VIRIDIPLANTAE

CHLOROPHYTA

PRASINOPHYCEAE

1pSymb2
0.901
1667
unnamed organism
1pSymb3
0.902
1663
unnamed organism
1pSymbi
0.893
1662
unnamed organism
Ttrs.convl
0.833
1687
Tetraselmis convolutae str. 208 from the North East Pacific Culture Collection
UNCLASSIFIED / UNALIGNED

TSU41900
0.857
1680
Tetraselmis sp. 18s ribosomal RNA gene, complete sequence.

Figure 3.2: Blast search results for finding most similar sequence with the Kochi Isolate

Taxonomy reports

Distribution of 34 Blast Hits on the Query Sequence

Sequences producing significant alignments:	Score (bits)	E Value
gi|7963626|gb|AF166379.1|AF166379 Prasinophyte symbiont of ...	3100	0.0
gi|7963629|gb|AF166381.1|AF166381 Prasinophyte symbiont of ...	3092	0.0
gi|7963628|gb|AF166380.1|AF166380 Prasinophyte symbiont of ...	3085	0.0
gi|450671|emb|X68484.1|SD16RRNA S.dubia 16S-like rRNA	2890	0.0
gi|18044|emb|X56105.1|CKRRN16L Chlorella kessleri 18S rRNA ...	2343	0.0
gi|19847958|emb|Y17470.1|CAC17470 Closteriopsis acicularis ...	2339	0.0
gi|18249|emb|X62441.1|CS16SLSSR Chlorella sorokiniana 18S r...	2000	0.0
gi|288915|emb|X72854.1|CSSSHRRNA Chlorella sp. (Ssh) gene f...	1984	0.0
gi|288912|emb|X72706.1|CSESHRRNA Chlorella sp. (Esh) gene f...	1980	0.0
gi|393466|emb|X74001.1|CSO18SRNA Chlorella sorokiniana 18S ...	1976	0.0

Figure 3.3: Alignment of 18S rDNA sequences.

TV1- *T. verrucosa* 163/3, TV4- *T. verrucosa* 66/46, TV2- *T. verrucosa* 66/18B, TV3- *T. verrucosa* 66/6, TC2- *T. chui* 66/21B, TG- *T. gracilis* 66/13, TC1- *T. chui* 8/6, TS1- *T. striata* 66/5, TA- *T. apiculata* 66/15, TN- *T. convolutae* (U05039), Tsp- *T.* sp. RG 07 (U41900), TS- *T. striata* (X70802), TS2- *T. striata* 66/16, SD1- *S. dubia,* SD2- *S. dubia* (X68484) KI- Kochi Isolate,

```
TV1     ATGTCTAAGTATAAACTGCTTATACTGTGAAACTGCGAATGGCTCATTAA-ATCA-GT-T 57
TV4     ATGTCTAAGTATAAACTGCTTATACTGTGAAACTGCGAATGGCTCATTAA-ATCA-GT-T 57
TV2     ATGTCTAAGTATAAACTGCTTATACTGTGAACCTGCGAATGGATCATTAA-ATCA-GT-T 57
TV3     ATGTCTAAGTATAAACTGCTTATACTGTGAAACTGCGAATGGCTCATTAA-ATCA-GT-T 57
TC2     ATGTCTAAGTATAAACTGCTTATACTGTGAACCTGCGAATGGATCATTAA-GTCATGTGT 59
TG      ATGTCTAAGTATAAACTGCTTATACTGTGAAACTGCGAATGGCTCATTAA-ATCA-GT-T 57
TC1     ATGTCTAAGTATAAACTGCTTATACTGTGAAACTGCGAATGGCTCATTAA-ATCA-GT-T 57
TS1     ATGTCTAAGTATAAACTGCTTATACTGTGAAACTGCGAATGGCTCATTAA-ATCA-GT-T 57
TN      ATGTCTAAGTATAAACTGCTTATACTGTGAAACTGCGAATGGCTCATTAA-ATCA-GT-T 57
Tsp     ATGTCTAAGTATAAACTGCTTATACTGTGAAACTGCGAATGGCTCATTAA-ATCA-GT-T 57
TS      ATGTCTAAGTATAAACTGCTTATACTGTGAAACTGCGAATGGCTCATTAA-ATCA-GT-T 57
TA      ATGTCTAAGTATAAACTGCTTATACTGTGAAACTGCGAATGGCTCATTAA-ATCA-GT-T 57
TS2     ATGTCTAAGTATAAACTGCTTATACTGTGAAACTGCGAATGGCTCATTAAGATCA-GT-T 58
SD1     ATGTCTAAGTATAAACTGCTTATACTGTGAAACTGCGAATGGCTCATTAA-ATCA-GT-T 57
SD2     ATGTCTAAGTATAAACTGCTTATACTGTGAAACTGCGAATGGCTCATTAA-ATCA-GT-T 57
KI      ATGTCTAAGTATAAACTGCTTATACTGTGAACCTGCGAATGGATCATTAA-ATCA-GT-T 57
        ******************************** ********** *******  *** ** *

TV1     ATAGTTTATTTGATGGTACCTACTACTCGGATAACCGTAGTAATTCTAGAGCTAATACGT 117
TV4     ATAGTTTATTTGATGGTACCTACTACTCGGATAACCGTAGTAATTCTAGAGCTAATACGT 117
TV2     ATAGTTTATTTGATGGTACCTACTACTCGGATAACCGTAGTAATTCTAGAGCTAATACGT 117
TV3     ATAGTTTATTTGATGGTACCTACTACTCGGATAACCGTAGTAATTCTAGAGCTAATACGT 117
TC2     ATAGTTTATTTGATGGTACCTACTACTCGGATAACCGTAGTAATTCTAGAGCTAATACGT 119
TG      ATAGTTTATTTGATGGTACCTACTACTCGGATAACCGTAGTAATTCTAGAGCTAATACGT 117
TC1     ATAGTTTATTTGATGGTACCTACTACTCGGATAACCGTAGTAATTCTAGAGCTAATACGT 117
TS1     ATAGTTTATTTGATGGTACCTACTACTCGGATAACCGTAGTAATTCTAGAGCTAATACGT 117
TN      ATAGTTTATTTGATGGTACCTACTACTCGGATAACCGTAGTAATTCTAGGGCTAATACGT 117
Tsp     ATAGTTTATTTGATGGTACCTACTACTCGGATAACCGTAGTAATTCTAGAGCTAATACGT 117
TS      ATAGTTTATTTGATGGTACCTACTACTCGGATAACCGTAGTAATTCTAGAGCTAATACGT 117
TA      ATAGTTTATTTGATGGTACCTACTACTCGGATAACCGTAGTAATTCTAGAGCTAATACGT 117
TS2     ATAGTTTATTTGATGGTACCTACTACTCGGATAACCGTAGTAATTCTAGAGCTAATACGT 118
SD1     ATAGTTTATTTGATGGTACCTACTACTCGGATAACCGTAGTAATTCTAGAGCTAATACGT 117
SD2     ATAGTTTATTTGATGGTACCTACTACTCGGATAACCGTAGTAATTCTAGAGCTAATACGT 117
KI      ATAGTTTATTTGATGGTACCTACTACTCGGATAACCGTAGTAATTCTAGAGCTAATACGT 117
        ********************************************** **********

TV1     GCGTAAATCCCGACTTCTGGAAGGGACGTATTTATTAGATTTAAGGCCAACCGAGCTCTG 177
TV4     GCGTAAATCCCGACTTCTGGAAGGGACGTATTTATTAGATTTAAGGCCAACCGAGCTCTG 177
TV2     GCGTAAATCCCGACTTCTGGAAGGGACGTATTTATTAGATTTAAGGCCAACCGAGCTCTG 177
TV3     GCGTAAATCCCGACTTCTGGAAGGGACGTATTTATTAGATTTAAGGCCAACCGAGCTCTG 177
TC2     GCGCAAATCCCGACTTCTGGAAGGGACGTATTTATTAGATTTAAGGCCGACCGAGCTTTG 179
TG      GCGCAAATCCCGACTTCTGGAAGGGACGTATTTATTAGATTTAAGGCCGACCGAGCTTTG 177
TC1     GCGCAAATCCCGACTTCTGGAAGGGACGTATTTATTAGATTTAAGGCCGACCGAGCTTTG 177
TS1     GCGTAAATCCCGACTTCTGGAAGGGACGTATTTATTAGATTTAAGGCCGACCGAGCTTTG 177
TN      GCGTAAATCCTGACTTCTGGAAGGGACGTATTTATTAGATTTAAGGCCGACCGAGCTTTG 177
Tsp     GCGTAAATCCCGACTTCTGGAAGGGACGTATTTATTAGATTTAAGGCCGACCGGGCTTTG 177
TS      GCGTAAATCCCGACTTCTGGAAGGGACGTATTTATTAGATTTAAGGCCGACCGAGCTTTG 177
TA      GCGTAAATCCCGACTTCTGGAAGGGACGTATTTATTAGATTTAAGGCCGACCGAGCTTTG 177
TS2     GCGTAAATCCCGACTTCTGGAAGGGACGTATTTATTAGATTTAAGGCCGACCGAGCTTTG 178
```

```
SD1     GCGTAAATCCCGACTTCTGGAAGGGACGTATTTATTAGATTTAAGGCCGACCGAGCTT-G 176
SD2     GCGTAAATCCCGACTTCTGGAAGGGACGTATTTATTAGATTTAAGGCCGACCGAGCTT-G 176
KI      GCGTAAATCCCGACTTCTGGAAGGGACGTATTTATTAGATTTAAGGCCAACCGAGCTTTG 177
        *** ****** ************************************ **** ***  *
TV1     CTCGTCTCTTGGTGAATCATGATAACTTCACGAATCGCATGGCCTCCGCG--CCGGCGAT 235
TV4     CTCGTCTCTTGGTGAATCATGATAACTTCACGAATCGCATGGCCTCCGCG--CCGGCGAT 235
TV2     CTCGTCTCTTGGTGAATCATGATAACTTCACGAATCGCATGGCCTCTGCG--CCGGCGAT 235
TV3     CTCGTCTCTTGGTGAATCATGATAACTTCACGAATCGCATGGCCTCTGCG--CCGGCGAT 235
TC2     CTCGTCTTGCGGTGAATCATGATAACTTCACGAATCGCATGGCCCTCGCG--CCGGCGAT 237
TG      CTCGTCTTGCGGTGAATCATGATAACTTCACGAATCGCATGGCCCTCGCG--CCGGCGAT 235
TC1     CTCGTCTTGCGGTGAATCATGATAACTTCACGAATCGCATGGCCCTCGCG--CCGGCGAT 235
TS1     CTCGTCTTGCGGTGAATCATGATAACTTCACGAATCGCATGGCCTCCGCG--CCGGCGAT 235
TN      CTCGTCTTGCGGTGAATCATGATAACTTCACGAATCGCATGGCCTCCGCGCGCCGGCGAT 237
Tsp     CTCGTCTTGCGGTGAATCATGATAACTTCACGAATCGCATGGCCTCCGCG--CCGGCGAT 235
TS      CTCGTCTTGCGGTGAATCATGATAACTTCACGAATCGCATGGCCTCCGCG--CCGGCGAT 235
TA      CTCGTCTTGCGGTGAATCATGATAACTTCACGAATCGCATGGCCTCCGCG--CCGGCGAT 235
TS2     CTCGTCTTGCGGTGAATCATGATAACTTCACGAATCGCATGGCCTCCGCG--CCGGCGAT 236
SD1     CTCGTCTTTCGGTGAATCATGATAACTTCACGAATCGCATAGCCTTTGTG--CTGGCGAT 234
SD2     CTCGTCTTTCGGTGAATCATGATAACTTCACGAATCGCATAGCCTTTGTG--CTGGCGAT 234
KI      CTCGTCTTTTGGTGAATCATGATAACTTCACGAATCGCATGGCCTT-GCG--CCGGCGAT 234
        *******   ****************************** ***   * *  * ******
TV1     GTTTCATTCAAATTTCTGCCCTATCAATTGGCGATGGTAGGATAGAGGCCTACCATGGTG 295
TV4     GTTTCATTCAAATTTCTGCCCTATCAATTGGCGATGGTAGGATAGAGGCCTACCATGGTG 295
TV2     GTTTCATTCAAATTTCTGCCCTATCAATTGGCGATGGTAGGATAGAGGCCTACCATGGTG 295
TV3     GTTTCATTCAAATTTCTGCCCTATCAATTGGCGATGGTAGGATAGAGGCCTACCATGGTG 295
TC2     ATTTCATTCAAATTTCTGCCCTATCAATTTGCGATGGTAGGATAGAGGCCTACCATGGTG 297
TG      ATTTCATTCAAATTTCTGCCCTATCAATTTGCGATGGTAGGATAGAGGCCTACCATGGTG 295
TC1     ATTTCATTCAAATTTCTGCCCTATCAATTTGCGATGGTAGGATAGAGGCCTACCATGGTG 295
TS1     GTTTCATTCAAATTTCTGCCCTATCAATTTGCGATGGTAGGATAGAGGCCTACCATGGTG 295
TN      GTTTCATTCAAATTTCTGCCCTATCAATTTGCGATGGTAGGATAGAGGCCTACCATGGTG 297
Tsp     GTTTCATTCAAATTTCTGCCCTATCAATTTGCGATGGTAGGATAGAGGCCTACCATGGTG 295
TS      GTTTCATTCAAATTTCTGCCCTATCAATTTGCGATGGTAGGATAGAGGCCTACCATGGTG 295
TA      GTTTCATTCAAATTTCTGCCCTATCAATTTGCGATGGTAGGATAGAGGCCTACCATGGTG 295
TS2     GTTTCATTCAAATTTCTGCCCTATCAATTTGCGATGGTAGGATAGAGGCCTACCATGGTG 296
SD1     GTTTCATTCAAATTTCTGCCCTATCAATTGGCGATGGTAGGATAGAGGCCTACCATGGTG 294
SD2     GTTTCATTCAAATTTCTGCCCTATCAATTGGCGATGGTAGGATAGAGGCCTACCATGGTG 294
KI      ATTTCATTCAAATTTCTGCCCTATCAATTGGCGATGGTAGGATAGAGGCCTACCATGGTG 294
         **************************** ******************************
TV1     TTAACGGGTGACGGAGGATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAATGGCTACC 355
TV4     TTAACGGGTGACGGAGGATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAATGGCTACC 355
TV2     TTAACGGGTGACGGAGGATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAATGGCTACC 355
TV3     TTAACGGGTGACGGAGGATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAATGGCTACC 355
TC2     GTAACGGGTGACGGAGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACC 357
TG      GTAACGGGTGACGGAGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACC 355
TC1     GTAACGGGTGACGGAGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACC 355
TS1     GTAACGGGTGACGGAGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACC 355
TN      GTAACGGGTGACGGAGAATTAGGGTTCGATTCCGGATAGGGAGCCTGAGAAACGGCTACC 357
Tsp     GTAACGGGTGACGGAGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACC 355
TS      GTAACGGGTGACGGAGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACC 355
TA      GTAACGGGTGACGGAGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACC 355
TS2     GTAACGGGTGACGGAGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACC 356
SD1     GTAACGGGTGACGGAGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACC 354
SD2     GTAACGGGTGACGGAGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACC 354
KI      TTAACGGGTGACGGGGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACC 354
         ************* * ******************** *************** *******
TV1     ACATCC-AAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTGATACAGGGAGGTAGTGA 414
TV4     ACATCC-AAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTGATACAGGGAGGTAGTGA 414
```

```
TV2    ACATCC-AAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTGATACAGGGAGGTAGTGA 414
TV3    ACATCCCAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTGATACAGGGAGGTAGTGA 415
TC2    ACATCC-AAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTGATACAGGGAGGTAGTGA 416
TG     ACATCC-AAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTGATACAGGGAGGTAGTGA 414
TC1    ACATCC-AAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTGATACAGGGAGGTAGTGA 414
TS1    ACATCC-AAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTGACACAGGGAGGTAGTGA 414
TN     ACATCC-AAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTGACACAGGGAGGTAGTGA 416
Tsp    ACATCC-AAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTGACACAGGGAGGTAGTGA 414
TS     ACATCC-AAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTGACACAGGGAGGTAGTGA 414
TA     ACATCC-AAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTGACACAGGGAGGTAGTGA 414
TS2    ACATCC-AAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTGACACAGGGAGGTAGTGA 415
SD1    ACATCC-AAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTGATACAGGGAGGTAGTGA 413
SD2    ACATCC-AAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTGATACAGGGAGGTAGTGA 413
KI     ACATCC-AAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCTGATACAGGGAGGTAGTGA 413
       ****** ************************************ ***************

TV1    CAATAAATAACAATACCGGGCTTTT-CAAGTCTGGTAA-TTGGAATGAGTACAATCTAAA 472
TV4    CAATAAATAACAATACCGGGCTTTT-CAAGTCTGGTAA-TTGGAATGAGTACAATCTAAA 472
TV2    CAATAAATAACAATACCGGGCTTTT-CAAGTCTGGTAA-TTGGAATGAGTACAATCTAAA 472
TV3    CAATAAATAACAATACCGGGCTTTT-CAAGTCTGGTAAATTGGAATGAGTACAATCTAAA 474
TC2    CAATAAATAACAATACCGGGCTTTT-CA-GTCTGGTAA-TTGGAATGAGTACAATCTAAA 473
TG     CAATAAATAACAATACCGGGCTTTT-CAAGTCTGGTAA-TTGGAATGAGTACAATCTAAA 472
TC1    CAATAAATAACAATACCGGGCTTTT-CA-GTCTGGTAA-TTGGAATGAGTACAATCTAAA 471
TS1    CAATAAATAACAATACCGGGCTTTT-CA-GTCTGGTAA-TTGGAATGAGTACAATCTAAA 471
TN     CAATAAATAACAATACCGGGCTTTT-CAAGTCTGGTAA-TTGGAATGAGTACAATCTAAA 474
Tsp    CAATAAATAACAATACCGGGCTTTT-CAAGTCTGGTAA-TTGGAATGAGTACAATCTAAA 472
TS     CAATAAATAACAATACCGGGCTTTT-CAAGTCTGGTAA-TTGGAATGAGTACAATCTAAA 472
TA     CAATAAATAACAATACCGGGCTTTT-CAAGTCTGGTAA-TTGGAATGAGTACAATCTAAA 472
TS2    CAATAAATAACAATACCGGGCTTTT-CAAGTCTGGTAA-TTGGAATGAGTACAATCTAAA 473
SD1    CAATAAATAACAATACCGGGCTTTTTCAAGTCTGGTAA-TTGGAATGAGTACAATCTAAA 472
SD2    CAATAAATAACAATACCGGGCTTTTTCAAGTCTGGTAA-TTGGAATGAGTACAATCTAAA 472
KI     CAATAAATAACAATACCGGGCTTCT-AAAGTCTGGTAA-TTGGAATGAGTACAATCTAAA 471
       ********************** *  * ********* ********************

TV1    TC-CCTTAACGAGGATCCATTGGAGGGCAAGTCTGGT-GCCAGCAGCCGCGGTAATTCCA 530
TV4    TC-CCTTAACGAGGATCCATTGGAGGGCAAGTCTGGT-GCCAGCAGCCGCGGTAATTCCA 530
TV2    TC-CCTTAACGAGGATCCATTGGAGGGCAAGTCTGGT-GCCAGCAGCCGCGGTAATTCCA 530
TV3    TC-CCTTAACGAGGATCCATTGGAGGGCAAGTCTGGT-GCCAGCAGCCGCGGTAATTCCA 532
TC2    CAACCTTAACGAGGATCCATTGGAGGGCAAGTCTGGT-GCCAGCAGCCGCGGTAATTCCA 532
TG     CAACCTTAACGAGGATCCATTGGAGGGCAAGTCTGGT-GCCAGCAGCCGCGGTAATTCCA 531
TC1    CAACCTTAACGAGGATCCATTGGAGGGCAAGTCTGGT-GCCAGCAGCCGCGGTAATTCCA 530
TS1    CAACCTTAACGAGGATCCATTGGAGGGCAAGTCTGGT-GCCAGCAGCCGCGGTAATTCCA 530
TN     CAACCTTAACGAGGATCCATTGGAGGGCAAGTCTGGTTGCCAGCAGCCGCG-TAATTCCA 533
Tsp    CAACCTTAACGAGGATCCATTGGAGGGCAAGTCTGGT-GCCAGCAGCCGCG-TAATTCCA 530
TS     CAACCTTAACGAGGATCCATTGGAGGGCAAGTCTGGT-GCCAGCAGCCGCGGTAATTCCA 531
TA     CAACCTTAACGAGGATCCATTGGAGGGCAAGTCTGGT-GCCAGCAGCCGCGGTAATTCCA 531
TS2    CAACCTTAACGAGGATCCATTGGAGGGCAAGTCTGGT-GCCAGCAGCCGCGGTAATTCCA 532
SD1    TC-CCTTAACGAGGATCCATTGGAGGGCAAGTCTGGT-GCCAGCAGCCGCGGTAATTCCA 530
SD2    TC-CCTTAACGAGGATCCATTGGAGGGCAAGTCTGGT-GCCAGCAGCCGCGGTAATTCCA 530
KI     TC-CCTTAACGAGGATCCATTGGAGGGCAAGTCTGGT-GCCAGCAGCCGCGGTAATTCCA 529
          ********************************** ************* ********

TV1    GCTCCAATAGCGTATATTTAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGGATTTCGGATG 590
TV4    GCTCCAATAGCGTATATTTAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGGATTTCGGATG 590
TV2    GCTCCAATAGCGTATATTTAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGGATTTCGGATG 590
TV3    GCTCCAATAGCGTATATTTAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGGATTTCGGATG 592
TC2    GCTCCAATAGCGTATATTTAAGTTGCTGCAGTTAAAAAGCTCGTAGTTGGATTTCGGATG 592
TG     GCTCCAATAGCGTATATTTAAGTTGCTGCAGTTAAAAAGCTCGTAGTTGGATTTCGGATG 591
TC1   GCTCCAATAGCGTATATTTAAGTTGCTGCAGTTAAAAAGCTCGTAGTTGGATTTCGGATG 590
TS1   GCTCCAATAGCGTATATTTAAGTTGCTGCAGTTAAAAAGCTCGTAGTTGGATTTCGGATG 590
```

```
TN    GCTCCAATAGCGTATATTTAAGTTGCTGCAGTTAAAAAGCTCGTAGTTGGATTTCGGATG 593
Tsp   GCTCCAATAGCGTATATTTAAGTTGCTGCAGTTAAAAAGCTCGTAGTTGGATTTCGGATG 590
TS    GCTCCAATAGCGTATATTTAAGTTGCTGCAGTTAAAAAGCTCGTAGTTGGATTTCGGATG 591
TA    GCTCCAATAGCGTATATTTAAGTTGCTGCAGTTAAAAAGCTCGTAGTTGGATTTCGGATG 591
TS2   GCTCCAATAGCGTATATTTAAGTTGCTGCAGTTAAAAAGCTCGTAGTTGGATTTCGGATG 592
SD1   GCTCCAATAGCGTATATTTAAGTTGCTGCAGTTAAAAAGCTCGTAGTTGGATTTCGGATG 590
SD2   GCTCCAATAGCGTATATTTAAGTTGCTGCAGTTAAAAAGCTCGTAGTTGGATTTCGGATG 590
KI    GCTCCAATAGCGTATATTTAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGGATTTCGGATG 589
      ************************* ********************************

TV1   GGACTTGCCGGTCCGTCGTTGCGATGTGCACTGGCCAGTCCTATCTTGTTGTCGGGGACT 650
TV4   GGACTTGCCGGTCCGTCGTTGCGATGTGCACTGGCCAGTCCTATCTTGTTGTCGGGGACT 650
TV2   GGACTTGCCGGTCCGTCGTTGCGATGTGCACTGGCCAGTCCTATCTTGTTGTCGGGGACT 650
TV3   GGACTTGCCGGTCCGTCGTTGCGATGTGCACTGGCCAGTCCTATCTTGTTGTCGGGGACT 652
TC2   GGATTTGCCGGTCCGCCGTTCCGGTGTGCACTGGCCAGTCTCATCTTGTTGTTGGGGACT 652
TG    GGATTTGCCGGTCCGCCGTTCCGGTGTGCACTGGCCAGTCTCATCTTGTTGTTGGGGACT 651
TC1   GGATTTGCCGGTCCGCCGTTCCGGTGTGCACTGGCCAGTCTCATCTTGTTGTTGGGGACT 650
TS1   GGATTTGCCGGTCCGCCGTTTCGGTGTGCACTGGCCAGTCCCATCTTGTTGTCGGGGACT 650
TN    GGATTTGCCGGTCCGCCGTTTCGGTGTGCACTGGCCAGTCCCATCTTGTTGTCGGGGACT 653
Tsp   GGATTTGCCGGTCCGCCGTTTCGGTGTGCACTGGCCAGTCCCATCTTGTTGTCGGGGACT 650
TS    GGATTTGCCGGTCCGCCGTTTCGGTGTGCACTGGCCAGTCCCATCTTGTTGTCGGGGACT 651
TA    GGATTTGCCGGTCCGCCGTTTCGGTGTGCACTGGCCAGTCCCATCTTGTTGTCGGGGACT 651
TS2   GGATTTGCCGGTCCGCCGTTTCGGTGTGCACTGGCCAGTCCCATCTTGTTGTCGGGGACT 652
SD1   GGGTTTGCCGGTCCGTCGTTGCGATGTGCACTGGCAAGTCCTATCTTGTTGTCGGGGACT 650
SD2   GGGTTTGCCGGTCCGTCGTTGCGATGTGCACTGGCAAGTCCTATCTTGTTGTCGGGGACT 650
KI    GGACCTGCCGGTCCGTCTTTGAGATGTGTACTGGCAAGTCCCATCTTGTTGTCGGGGACT 649
      **   ********** * **  * **** ****** ****  ********** *******

TV1   AGCTCCTGGGCTTCACTGTCC-GGGACTAGGAGCTGACGAGGTTACTTTGAGTAAATTAG 709
TV4   AGCTCCTGGGCTTCACTGTCC-GGGACTAGGAGCTGACGAGGTTACTTTGAGTAAATTAG 709
TV2   AGCTCCTGGGCTTCACTGTCC-GGGACTAGGAGCTGACAAGGT-ACTTTGAGTAA-TTAT 707
TV3   AGCTCCTGGGCTTCACTGTCC-GGGACTAGGAGCTGACGAGGTTACTTTGAGTAAATTAG 711
TC2   AGCTCCTGGGCTTNACTGTCC-GGGACTAGGAGCTGACGAGGTTACTTTGAGTAAATTAG 711
TG    AGCTCCTGGGCTTCACTGTCC-GGGACTAGGAGCTGACGAGGTTACTTTGAGTAAATTAG 710
TC1   AGCTCCTGGGCTTCACTGTCC-GGGACTAGGAGCTGACGAGGTTACTTTGAGTAAATTAG 709
TS1   AGCTCCTGGGCTTCACTGTCC-GGGACTAGGAGCTGACGAGGTTACTTTGAGTAAATTAG 709
TN    AGCTCCTGGGCTTCACTGTCC-GGGACTAGGAGCTGACGAGGTTACTTTGAGTAAATTAG 712
Tsp   AGCTCCTGGGCTTCACTGTCC-GGGACTAGGAGCTGACGAGGTTACTTTGAGTAAATTAG 709
TS    AGCTCCTGGGCTTCACTGTCC-GGGACTAGGAGCTGACGAGGTTACTTTGAGTAAATTAG 710
TA    AGCTCCTGGGCTTCACTGTCC-GGGACTAGGAGCTGACGAGGTTACTTTGAGTAAATTAG 710
TS2   AGCTCCTGGGCTTCACTGTCC-GGGACTAGGAGCTGACGAGGTTACTTTGAGTAAATTAG 711
SD1   AGCTCCTGGGCTTCACTGTCC-GGGACTAGGAGCTGACGAGGTTACTTTGAGTAAATTAG 709
SD2   AGCTCCTGGGCTTCACTGTCC-GGGACTAGGAGCTGACGAGGTTACTTTGAGTAAATTAG 709
KI    AGCTCCTGGGCTTCACTGTCCCGGGACTAGGAGCCGACGAAGTTACTTTGAGTAAATTAG 709
      ************* ******* ************ *** * ** *********** ***

TV1   AGTGTTCAAAGCAAGCCTACGCTCTGAATACATTAGCATGGAATAACACGATAGGACTCT 769
TV4   AGTGTTCAAAGCAAGCCTACGCTCTGAATACATTAGCATGGAATAACACGATAGGACTCT 769
TV2   AATGTTCAAGC--AGCCTACGCTCTGA-TACATTAGCATGGAATAACACGATAGGACTCT 764
TV3   AGTGTTCAAAGCAAGCCTACGCTCTGAATACATTAGCATGGAATAACACGATAGGACTCT 771
TC2   AGTGTTCAAAGCAAGCCTACGCTCTGAATACATTAGCATGGAATAACATGATAGGACTCT 771
TG    AGTGTTCAAAGCAAGCCTACGCTCTGAATACATTAGCATGGAATAACATGATAGGACTCT 770
TC1   AGTGTTCAAAGCAAGCCTACGCTCTGAATACATTAGCATGGAATAACATGATAGGACTCT 769
TS1   AGTGTTCAAAGCAAGCCTACGCTCTGAATACATTAGCATGGAATAACATGATAGGACTCT 769
TN    AGTGTTCAAAGCAAGCCTACGCTCTGAATACATTAGCATGGAATAACATGATAGGACTCT 772
Tsp   AGTGTTCAAAGCAAGCCTACGCTCTGAATACATTAGCATGGAATAACATGATAGGACTCT 769
TS    AGTGTTCAAAGCAAGCCTACGCTCTGAATACATTAGCATGGAATAACATGATAGGACTCT 770
TA    AGTGTTCAAAGCAAGCCTACGCTCTGAATACATTAGCATGGAATAACATGATAGGACTCT 770
TS2   AGTGTTCAAAGCAAGCCTACGCTCTGAATACATTAGCATGGAATAACATGATAGGACTCT 771
SD1   AGTGTTCAAAGCAAGCCTACGCTCTGAATACATTAGCATGGAATAACACGATAGGACTCT 769
```

```
SD2   AGTGTTCAAAGCAAGCCTACGCTCTGAATACATTAGCATGGAATAACACGATAGGACTCT 769
KI    AGTGTTCAAAGCAAGCCTACGCTCTGAATATATTAGCATGGAATAACACGATAGGACTCT 769
      * *******    ************** ** ***************** ***********
TV1   GGCTTATCCTGTTGGTCTGTGAGACCAGAGTAATGATTAAGAGGGACAGTCGGGGACATT 829
TV4   GGCTTATCCTGTTGGTCTGTGAGACCAGAGTAATGATTAAGAGGGACAGTCGGGGACATT 829
TV2   GGCTTATCCTGTTGGTCTGTGAGACCAGAGTAATGATTAAGAGGGACAGTCGGGGACATT 824
TV3   GGCTTATCCTGTTGGTCTGTGAGACCAGAGTAATGATTAAGAGGGACAGTCGGGGACATT 831
TC2   GGCTTATCTTGTTGGTCTGTGAGACCAGAGTAATGATTAAGAGGGACAGTCGGGGACATT 831
TG    GGCTTATCTTGTTGGTCTGTGAGACCAGAGTAATGATTAAGAGGGACAGTCGGGGACATT 830
TC    GGCTTATCTTGTTGGTCTGTGAGACCAGAGTAATGATTAAGAGGGACAGTCGGGGACATT 829
TS1   GGCTTATCTTGTTGGTCTGTGAGACCAGAGTAATGATTAAGAGGGACAGTCGGGGGCATT 829
TN    GGCTTATCTTGTTGGTCTGTGAGACCAGAGTAATGATTAAGAGGGACAGTCGGGGGCATT 832
Tsp   GGCTTATCTTGTTGGTCTGTGAGACCAGAGTAATGATTAAGAGGGACAGTCGGGGGCATT 829
TS    GGCTTATCTTGTTGGTCTGTGAGACCAGAGTAATGATTAAGAGGGACAGTCGGGGGCATT 830
TA    GGCTTATCTTGTTGGTCTGTGAGACCAGAGTAATGATTAAGAGGGACAGTCGGGGGCATT 830
TS2   GGCTTATCTTGTTGGTCTGTGAGACCAGAGTAATGATTAAGAGGGACAGTCGGGGG-ATT 830
SD1   GGCTTATCTTGTTGGTCTGTGAGACCAGAGTAATGATTAAGAGGGACAGTCGGGGACATT 829
SD2   GGCTTATCTTGTTGGTCTGTGAGACCAGAGTAATGATTAAGAGGGACAGTCGGGGACATT 829
KI    GGCTTATCTTGTTGGTCTGTGAGACCAGAGTAATGATTAAGAGGGACAGTCGGGGACATT 829
      ******** ******************************************  ***
TV1   CGTATTTCATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGACGAACTTCTGCGAAAGC 889
TV4   CGTATTTCATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGACGAACTTCTGCGAAAGC 889
TV2   CGTATTTCATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGACGAACTTCTGCGAAAGC 884
TV3   CGTATTTCATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGACGAACTTCTGCGAAAGC 891
TC2   CGTATTTCATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGACGAACTTCTGCGAAAGC 891
TG    CGTATTTCATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGACGAACTTCTGCGAAAGC 890
TC1   CGTATTTCATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGACGAACTTCTGCGAAAGC 889
TS1   CGTATTTCATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGACGAACTTCTGCGAAAGC 889
TN    CGTATTTCATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGACGAACTTCTGCGAAAGC 892
Tsp   CGTATTTCATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGACGAACTTCTGCGAAAGC 889
TS    CGTATTTCATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGACGAACTTCTGCGAAAGC 890
TA    CGTATTTCATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGACGAACTTCTGCGAAAGC 890
TS2   CGTATTTCATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGACGAACTTCTGCGAAAGC 890
SD1   CGTATTTCATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGACGAACTTCTGCGAAAGC 889
SD2   CGTATTTCATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGACGAACTTCTGCGAAAGC 889
KI    CGTATTTCATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGACGAACTTCTGCGAAAGC 889
      ************************************************************
TV1   ATTT-GTCAAGGATGTTTTCATTAATCAAGAAC-GAAAGTTGGGGG-CTCGAAGACGATT 946
TV4   ATTT-GTCAAGGATGTTTTCATTAATCAAGAAC-GAAAGTTGGGGG-CTCGAAGACGATT 946
TV2   ATTT-GTCAAGGATGTTTTCATTAATCAAGAAC-GAAAGTTGGGGG-CTCGAAGACGATT 941
TV3   ATTT-GTCAAGGATGTTTTCATTAATCAAGAAC-GAAAGTTGGGGG-CTCGAAGACGATT 948
TC2   ATTT-GTCAAGGATGTTTTCATTAATCA-GAAC-GAAAGTTGGGGG-CTCGAAGACGATT 947
TG    ATTT-GTCAAGGATGTTTTCATTAATCAAGAAC-GAAAGTTGGGGG-CTCGAAGACGATT 947
TC1   ATTT-GTCAAGGATGTTTTCATTAATCAAGAAC-GAAAGTTGGGGGGCTCGAAGACGATT 947
TS1   ATTT-GTCAAGGATGTTTTCATTAATCAAGAAC-GAAAGTTGGGGG-CTCGAAGACGATT 946
TN    ATTTTGTCAAGGATGTTTTCATTAATCAACAACCGAAAGTTGGGGG-CTCGAAGACGATT 951
Tsp   ATTT-GTCAAGGATGTTTTCATTAATCAAGAAC-GAAAGTTGGGGG-CTCGAAGACGATT 946
TS    ATTT-GTCAAGGATGTTTTCATTAATCAAGAAC-GAAAGTTGGGGG-CTCGAAGACGATT 947
TA    ATTT-GTCAAGGATGTTTTCATTAATCAAGAAC-GAAAGTTGGGGG-CTCGAAGACGATT 947
TS2   ATTT-GTCAAGGATGTTTTCATTAATCAAGAAC-GAAAGTTGGGGG-CTCGAAGACGATT 947
SD1   ATTT-GTCAAGGATGTTTTCATTAATCAAGAAC-GAAAGTTGGGGG-CTCGAAGACGATT 946
SD2   ATTT-GTCAAGGATGTTTTCATTAATCAAGAAC-GAAAGTTGGGGG-CTCGAAGACGATT 946
KI    ATTT-GTCAAGGATGTTTTCATTAATCAAGAAC-GAAAGTTGGGGG-CTCGAAGACGATT 946
      **** **********************  *** ************ *************


TV1   AGATACCGTCCTAGTCTCAACCATAAACGATGCCGACTAGGGATTGGCAGACGTTTTTTT 1006
```

```
TV4   AGATACCGTCCTAGTCTCAACCATAAACGATGCCGACTAGGGATTGGCAGACGTTTTTTT 1006
TV2   AGATACCGTCCTAGTCTCAACCATAAACGATGCCGACTAGGGATTGGCAGACGTTTTTTT 1001
TV3   AGATACCGTCCTAGTCTCAACCATAAACGATGCCGACTAGGGATTGGCAGACGTTTTTTT 1008
TC2   AGATACCGTCCTAGTCTCAACCATAAACGATGCCGACTAGGGATTGGCAGACGTTTTTTT 1007
TG    AGATACCGTCCTAGTCTCAACCATAAACGATGCCGACTAGGGATTGGCAGACGTTTTTTT 1007
TC1   AGATACCGTCCTAGTCTCAACCATAAACGATGCCGACTAGGGATTGGCAGACGTTTTTTT 1007
TS1   AGATACCGTCCTAGTCTCAACCATAAACGATGCCGACTAGGGATTGGCAGACGTTTTTTT 1006
TN    AGATACCGTCCTAGTCTCAACCATAAACGATGCCGACTAGGGATTGGCAGACGTTTTTTT 1011
Tsp   AGATACCGTCCTAGTCTCAACCATAAACGATGCCGACTAGGGATTGGCAGACGTTTTTTT 1006
TS    AGATACCGTCCTAGTCTCAACCATAAACGATGCCGACTAGGGATTGGCAGACGTTTTTTT 1007
TA    AGATACCGTCCTAGTCTCAACCATAAACGATGCCGACTAGGGATTGGCAGACGTTTTTTT 1007
TS2   AGATACCGTCCTAGTCTCAACCATAAACGATGCCGACTAGGGATTGGCAGACGTTTTTTT 1007
SD1   AGATACCGTCCTAGTCTCAACCATAAACGATGCCGACTAGGGATTGGCAGATGTTTTTTT 1006
SD2   AGATACCGTCCTAGTCTCAACCATAAACGATGCCGACTAGGGATTGGCAGATGTTTTTTT 1006
KI    AGATACCGTCCTAGTCTCAACCATAAACGATGCCGACTAGGGATTGGCAGATGTTTTTTT 1006
      ********************************************** ********

TV1   GATGACTCTGCCAGC-ACCTTATGAAAAATCAAAGTTTTTGGGTTCCGGGGGGAGTTTGG 1065
TV4   GATGACTCTGCCAGCCACCTTATGAAAAATCAAAGTTTTTGGGTTCCGGGGGGAGTTTGG 1066
TV2   GATGACTCTGCCAGC-ACCTTATGAGAAATCAAAGTTTTTGGGTTCCGGGGGGAGTATGG 1060
TV3   GATGACTCTGCCAGC-ACCTTATGAGAAATCAAAGTTTTTGGGTTCCGGGGGGAGTATGG 1067
TC2   GATGACTCTGCCAGC-ACCTTATGAGAAATCAAAGTTTTTGGGTTCCGGGGGGAGTATGG 1066
TG    GATGACTCTGCCAGC-ACCTTATGAGAAATCAAAGTTTTTGGGTTCCGGGGGGAGTATGG 1066
TC1   GATGACTCTGCCAGC-ACCTTATGAGAAATCAAAGTTTTTGGGTTCCGGGGGGAGTATGG 1066
TS1   GATGACTCTGCCAGC-ACCTTATGAGAAATCAAAGTTTTTGGGTTCCGGGGGGAGTATGG 1065
TN    GATGACTCTGCCAGC-ACCTTATGAGAAATCAAAGTTTTTGGGTTCCGGGGGGAGTATGG 1070
Tsp   GATGACTCTGCCAGC-ACCTTATGAGAAATCAAAGTTTTTGGGTTCCGGGGGGAGTATGG 1065
TS    GATGACTCTGCCAGC-ACCTTATGAGAAATCAAAGTTTTTGGGTTCCGGGGGGAGTATGG 1066
TA    GATGACTCTGCCAGC-ACCTTATGAGAAATCAAAGTTTTTGGGTTCCGGGGGGAGTATGG 1066
TS2   GATGACTCTGCCAGC-ACCTTATGAGAAATCAAAGTTTTTGGGTTCCGGGGGGAGTATGG 1066
SD1   GATGACTCTGCCAGC-ACCTTATGAGAAATCAAAGTTTTTGGGTTCCGGGGGGAGTATGG 1065
SD2   GATGACTCTGCCAGC-ACCTTATGAGAAATCAAAGTTTTTGGGTTCCGGGGGGAGTATGG 1065
KI    GATGACTCTGCCAGC-ACCTTATGAGAAATCAAAGTTTTTGGGTTCCGGGGGGAGTATGG 1065
      *************** ********* ****************************** ***

TV1   TC-GCAGGGCTGAAAACTTAAAGGAATTGACGGAAGG--CACCACCAGGCGTGG--AGCC 1120
TV4   TCCGCAAGGCTGAAA-CTTAAAGGAATTGACGGAAGGGCCACCACCAGGCGTGG--AGCC 1123
TV2   TC-GCAAGGCTGAAA-CTTAAAGGAATTGACGGAAGGG-CACCACCAGGCGTGG--AGCC 1115
TV3   TC-GCAAGGCTGAAA-CTTAAAGGAATTGACGGAAGGG-CACCACCAGGCGTGG--AGCC 1122
TC2   TC-GCAAGGCTGAAA-CTTAAAGGAATTGACGGAAGGG-CACCACCAGGCGTGG--AGCC 1121
TG    TC-GCAAGGCTGAAA-CTTAAAGGAATTGACGGAAGGG-ACACACCAGGCGTGG--AGCC 1121
TC1   TC-GCAAGGCTGAAA-CTTAAAGGAATTGACGGAAGGG-CACCACCAGGCGTGG--AGCC 1121
TS1   TC-GCAAGGCTGAAA-CTTAAAGGAATTGACGGAAGGG-CACCACCAGGCGTGG--AGCC 1120
TN    TC-GCAAGGCTGAAA-CTTAAAGGAATTGACGGAAGGG-CACCACCAGGCGTGGGCAGCC 1127
Tsp   TC-GCAAGGCTGAAA-CTTAAAGGAATTGACGGAAGGG-CACCACCAGGCGTGG--AGCC 1120
TS    TC-GCAAGGCTGAAA-CTTAAAGGAATTGACGGAAGGG-CACCACCAGGCGTGG--AGCC 1121
TA    TC-GCAAGGCTGAAA-CTTAAAGGAATTGACGGAAGGG-CACCACCAGGCGTGG--AGCC 1121
TS2   TC-GCAAGGCTGAAA-CTTAAAGGAATTGACGGAAGGG-CACCACCAGGCGTGG--AGCC 1121
SD1   TC-GCAAGGCTGAAA-CTTAAAGGAATTGACGGAAGGG-CACCACCAGGCGTGG---AGC 1119
SD2   TC-GCAAGGCTGAAA-CTTAAAGGAATTGACGGAAGGG-CACCACCAGGCGTGG---AGC 1119
KI    TC-GCAAGGCTGAAA-CTTAAAGGAATTGACGGAAGGG-CACCACCAGGCGTGG--AGCC 1120
      ** *** ******** *********************     ************     *

TV1   TGCGGCTTATTTTGACTCA-CACGGGAAAACTTA-CCAGGTCCAGACATAGTGAGGATTG 1178
TV4   TGCGGTTTAATTTGACTCAACACGGGAAAACTTA-CCAGGTCCAGACATAGTGAGGATTG 1182
TV2   TGCGGCTTAATTTGACTCAACACGGGAAAACTTA-CCAGGTCCAGACATAGTGAGGATTG 1174
TV3   TGCGGCTTAATTTGACTCAACACGGGAAAACTTA-CCAGGTCCAGACATAGTGAGGATTG 1181
TC2   TGCGGCTTAATTTGACTCAACACGGGAAAACTTA-CCAGGTCCAGACATAGTGAGGATTG 1180
TG    TGCGGCTTAATTTGACTCAACACGG-AAAACTTA-CCAGGTCCAGACATAGTGAGGATTG 1179
TC1   TGCGGCTTAATTTGACTCAACACGGGAAAACTTA-CCAGGTCCAGACATAGTGAGGATTG 1180
```

```
TS1   TGCGGCTTAATTTGACTCAACACGGGAAAACTTA-CCAGGTCCAGACATAGTGAGGATTG 1179
TN    TGCGGCTTAATTTGACTCAACACGGGAAAACTTAACCAGGTCCAGACATAGTGAGGATTG 1187
Tsp   TGCGGCTTAATTTGACTCAACACGGGAAAACTTA-CCAGGTCCAGACATAGTGAGGATTG 1179
TS    TGCGGCTTAATTTGACTCAACACGGGAAAACTTA-CCAGGTCCAGACATAGTGAGGATTG 1180
TA    TGCGGCTTAATTTGACTCAACACGGGAAAACTTA-CCAGGTCCAGACATAGTGAGGATTG 1180
TS2   TGCGGCTTAATTTGACTCAACACGGGAAAACTTA-CCAGGTCCAGACATAGTGAGGATTG 1180
SD1   TGCGGCTTAATTTGACTCAACACGGGGAAACTTA-CCAGGTCCAGACATAGTGAGGATTG 1178
SD2   TGCGGCTTAATTTGACTCAACACGGGGAAACTTA-CCAGGTCCAGACATAGTGAGGATTG 1178
KI    TGCGGCTTAATTTGACTCAACACGGGAAAACTTA-CCAGGTCCAGACATAGTGAGGATTG 1179
      ***** *** ********* *****  ******* ************************

TV1   ACAGATTGAGAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGTTGGT 1238
TV4   ACAGATTGAGAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGTTGGT 1242
TV2   ACAGATTGAGAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGTTGGT 1234
TV3   ACAGATTGAGAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGTTGGT 1241
TC2   ACAGATTGAGAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGTTGGT 1240
TG    ACAGATTGAGAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGTTGGT 1239
TC1   ACAGATTGAGAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGTTGGT 1240
TS1   ACAGATTGAGAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGTTGGT 1239
TN    ACAGATTGAGAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGTTGGT 1247
Tsp   ACAGATTGAGAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGTTGGT 1239
TS    ACAGATTGAGAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGTTGGT 1240
TA    ACAGATTGAGAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGTTGGT 1240
TS2   ACAGATTGAGAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGTTGGT 1240
SD1   ACAGATTGAGAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGTTGGT 1238
SD2   ACAGATTGAGAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGTTGGT 1238
KI    ACAGATTGAGAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGTTGGT 1239
      ************************************************************

TV1   GGGTTGCCTTGTCAGGTTGATTCCGGTAACGAACGAGACCTCAGCCTGCTAAATAGTTAC 1298
TV4   GGGTTGCCTTGTCAGGTTGATTCCGGTAACGAACGAGACCTCAGCCTGCTAAATAGTTAC 1302
TV2   GGGTTGCCTTGTCAGGTTGATTCCGGTAACGAACGAGACCTCAGCCTGCTAAATAGTTAC 1294
TV3   GGGTTGCCTTGTCAGGTTGATTCCGGTAACGAACGAGACCTCAGCCTGCTAAATAGTTAC 1301
TC2   GGGTTGCCTTGTCAGGTTGATTCCGGTAACGAACGAGACCTCAGCCTGCTAAATAGTTAC 1300
TG    GGGTTGCCTTGTCAGGTTGATTCCGGTAACGAACGAGACCTCAGCCTGCTAAATAGTTAC 1299
TC1   GGGTTGCCTTGTCAGGTTGATTCCGGTAACGAACGAGACCTCAGCCTGCTAAATAGTTAC 1300
TS1   GGGTTGCCTTGTCAGGTTGATTCCGGTAACGAACGAGACCTCAGCCTGCTAAATAGTTAC 1299
TN    GGGTTGCCTTGTCAGGTTGATTCCGGTAACGAACGAGACCTCAGCCTGCTAAATAGTTAC 1307
Tsp   GGGTTGCCTTGTCAGGTTGATTCCGGTAACGAACGAGACCTCAGCCTGCTAAATAGTTAC 1299
TS    GGGTTGCCTTGTCAGGTTGATTCCGGTAACGAACGAGACCTCAGCCTGCTAAATAGTTAC 1300
TA    GGGTTGCCTTGTCAGGTTGATTCCGGTAACGAACGAGACCTCAGCCTGCTAAATAGTTAC 1300
TS2   GGGTTGCCTTGTCAGGTTGATTCCGGTAACGAACGAGACCTCAGCCTGCTAAATAGTTAC 1300
SD1   GGGTTGCCTTGTCAGGTTGATTCCGGTAACGAACGAGACCTCAGCCTGCTAAATAGTTAC 1298
SD2   GGGTTGCCTTGTCAGGTTGATTCCGGTAACGAACGAGACCTCAGCCTGCTAAATAGTTAC 1298
KI    GGGTTGCCTTGTCAGGTTGATTCCGGTAACGAACGAGACCTCAGCCTGCTAAATAGTTAC 1299
      ************************************************************

TV1   TCCTACTTTGGTAGGAGGCGAACTTCTTAGAGGGACTATTGGCGTTTAGCCAATGGAAGT 1358
TV4   TCCTACTTTGGTAGGAGGCGAACTTCTTAGAGGGACTATTGGCGTTTAGCCAATGGAAGT 1362
TV2   TCCTACCTTGGTAGGAGGCGAACTTCTTAGAGGGACTATTGGCGTTTAGCCAATGGAAGT 1354
TV3   TCCTACCTTGGTAGGAGGCGAACTTCTTAGAGGGACTATTGGCGTTTAGCCAATGGAAGT 1361
TC2   TCCTACTTTGGTAGGAGGTGAACTTCTTAGAGGGACTATTGGCGTTTAGCCAATGGAAGT 1360
TG    TCCTAGTTTGGTAGGAGGTGAACTTCTTAGAGGGACTATTGGCGTTTAGCCAATGGAAGT 1359
TC1   TCCTACTTTGGTAGGAGGTGAACTTCTTAGAGGGACTATTGGCGTTTAGCCAATGGAAGT 1360
TS1   TCCTACTTTGGTAGGAGGTGAACTTCTTAGAGGGACTATTGGCGTTTAGCCAATGGAAGT 1359
TN    TCCTACTTTGGTAGGAGGTGAACTTCTTAGAGGGACTATTGGCGTTTAGCCAATGGAAGT 1367
Tsp   TCCTACTTTGGTAGGAGGTGAACTTCTTAGAGGGACTATTGGCGTTTAGCCAATGGAAGT 1359
TS    TCCTACTTTGGTAGGAGGTGAACTTCTTAGAGGGACTATTGGCGTTTAGCCAATGGAAGT 1360
TA    TCCTACTTTGGTAGGAGGTGAACTTCTTAGAGGGACTATTGGCGTTTAGCCAATGGAAGT 1360
TS2   TCCTACTTTGGTAGGAGGTGAACTTCTTAGAGGGACTATTGGCGTTTAGCCAATGGAAGT 1360
```

```
SD1   TGCTACCTTGGTAGCTGGCGAACTTCTTAGAGGGACTATTGGCGTTTAGCCAATGGAAGT 1358
SD2   TGCTACCTTGGTAGCTGGCGAACTTCTTAGAGGGACTATTGGCGTTTAGCCAATGGAAGT 1358
KI    TCCTACTTTGGTAGGTGGCAAACTTCTTAGAGGGACTATTGGCGTTTAGCTAATGGAAGT 1359
      * ***  *******  **  ****************************** *********
TV1   GTGAGGCAATAACAGGTCTGTGATGCCCTTAGATGTTCTGGGCCGCACGCGCGCTACACT 1418
TV4   GTGAGGCAATAACAGGTCTGTGATGCCCTTAGATGTTCTGGGCCGCACGCGCGCTACACT 1422
TV2   GTGAGGCAATAACAGGTCTGTGATGCCCTTAGATGTTCTGGGCCGCACGCGCGCTACACT 1414
TV3   GTGAGGCAATAACAGGTCTGTGATGCCCTTAGATGTTCTGGGCCGCACGCGCGCTACACT 1421
TC2   GTGAGGCAATAACAGGTCTGTGATGCCCTTAGATGTTCTGGGCCGCACGCGCGCTACACT 1420
TG    GTGAGGCAATAACAGGTCTGTGATGCCCTTAGATGTTCTGGGCCGCACGCGCGCTACACT 1419
TC1   GTGAGGCAATAACAGGTCTGTGATGCCCTTAGATGTTCTGGGCCGCACGCGCGCTACACT 1420
TS1   GTGAGGCAATAACAGGTCTGTGATGCCCTTAGATGTTCTGGGCCGCACGCGCGCTACACT 1419
TN    GTGAGGCAATAACAGGTCTGTGATGCCCTTAGATGTTCTGGGCCGCACGCGCGCTACACT 1427
Tsp   GTGAGGCAATAACAGGTCTGTGATGCCCTTAGATGTTCTGGGCCGCACGCGCGCTACACT 1419
TS    GTGAGGCAATAACAGGTCTGTGATGCCCTTAGATGTTCTGGGCCGCACGCGCGCTACACT 1420
TA    GTGAGGCAATAACAGGTCTGTGATGCCCTTAGATGTTCTGGGCCGCACGCGCGCTACACT 1420
TS2   GTGAGGCAATAACAGGTCTGTGATGCCCTTAGATGTTCTGGGCCGCACGCGCGCTACACT 1420
SD1   GTGAGGCAATAACAGGTCTGTGATGCCCTTAGATGTTCTGGGCCGCACGCGCGCTACACT 1418
SD2   GTGAGGCAATAACAGGTCTGTGATGCCCTTAGATGTTCTGGGCCGCACGCGCGCTACACT 1418
KI    GTGAGGCAATAACAGGTCTGTGATGCCCTTAGATGTTCTGGGCCGCACGCGCGCTACACT 1419
      ************************************************************
TV1   GATGCATTCAAC-GAGCCTAGCCTTGACCGAGAGGTCCGGGTAATCTTTGAAACTGCATC 1477
TV4   GATGCATTCAAC-GAGCCTAGCCTTGACCGAGAGGTCCGGGTAATCTTTGAAACTGCATC 1481
TV2   GATGCATTCAAC-GAGCCTAGCCTTGACCGAGAGGTCCGGGTAATCTTTGAAACTGCATC 1473
TV3   GATGCATTCAAC-GAGCCTAGCCTTGACCGAGAGGTCCGGGTAATCTTTGAAACTGCATC 1480
TC2   GATGCATTCAAC-GAGCCTAACCTTGACCGAGAGGTCCGGGTAATCTTTGAAACTGCATC 1479
TG    GATGCATTCAACAGAGCCTAACCTTGACCGAGAGGTCCGGGTAATCTTTGAAACTGCATC 1479
TC1   GATGCATTCAAC-GAGCCTAACCTTGACCGAGAGGTCCGGGTAATCTTTGAAACTGCATC 1479
TS1   GATGCATTCAAC-GAGCCTAGCCTTGACCGAGAGGTCCGGGTAATCTTTGAAACTGCATC 1478
TN    GATGCATTCAAC-GAGCCTAGCCTTGACCGAGAGGTCCGGGTAATCTTTGAAACTGCATC 1486
Tsp   GATGCATTCAAC-GAGCCTAGCCTTGACCGAGAGGTCCGGGTAATCTTTGAAACTGCATC 1478
TS    GATGCATTCAAC-GAGCCTAGCCTTGACCGAGAGGTCCGGGTAATCTTTGAAACTGCATC 1479
TA    GATGCATTCAAC-GAGCCTAGCCTTGACCGAGAGGTCCGGGTAATCTTTGAAACTGCATC 1479
TS2   GATGCATTCAAC-GAGCCTAGCCTTGACCGAGAGGTCCGGGTAATCTTTGAAACTGCATC 1479
SD1   GATGCATTCAAC-GAGCCTAGCCTTGACCGAGAGGTCCGGGTAATCTTTGAAACTGCATC 1477
SD2   GATGCATTCAAC-GAGCCTAGCCTTGACCGAGAGGTCCGGGTAATCTTTGAAACTGCATC 1477
KI    GATGCATTCACC-GAGCCTAGCCTTGACCGAGAGGTCCGGGTAATCTTTGAAACTGCATC 1478
      ********** * ******* ************************************
TV1   GTGATGGGGCTAGATTATTGCAATTATT---------AATCTTCAACGAGGAATGCCTAG 1528
TV4   GTGATGGGGCTAGATTATTGCAATTATT---------AATCTTCAACGAGGAATGCCTAG 1532
TV2   GTGATGGGGCTAGATTATTGCAATTATT---------AATCTTCAACGAGGAATGCCTAG 1524
TV3   GTGATGGGGCTAGATTATTGCAATTATT---------AATCTTCAACGAGGAATGCCTAG 1531
TC2   GTGATGGGGCTAGATTATTGCAATTATT---------AATCTTCAACGAGGAATGCCTAG 1530
TG    GTGATGGGGCTAGATTATTGCAATTATTGCAATTATTAATCTTCAACGAGGAATGCCTAG 1539
TC1   GTGATGGGGCTAGATTATTGCAATTATT---------AATCTTCAACGAGGAATGCCTAG 1530
TS1   GTGATGGGGCTAGATTATTGCAATTATT---------AATCTTCAACGAGGAATGCCTAG 1529
TN    GTGATGGGGCTAGATTATTGCAATTATT---------AATCTTCAACGAGGAATGCCTAG 1537
Tsp   GTGATGGGGCTAGATTATTGCAATTATT---------AATCTTCAACGAGGAATGCCTAG 1529
TS    GTGATGGGGCTAGATTATTGCAATTATT---------AATCTTCAACGAGGAATGCCTAG 1530
TA    GTGATGGGGCTAGATTATTGCAATTATT---------AATCTTCAACGAGGAATGCCTAG 1530
TS2   GTGATGGGGCTAGATTATTGCAATTATT---------AATCTTCAACGAGGAATGCCTAG 1530
SD1   GTGATGGGGCTAGATTATTGCAATTATT---------AATCTTCAACGAGGAATGCCTAG 1528
SD2   GTGATGGGGCTAGATTATTGCAATTATT---------AATCTTCAACGAGGAATGCCTAG 1528
KI    GTGATGGGGCTAGATTATTGCAATTATT---------AATCTTCAACGAGGAATGCCTAG 1529
      ***************************         ***********************
TV1   TAAGCGTGATTCATCAAA-TCGCGTTGATTACGTCCCTGCCCTTTGTACACACCGCCCGT 1587
TV4   TAAGCGTGATTCATCAAA-TCGCGTTGATTACGTCCCTGCCCTTTGTACACACCGCCCGT 1591
```

```
TV2   TAAGCGTGATTCATCAAA-TCGCGTTGATTACGTCCCTGCCCTTTGTACACACCGCCCGT 1583
TV3   TAAGCGTGATTCATCAAA-TCGCGTTGATTACGTCCCTGCCCTTTGTACACACCGCCCGT 1590
TC2   TAAGCGTGATTCATCAAA-TCGCGTTGATTACGTCCCTGCCCTTTGTACACACCGCCCGT 1589
TG    TAAGCGTGATTCATCAAAATCGCGTTGATTACGTCCCTGCCCTTTGTACACACCGCCCGT 1599
TC1   TAAGCGTGATTCATCAAA-TCGCGTTGATTACGTCCCTGCCCTTTGTACACACCGCCCGT 1589
TS1   TAAGCGTGATTCATCAGA-TCGCGTTGATTACGTCCCTGCCCTTTGTACACACCGCCCGT 1588
TN    TAAGCGTGATTCATCAGA-TCGCGTTGATTACGTCCCTGCCCTTTGTACACACCGCCCGT 1596
Tsp   TAAGCGTGATTCATCAGA-TCGCGTTGATTACGTCCCTGCCCTTTGTACACACCGCCCGT 1588
TS    TAAGCGTGATTCATCAGA-TCGCGTTGATTACGTCCCTGCCCTTTGTACACACCGCCCGT 1589
TA    TAAGCGTGATTCATCAGA-TCGCGTTGATTACGTCCCTGCCCTTTGTACACACCGCCCGT 1589
TS2   TAAGCGTGATTCATCAGA-TCGCGTTGATTACGTCCCTGCCCTTTGTACACACCGCCCGT 1589
SD1   TAAGCGTGATTCATCAAA-TCGCGTTGATTACGTCCCTGCCCTTTGTACACACCGCCCGT 1587
SD2   TAAGCGTGATTCATCAAA-TCGCGTTGATTACGTCCCTGCCCTTTGTACACACCGCCCGT 1587
KI    TAAGCGTGATTCATCAAA-TCGCGTTGATTACGTCCCTGCCCTTTGTACACACCGCCCGT 1588
      ********************* ************************************** 
TV1   CGCTCCTACCGATTGAGTGTGTTGGTGAGGAGTTCGGATTGGCTCTTAGTGGTGGTTC-G 1646
TV4   CGCTCCTACCGATTGAGTGTGTTGGTGAGGAGTTCGGATTGGCTCTTAGTGGTGGTTC-G 1650
TV2   CGCTCCTACCGATTGAGTGTGTTGGTGAGGAGTTCGGATTGGCTCTTAGTGGTGGTTC-G 1642
TV3   CGCTCCTACCGATTGAGTGTGTTGGTGAGGAGTTCGGATTGGCTCTTAGTGGTGGTTC-G 1649
TC2   CGCTCCTACCGATTGAATGTGTTGGTGAGGAGTTCGGATTGGCAGTTAGTGGTGGTTCAG 1649
TG    CGCTCCTACCGATTGAATGTGTTGGTGAGGAGTTCGGATTGGCAGTTAGTGGTGGTTCCG 1659
TC1   CGCTCCTACCGATTGAATGTGTTGGTGAGGAGTTCGGATTGGCAGTTAGTGGTGGTTCCG 1649
TS1   CGCTCCTACCGATTGAATGTGTTGGT-AGGAGTTTCGGATGGCAGTT-GTGG-GGTTC-- 1643
TN    CGCTCCTACCGATTGAATGTGTTGGTGAGGAGTTCGGATTGGCAGTTTGTGGTGGTTC-G 1655
Tsp   CGCTCCTACCGATTGAATGTGTTGGTGAGGAGTTCGGATTGGCAGTTTGTGGTGGTTC-G 1647
TS    CGCTCCTACCGATTGAATGTGTTGGTGAGGAGTTCGGATTGGCAGTTTGTGGTGGTTC-G 1648
TA    CGCTCCTACCGATTGAATGTGTTGGTGAGGAGTTCGGATTGGCAGTTTGTGGTGGTTC-G 1648
TS2   CGCTCCTACCGATTGAATGTGTTGGTGAGGAGTTCGGATTGGCAGTTTGTGGTGGTTC-G 1648
SD1   CGCTCCTACCGATTGAGTGTGTTGGTGAAGAGTTCGGATTGGTACCAGATGGTGGTTC-G 1646
SD2   CGCTCCTACCGATTGAGTGTGTTGGTGAAGAGTTCGGATTGGTACCAGATGGTGGTTC-G 1646
KI    CGCTCCTACCGATTGAATGTGTTGGTGAGGAGTTTGGATTGGCAACTGGAGGTGGTTT-T 1647
      ********************* ********* * *****  *  ***        ** **** 
TV1   CCACCACCTAGAGCC-ATGAGAAGTTCTCCAA--GTCCGCCCCACTTAGAGGAAGGAGAA 1703
TV4   CCACCACCTAGAGC--ATGAGAAGTTCTCCAA--GTCCGCCCCACTTAGAGGAAGGAGAA 1706
TV2   CCACCACCTAGAGC---TGAGAAGTTCTCCAA--A-CCGCCCCACTTAGAGGAAGGAGAA 1696
TV3   CCACCACCTAGAGC---TGAGAAGTTCTCCAA--A-CCGCCCCACTTAGAGGAAGGAGAA 1703
TC2   CCACTGCCC-GCACA--GAGGAAGTTATGCT---GTCCGCCCCATTTAGAG-AAGCAGAA 1702
TG    CCACTGCCTTGCACA--AGAGAAGTTCTCCCCAAGTCCGCCCCATTTAGAGGAAGGAGAA 1717
TC1   CCACTGCCT-GCACA--GA-GAAGTTATCCA---ATCCGCCCCATTTAGAGGAAGGAGAA 1702
TS1   CCACTGCCTTACAGC--TGAGAAGTC--TCA---AACCGCCCCAT----AAGAAGGAGA- 1691
TN    CCACTGC-TTACAGC--TGAGAAGTTCTCCA---AACCGCCCCATTTAGAGGAAGGAGAA 1709
Tsp   CCACTGC-TTACAGC--TGAGAAGTTCTCCA---AACCGCCCCATTTAGAGGAAGGAGAA 1701
TS    CCACTGC-TTACAGC--TGAGAAGTTCTCCA---AACCGCCCCATTTAGAGGAAGGAGAA 1702
TA    CCACTGC-TTACAGC--TGAGAAGTTCTCCA---AACCGCCCCATTTAGAGGAAGGAGAA 1702
TS2   CCACTGC-TTACAGCCATGAGAAGTTCTCCA---AACCGCCCCATTTAGAGGAAGGAGAA 1704
SD1   CCACCGT-CTGTAGC--TGAGAAGTTCTTTA---AACCGCCCCACTTAGAGGAAGGAGAA 1700
SD2   CCACCGT-CTGTAGC--TGAGAAGTTCTTTA---AACCGCCCCACTTAGAGGAAGGAGAA 1700
KI    CCACCGCCTATTGCT---GAGAAGTTCTCCA---AACCGCCCCATTTAGAGGAAGGAGAA 1701
      ****               *****          ********     *  *** *** 
```

Figure 3.4: Phylogeny of *Tetraselmis* within Prasinophyceae based on 18S rDNA sequence comparisons inferred with Neighbor joining method.

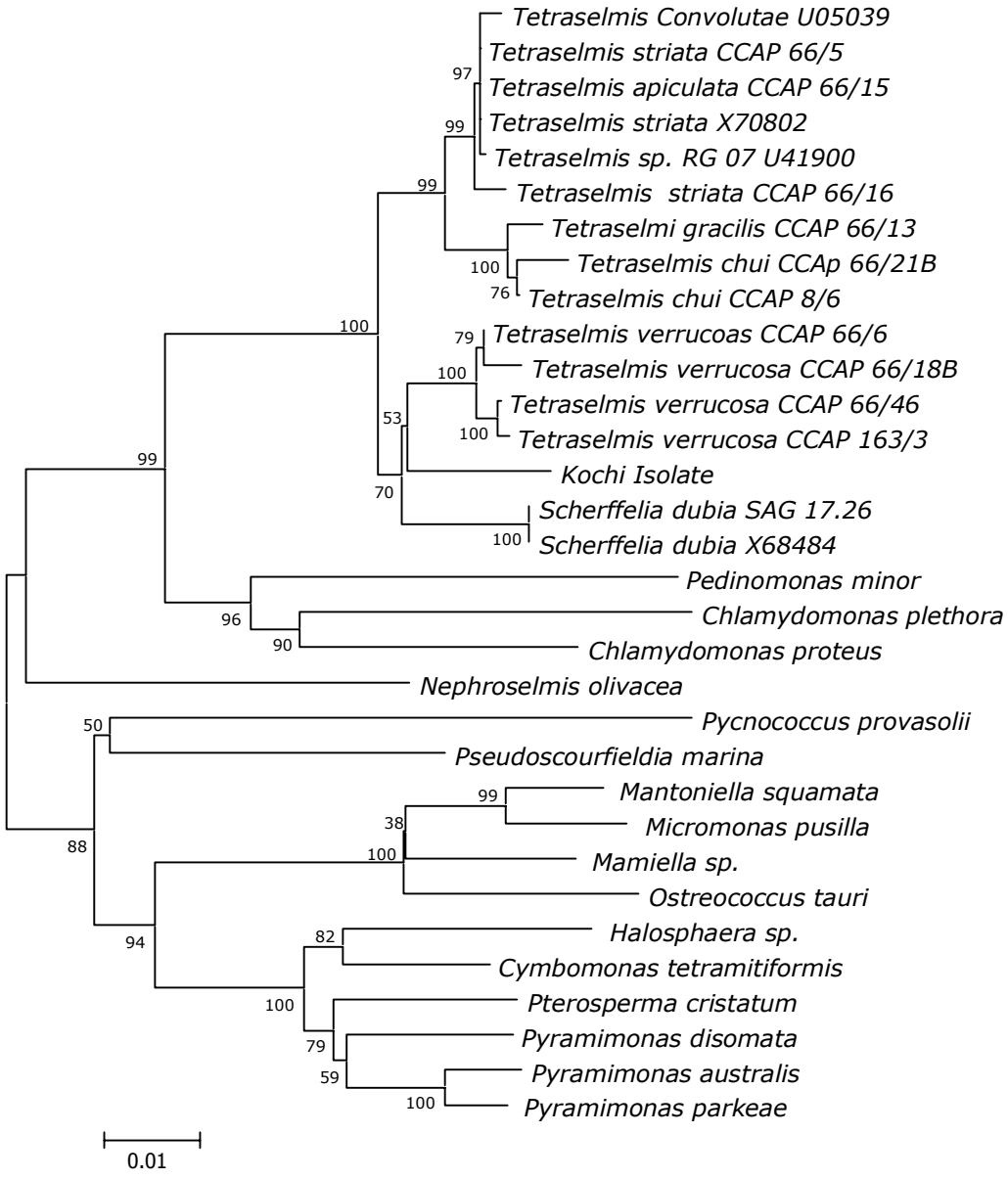
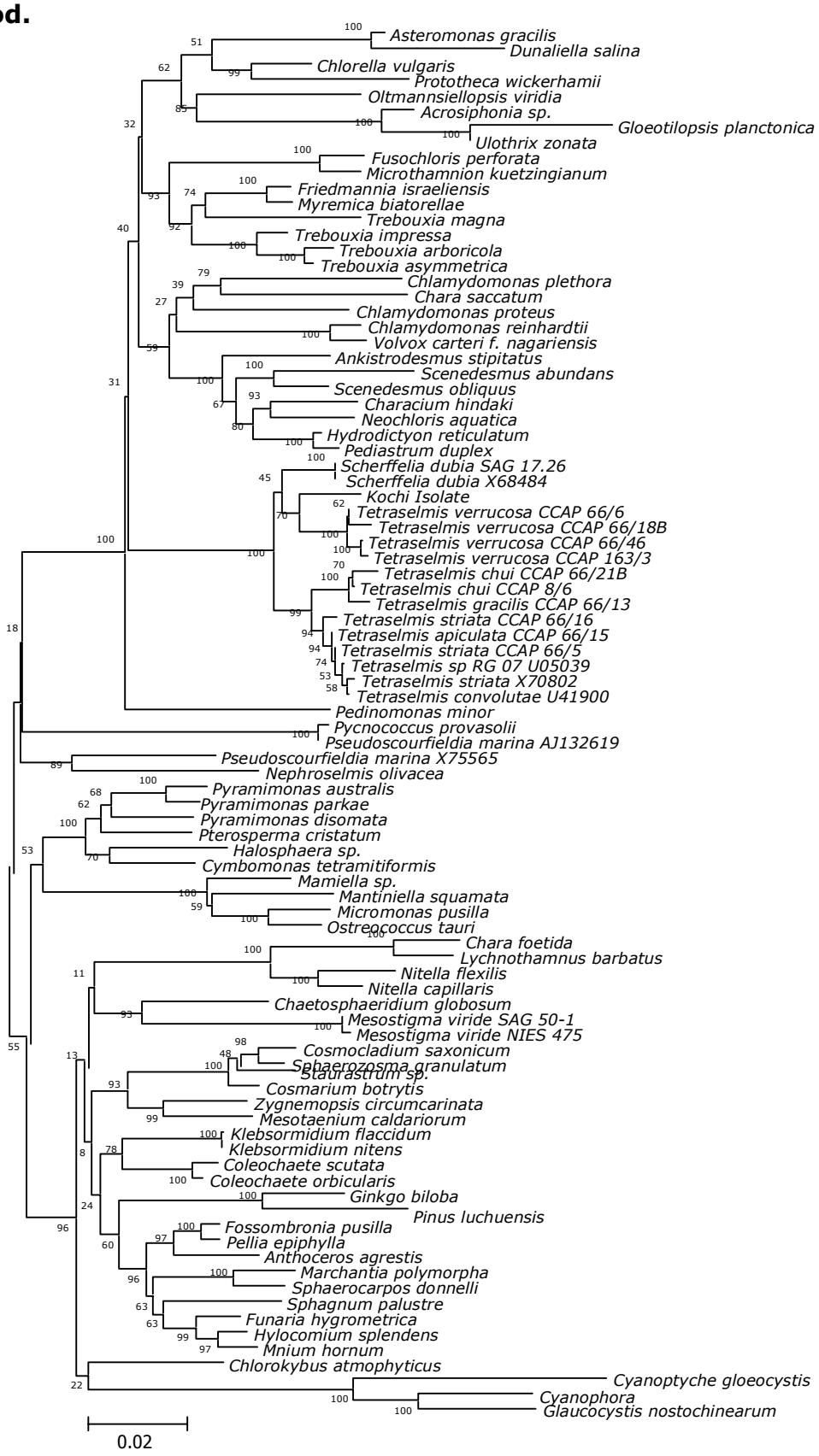
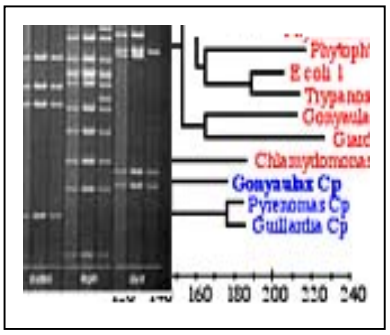


Figure 3.5: Phylogeny of *Tetraselmis* within other green algae based on 18S rDNA sequence comparisons inferred with Neighbor joining method.





CHAPTER 4

DNA FINGERPRINTING OF THE PRASINOPHYTE FLAGELLATE ISOLATED FROM KOCHI BACKWATER AND ITS1-5.8S-ITS2 rDNA SEQUENCE VARIATON IN THE GENUS *TETRASELMIS*.

1. ABSTRACT

DNA fingerprinting was carried out using ISSR primers. A total of 100 primers were screened. Fifteen dinucleotide and one trinucleotide repeat primers gave clear and reproducible banding patterns. The average band size ranged between 200- 1700 bp.

Cluster analysis showed that the five species of *Tetraselmis* separated into three clusters. The Kochi isolate separated out from these five species. The first cluster consisted of the two strains of *T. chui* grouped with *T. gracilis*. The results suggested that *T. chui* CCAP 8/6, CCAP 66/21B and *T. gracilis* CCAP 66/13 might be the same species. The second cluster comprised of two strains *T. striata* and *T. apiculata*. The strains of *T. striata* CCAP 66/5, CCAP 66/16 and *T. apiculata* CCAP 66/15 were extremely closely related with a bootstrap values of more than 99% and may be the same species. The four strains of *T. verrucosa* formed the third cluster. The species of *Chlamydomonas* and *Pedinomonas minor* grouped into a separate cluster. The Kochi isolate separated at equal distances from both the clusters and formed sister branch with *Scherffelia dubia*.

ITS sequences of *Tetraselmis* were more than 96% similar within the species whereas sequence similarity ranged from 60 to 83% between species. The highest sequence similarity of 83% was observed in ITS1 region of *T. chui-T. gracilis* and *T. striata-T. apiculata*. The sequence similarity between *T. verrucosa* and *T. chui* or *T. striata* ranged between 65-75% in ITS1 and 60-70% in ITS2. The Kochi isolate was almost equidistant from *T. chui-T. gracilis*, *T. striata-T. apiculata* and *T. verrucosa* groups. The isolate showed 68-75% sequence similarity in ITS1 and 60-70% sequence similarity in ITS2 with that of other *Tetraselmis* species. ITS sequence results were comparable with ISSR analysis and both the analyses resolved same grouping of species and strains.

2. INTRODUCTION

Species level studies in algal systematics have developed mostly from morphological observations, and taxonomy is still strongly based on it. However, sometimes it is difficult to make taxonomic decisions at generic or species level based on morphological observations alone. Also species relationship and genetic variation between and within the species cannot be assessed using morphological markers. Knowledge about intra-specific genomic variation is essential because it indicates the size of the genetic pool within a species and also helps to assess the genetic variation between the species. Among classical methods, isozyme analyses have been used for inferring genetic divergence between and within related species or between and within a population (Cheney 1985; Huber and Lewin 1986; Sosa and Garcia-Reina 1992 and 1993).

Earlier molecular studies in algal systematics involved measurement of DNA base composition, DNA:DNA relatedness through in-vitro hybridization and restriction fragment length polymorphism (RFLP) (Stam, and Venema, 1977; Olsen *et al*., 1987; Stam *et al*., 1987; Bot *et al*., 1989 a, b; Scholfield *et al*., 1991). RFLPs have been used to explore phylogenetic relationships at the population, species and generic level in Gracilaria (Goff and Coleman, 1988; Carroll, 1989), Pandorina (Moore and Coleman, 1987) and within the laminariales (Fain 1986, Fain *et al*., 1988; Bhattacharya and Druehl 1989 and Druehl 1989).

The Polymerase Chain Reaction (PCR) has made it possible to access many regions in the genome and detect genetic variations using both DNA fingerprinting and sequencing of specific region in the genome. Distantly related taxa are studied using conserved DNA regions in the genome whereas variable regions are studied at or below the species level. DNA fingerprinting uses these highly variable regions to differentiate among closely related individuals.

The term DNA fingerprinting was introduced by Jeffreys (Jeffreys, 1985) to describe a method for simultaneous detection of variable DNA loci

by hybridization of multilocus probes to electrophoretically separated restriction fragments. DNA typing has rapidly become the primary method for identifying and distinguishing individuals, species and populations. It is also used in forensic studies.

In recent years, DNA markers are being used by phycologists to study genetic relationships at population, genus and species level. Randomly amplified polymorphic DNA (RAPD) has been used widely to study algal relationships. For example this marker has been used to study interspecific variation in *Porphyra* (Rhodophyta) (Dutcher and Kapraun, 1994), genetic similarities among *Gelidium* populations (Rhodophyta) (Alberto *et al*., 1997) and *Postelsia* (Phaeophyta) (Coyer *et al.*, 1997). A more recent technique of amplified fragment length polymophism (AFLP) using RFLP and PCR based technique together has been used in *Chondrus crispus* (Rhodophyta) for population studies (Donaldson *et al*., 1998).

DNA polymorphism detected by inter simple sequence repeat (ISSR) also offers a potential tool for genome fingerprinting (Zietkiewicz *et al.*, 1994). ISSR markers have been widely used in studies on genetic diversity of crop plants such as barley (Hoz *et al*., 1996), maize (Kantety *et al*., 1995) and finger millet (Salimath *et al.*, 1995). They have also been used for diversity analysis of the Mycorrhiza *Suillus grevillei* (Zhou *et al*., 1999) and in distinguishing gametophytes of strains of the Rhodophycean alga *Batracospermum boryanum* (Vis 1999). However the best molecular resolution for taxonomic investigation is achieved by DNA or rRNA sequencing. Ribosomal genes are well characterized, ubiquitous and easily accessible by PCR (White *et al*., 1990). The sequences form a mosaic pattern of conserved and variable regions, which make them attractive for taxonomic identification and phylogenetic studies.

Internal transcribed spacers of ribosomal DNA (ITS1 and ITS2) have variable sequences. These sequences have been used in a wide variety of marine organisms including several species of algae such as in *Cladophorosis memrbranacea* (Chlorophyata) for biogeographic analysis (Kooistra *et al*., 1992); in tracking the dispersal routes of *Acrosiphonia arcta* (Chlorophyata)

(van Oppen *et al*., 1994); in studying the evolutionary history of family Fucaceae (Phaeophyceae) (Serrão *et al*., 1999) and for identification of *Chlorella* strains (Cozzolino *et al*., 1999). They have been used to study inter and intra-specific genetic variation in *Caulerpa* (Pillmann *et al.*, 1997) and phylogeographic grouping of *C. taxifolia* and *C. mexicana* (Olsen *et al*., 1998) and C*. racemosa* (Fama *et al*., 2000). Coleman and Mai (1997) have used rDNA-ITS sequences to find out close relatives of *Chlamydomonas reinhardtii*.

In the genus *Tetraselmis* subgeneric and species level identification has always been difficult (Butcher 1959; Norris *et al.*, 1980). The species belonging to the genus mostly have separated on the basis of cell size, shape and symmetry of the cell, position of eyespot. Hori *et al*., (1982, 1983 and 1986) used pyrenoid ultrastructure to separate the species and classified the genus into four subgenera (Table 1.2, Chapter 1). Marin *et al*., (1993) used the ultrastructure of flagellar hairs to separate the species and subdivided the genus into four strain specific flagellar types (Table 1.3, Chapter 1). However as discussed in chapter 1 neither pyrenoid structure nor flagellar hairs can be used as marker for species identification. Hence it becomes difficult to position new isolate in any taxonomically acceptable manner.

In this chapter we present, results based on ISSR profiles and rDNA ITS1-5.8S-ITS2 sequences to study the genetic variability in the genus *Tetraselmis*. Taxonomic grouping of the Kochi isolate, identified as *Tetraselmis kochinensis* (?) NCIM 7001 is also determined using these protocols.

3. Materials and Methods

Materials

List of algae used in these studies is given in 2, Table 2.1 (Page). Taq DNA polymerase was purchased from Perkin Elmer (USA), ISSR PCR primers (set # 9) from University of British Columbia (Canada), dNTPs and cloning kit from Promega (USA). *E. coli* XL1 blue strain was from Stratagene (Switzerland). DNase free RNase, CTAB and PVP were Sigma (USA) products.

IPTG and x-Gal were purchased from Bangalore Genie (India). Other chemicals used were of Molecular Biology Grade.

Methods

ISSR-PCR Amplification and Optimization of PCR Conditions

A set of 100 primers (UBC set # 9) was procured from University of British Columbia, Canada. These primers were 12-22 nucleotides in length and included various di, tri, tetra and penta nucleotide repeat motifs. The dinucleotide repeat primers were anchored at 5' or 3' with one or two selected nucleotides.

PCR conditions were optimized with respect to concentration of template DNA, enzyme and primer. Polymerase chain reactions were carried out in 10 mM TrisCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.15 mM dNTPs, 2% formamide, 0.5 mM spermidine, 1 unit of Taq DNA polymerase, 15 picomoles of primer, 60 ng of template DNA in 20 μl volume for 45 cycles. After initial denaturation at 94^0C for 5 min, each cycle comprised 1 min denaturation at 94^0C, 45 seconds annealing at 50^0C, 2 min extension at 72^0C with final extension at 72^0C for 5 min at the end of 45 cycles. All amplification reactions were performed in a PTC-200 DNA engine thermal cycler (M. J. Research, USA). The use of formamide in the reaction mixture was critical to avoid a high background and smearing. An initial screening of the 100 primers was done where DNA from *Tetraselmis chui* CCAP 8/6, *Chlamydomonas moewusii* CCAP 11/11 and the Kochi isolate were amplified with each primer. Amplified PCR products were electrophoresed on 2% agarose gel in 0.5X TAE buffer, pH 8.0 and visualised by ethidium bromide staining and scored for the presence (1) or absence (0) of bands.

The binary data was used to construct a dendrogram and the cluster analysis was based on Unweighted Pair Group Method for Arthmetic mean (UPGMA) with software package NTSYS-pc (Rolhf, 1989) using Dice coefficient. Genetic distance was computed using WINDIST whereas bootstrapping of the data was done using WINBOOT software (Yap and Nelson, 1996) to determine the confidence limits of the dendrograms.

Southern Blotting and Hybridization

After gel electrophoresis, ISSR-PCR products obtained with primer $(AG)_8YC$ were blotted onto nylon membrane (Sambrook *et al*., 1989). The monomorphic bands were eluted from the individual lanes using low melting agarose gel. These bands were then used as probes for hybridization. The probes were radiolabeled with α-P^{32} dATP using random priming method. Hybridization reaction was carried out at 16 –18 hrs. in 50% formamide, 6X SSPE, 0.1% SDS 1% milk powder at 42^0C. After hybridization blots were washed with 3X SSPE and 0.1 % SDS for 30 min at room temperature followed by hot wash at 42^0C with 2X SSPE, 0.5% SDS for 5 min. The signals obtained were visualized by autoradiography (Sambrook *et al*., 1989).

Cloning and Sequencing of the ISSR Product

A monomorphic band obtained with primer $(AG)_8YC$ was eluted from the agarose gel slice by freeze-thaw method and eluted DNA was re-amplified using same primer as described previously in a 50 µl reaction. The amplified reaction mixture was extracted once with chloroform: isoamyl alcohol 24:1 mixture and precipitated with two volumes of chilled ethanol.

Reamplified DNA was ligated to PCR-product cloning vector pGEM-T easy from the commercially available cloning kit (Promega). The ligation reaction was carried out at 16^0C for 16 hrs in 10 µl reaction volume which contained 50 ng of insert DNA, 1 unit of T4 DNA ligase, 50 ng of pGEM-T easy vector and 5x cloning buffer. The ligated mixture was transformed into the *E. coli* strain XL-1 Blue (Stratagene, Switzerland). The transformed cells were plated on amphicillin, x-Gal-IPTG-LB (Luria Broth) agar medium and incubated at 37^0C for 16 to 18 hrs and at 4^0C for 2 hrs. Resulting white colonies on x-Gal-IPTG plates were screened for the presence of an insert by restricting the recombinant plasmid using appropriate enzymes and also by hybridization with earlier blot.

Plasmid DNA was prepared by alkaline lysis method. Positive colonies for the presence of insert were chosen and grown in a 10 ml LB medium with amphicillin (Sambrook *et al*., 1989). The cells were harvested by centrifugation at 5000 rpm for 10 min., suspended in GTE buffer (50 mM glucose, 25 mM TrisCl pH 8.0, 10 mM EDTA pH 8.0) and after vortexing incubated at room temperature for 10 min. Then double volume of solution II (1% SDS, 0.2 M NaOH) was added to the above cell suspension, the contents were mixed well and kept on ice for 10 min. The suspension was neutralized by adding half volume of solution III (60 ml 5 M potassium acetate, 11.5 ml glacial acetic acid, 28.5 ml water), mixed well by inversion, kept on ice for 10 min. and centrifuged at 10,000 rpm for 10 min. to pellet out cell debris and chromosomal DNA. To the supernatant equal volume of phenol was added which was then centrifuged as above followed by treatment with equal volume of a mixture of phenol: chloroform: isoamyl alcohol (25:24:1) and lastly with chloroform: isoamyl alcohol (24:1).

The DNA was then precipitated by adding 1/10 volume of 3M sodium acetate (pH 5.2) and two and half volumes of ethanol. The DNA precipitate was washed with 70% ethanol, centrifuged and dissolved in TE buffer (10 mM TrisCl, 1 mM EDTA pH 8.0). To this RNase treatment was given (10 μg/ml) for 30 min at 37^0C.

Both strands were sequenced using T7 and SP6 RNA polymerase primers using Big Dye terminator Kit (ABI-Perkin Elmer USA) on ABI-PRISM automated 310 DNA sequencer.

Amplification, Cloning and Sequencing of, ITS1-5.8S-ITS2 rDNA

For the amplification ITS1-5.8S-ITS2 rDNA, primers used were ITS1- 5'-TCC GTA GAA CCT GCG G- 3' and ITS4- 5' TCC TCC GCT TAT TGA TAT GC 3' (White *et al*., 1990). PCR reaction contained 10 mM TrisCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.15 mM dNTPs, 1 unit of Taq DNA polymerase, 10 picomoles of primer, 100 ng of template DNA in 25 μl volume for 35 cycles. After initial denaturation at 94^0C for 5 min, each cycle comprised 1 min denaturation at 94^0C, 45 seconds annealing at 50^0C, 2 min extension at 72^0C

with final extension at 72^0C for 5 min at the end of 35 cycles. All amplification reactions were performed in a PTC-200 DNA Engine thermalcycler (M. J. Research, USA). The amplified PCR products were electrophoresed on 2% agarose gel in 0.5X TAE buffer, pH 8.0 and visualised by ethidium bromide staining

After purification the PCR products were ligated with pGEMT easy plasmid vector (Promega) as described above. The recombinant plasmids were used to transform *E. coli* XL1 blue strains. Transformants were selected by Blue/White screening. The plasmid DNA was prepared as described above. The colonies were checked for the presence of insert by amplifying the recombinant plasmid DNA using ITS1 and ITS4 primers as described earlier.

The cloned fragments were sequenced for both the strands with T7 and SP6 universal RNA polymerase primers using Big Dye terminator Kit (ABI-Perkin Elmer USA) on ABI-PRISM automated 310 DNA sequencer. For each sample at least two colonies were sequenced.

Sequence Analysis

The resulting sequences were aligned using CLUSTAL W program www.ebi.ac.uk\clustalw. The entire ITS1-5.8S-ITS2 region was used in master alignment. The genetic distances for ITS1, ITS2 and 5.8S regions were calculated separately according to the two-parameter model of Kimura (1980) using Mega 2 software (Kumar *et al*., 2001). The phylogenetic analysis was using in Neighbor-Joining and UPGMA method with 1000 bootstrap replications. The sequences from database are *Chlamydomonas* species (AJ297808) and *Chlamydomonas reinhardtii* (X65621).

4. RESULTS AND DISCUSSION

ISSR fingerprinting

Reproducible banding patterns were obtained with 60 ng template DNA, 1 unit of Taq DNA polymerase and 15 picomoles of primer. Out of 100 primers screened sixteen primers gave clear and repeatable amplification profiles, which were then used for amplifying DNA from all the species listed

in Table 2.1 (Chapter 2). High concentrations of template DNA and primers were used in order to reduce methodological errors in the analysis. Smith *et al*., (1994) and Oppen *et al*., (1996) have discussed, in case of RAPD analysis, that the presence or absence of certain bands is dependent on the presence or absence of other bands. This source of error, which may occur due to competition for the priming site, can be overcome by the use of high concentrations of template DNA and primers.

Sixteen primers including 15 dinucleotide and one trinucleotide repeat amplified a total of 536 loci. The average band size ranged between 100 bp to 1600 bp (Table 4.1). The amplification profile generated by these primers demonstrated a high degree of polymorphism where 535 of the 536 (99.81 %) loci were polymorphic. No two amplification profiles were identical, however some bands were monomorphic within same species as discussed later.

Out of 11 trinuclotide repeats tested, only $(ATG)_6$ gave amplification. The bands ranged from 400 to 1500 bp. As seen from the amplification profile (Fig 4.1) both the *T. chui* strains showed identical banding patterns which were different from the banding patterns of all other species. These two strains can be distinguished from all other *Tetraselmis* species tested on the basis of these results.

AG and GA repeats gave amplification patterns irrespective of the anchoring nucleotide whereas CA, CT, GT, and TG repeats amplified algal DNA only when used in conjunction with specific anchors (Table 4.1). Amplification patterns generated by $(GT)_8$ and $(TG)_8$ were always accompanied by a background smear although banding patterns were replicable. $(CA)_8$ and $(CT)_8$ amplified reproducible polymorphic patterns. None of the AC, AT and TA repeats amplified algal DNA indicating that these repeats might have been beyond the range of amplification by Taq DNA polymerase or might be absent in these genomes. The results indicate that (GA), (AG), (CA) and (CT) repeats are most suitable for fingerprinting the *Tetraselmis* genome.

The amplification of algal DNA using the primer $(AG)_8$ anchored with YC is depicted in Figure 4.2. This primer amplified a 900 bp band, which was present in all the species tested and was the only monomorphic band obtained among the species examined. In order to check whether there is a sequence similarity of this 900 bp band between species, hybridization studies were carried out. The results showed that the band eluted from *T. chui* 8/6 (Lane 1, Fig. 4.2) hybridized with all the other 900 bp bands amplified using this primer (Fig 4.3). This suggested that these bands had similar sequences. The nucleotide sequence of this band was determined (Fig 4.4) and deposited in the EMBL database. This band was also present in *Pedinomonas minor* and three *Chlamydomonas* species, indicating it is not a *Tetraselmis* specific fragment.

A BLAST search comparing this 900 bp sequence with all available nucleotide sequence databases and complete genome sequence databases listed at http://www.ncbi.nlm.nih.gov/BLAST/producttable.html, suggested that there was no significant sequence similarity with any other reported nucleotide sequences. Furthermore deduced amino acid sequences using different Open Reading Frame's (ORF) in the 900 bp sequence did not show homology with that of any proteins from the SWISSPROT database. These results suggested that the fragment might represent either a noncoding region or refer to an excised part of a zymogen protein. These results are not discussed further.

Dendrogram and Species Relationship

The amplification patterns of the 16 samples analysed using 16 ISSR primers were used to compute the similarity matrix (Table 4.2), which was then used for cluster analysis (Fig 4.5). There were three major clusters within the *Tetraselmis* species. The Kochi isolate and *Scherffelia dubia* separated from these clusters at equal distances.

The first cluster was that of two *T. chui* strains CCAP 8/6, CCAP 66/21B grouped with *T. gracilis* CCAP 66/13 with 100 % bootstrap value indicated a very strong grouping (Fig 4.5). This suggested that two strains of

T. chui CCAP 8/6, CCAP 66/21B and *T. gracilis* CCAP 66/13 are closely related. Butcher (1959) has separated, *T. chui* Butcher from *T*. *gracilis* Kylin (Kylin 1935), on the basis that *T. chui* has an acute and slightly curved posterior end and a posterior stigma. However, the micrograph of *T. gracilis* by Norris *et al*., (1980) also shows that the posterior end of this species is tapering and slightly curved although they did not give any further description of the species. Secondly, Hori *et al*., (1986) pointed out that the position of eyespot in *T. chui* may vary from central to posterior. Butcher (1959) has also emphasized that these two species are closely related. On the basis of the ISSR dendrogram reported here it appears that *T. chui* CCAP 8/6, CCAP 66/21B and *T. gracilis* CCAP 66/13 are the same species or atleast extremely closely related. Examination of more isolates of the two apparently different species is necessary to resolve this issue.

The second group was *T. striata* CCAP 66/5*, T. apiculata* CCAP 66/15 and *T. striata* CCAP 66/16. *T. striata* CCAP 66/5 was closer to *T. apiculata* CCAP 66/15 than to the second strain of *T. striata* CCAP 66/16. The similarity index value between *T*. *striata* CCAP 66/5 and *T. apiculata* CCAP 66/15 was 0.55 whereas it was 0.32 between *T. striata* CCAP 66/5 and *T. striata* CCAP 66/16 (Table 4.2). Butcher (1959) used the presence of the vertical lines of granular bodies in the cell of *T. striata* as one of the characteristics to separate it from *T. apiculata*. However Hori *et al*., (1986) have not found such granular lines in laboratory cultures of *T. striata* and stated that the granular line may be caused by environmental conditions or aging. The clustering of these strains in Fig 3.5 suggest that strains of *T*. *striata* CCAP 66/5, CCAP 66/16 and *T. apiculata* CCAP 66/15 are extremely closely related with a bootstrap values of more than 99% and may be the same species.

The cluster analysis showed that though related *T. chui*-*T. gracilis* and *T. striata* -*T. apiculata* formed two separate groups with a bootstrap value of 78.6 % (Fig 4.5). Hori *et al*., (1986) have placed *T. chui* and *T. striata* under one subgenus on the basis of pyrenoid ultrastructure, whereas Marin *et al*., (1993) on the basis of ultrastructure of flagellar hairs, separated

them into two different flagellar types. Our results support the grouping of Marin *et al*., (1993) in that though *T. chui-T. gracilis* and *T. striata-T. apiculata* groups are related they form separate clusters with ISSR markers.

The third group was *T. verrucosa* group in which *T. verrucosa* 66/18B was closer to *T. verrucosa* 66/6 and *T. verrucosa* 66/46 was closer to *T. verrucosa* 163/3 (Fig 4.5). The three strains of *T. verrucosa* 66/18B, 66/6, 66/46 were placed under *T. rubens* by Butcher (1959) because of the reddish colored chloroplast due to accumulation of haematochrome pigment. The verrucose posterior end distinguishes *T*. *verrucosa* from other *Tetraselmis* species (Butcher 1959). Hori *et al*., (1983) found that the verrucose posterior end of this strain is not a constant feature in laboratory cultures of the organism and hence cannot be used to separate this species from *T. rubens*. They therefore transferred *T*. *rubens* to *T. verrucosa*. ISSR analysis indicated that these four strains are closely related and belong to one species and the DNA marker data thus support the view of Hori *et al*., (1982).

The Kochi isolate diverged from the three *Tetraselmis* clusters with a bootstrap value of 79.7% and clustered with *Scherffelia dubia* another genus from the order Chlorodendrales although the low bootstrap value of 64%, indicated that the grouping was very weak and may change with addition of more taxa. On the basis of these results it appears that the Kochi isolate is genetically different from the other *Tetraselmis* species studied here.

The three *Chlamydomonas* species and *Pedinomonas minor* formed a separate cluster where two fresh water species*, C. moewusii* and *C. proteus* appeared to be more closely related with bootstrap value of 64.4% and *C. plethora* which separated out from these two species with 60.1%.

There are two points to be noted in the analysis of the dendrogram from ISSR banding patterns. The first is the occurrence of same molecular weight bands in distantly related taxa. Agarose gels do not have a very high resolution power and small differences in number of nucleotides would not separate on these gels. Furthermore the same molecular weight band does not imply similarity of sequences.

The second point relates to the statistical analysis of the data set which result in production of the dendrogram. Felsenstein (1985) suggested that only groups with bootstrap values of 95% or greater be considered significant and/or monophyletic. In the present analysis it was observed that the bootstrap values were low at certain nodes even at intraspecific level. For example it was 73% for *T. verrucosa* CCAP 163/3 and *T. verrucosa* CCAP 66/46. This might be due to either low sample size in the present data set. In spite of these uncertainties, however there were clearly differentiated groupings within the Chlorodendrales where the *T. chui-T. gracilis* separated out from the *T. striata-T. apiculata* and both were distinguished from the four *T. verrucosa* strains. The Kochi isolate diverged from these groups and clustered with *S. dubia*. These results were tested by amplifying and sequencing ITS1-5.8S-ITS2 rDNA region from these strains.

ITS sequences are widely used in phylogenetic analysis of related taxa, population studies and species identification (Lee and Taylor 1990; Kooistra *et al*., 1992; Gardes and Bruns 1993; van Oppen *et al*., 1994; Serrão *et al*., 1999; Fama *et al*., 2000; Chen *et al*., 2001; Ko and Jung 2002).

Table 4.1: Inter simple sequence repeat primers, number of loci amplified and size range of band that they produce

Primer	Number of loci amplified	Band Sizes (bp)
$(AG)_8C$	4-8	300-1300
$(AG)_8G$	4-9	200-1000
$(AG)_8T$	3-10	200-1400
$(AG)_8YC$	1-13	220-1500
$(AG)_8YT$	4-10	250-1400
$(GA)_8C$	2-7	350-1400
$(GA)_8T$	2-6	400-1500
$(GA)_8YC$	3-11	100-1200
$(GA)_8YG$	5-9	100-1300
$BHB(GA)_7$	1-8	200-100
$(CA)_8RG$	2-11	200-1500
$(CA)_8RT$	1-7	350-1500
$(CT)_8RC$	5-10	100-1300
$(GT)_8YC$	2-10	200-1000
$(TG)_8RC$	0-8	300-1300
$(ATG)_6$	3-8	400-1500

Figure 4.1: Agarose gel pattern of PCR products amplified with primer $(ATG)_6$

M-marker ϕX 174 DNA Hae III digest (from top to bottom in bp 1353, 1078, 872, 603, 310, 270), Lane 1-16 are *T. chui* 8/6, *T. chui* 66/21B*, T. striata* 66/5*, T. striata* 66/16*, T. apiculata* 66/15*, T. verrucosa* 163/3*, T. verrucosa* 66/18B*, T. verrucosa* 66/*6, T. verrucosa* 66/46*, T. gracilis* 66/13*,* Kochi isolate*, S. dubia, Pedinomonas minor*1965/3B*, C. moewusii* 11/11*, C. proteus* 11/21*, C. plethora* 11/86B*.*

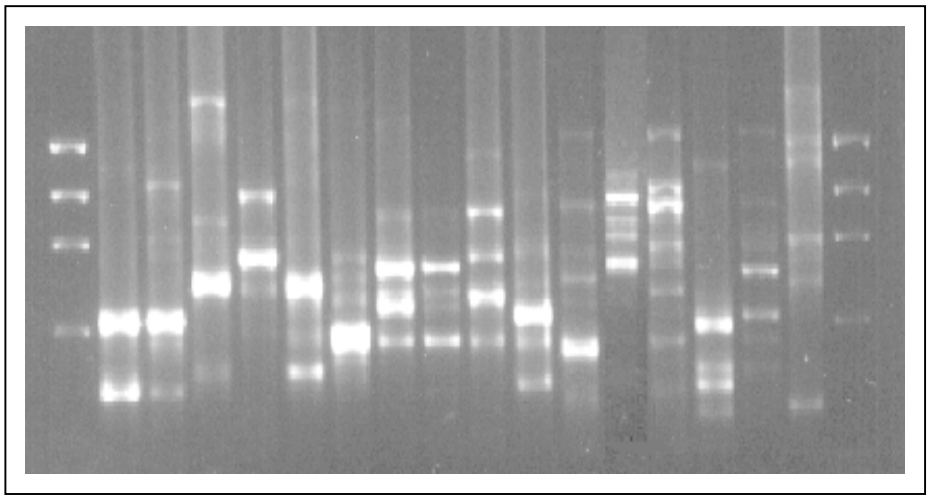


Figure 4.2: Agarose gel pattern of PCR products amplified with primer $(AG)_8YC$

M-marker ϕX 174 DNA Hae III digest (from top to bottom in bp 1353, 1078, 872, 603, 310, 270), Lane 1-16 are *T. chui* 8/6, *T. chui* 66/21B*, T. striata* 66/5*, T. striata* 66/16*, T. apiculata* 66/15*, T. verrucosa* 163/3*, T. verrucosa* 66/18B*, T. verrucosa* 66/6*, T. verrucosa* 66/46*, T. gracilis* 66/13*,* Kochi isolate*, S. dubia, Pedinomonas minor* 1965/3B*, C. moewusii* 11/11*, C. proteus* 11/21*, C. plethora* 11/86B*.*

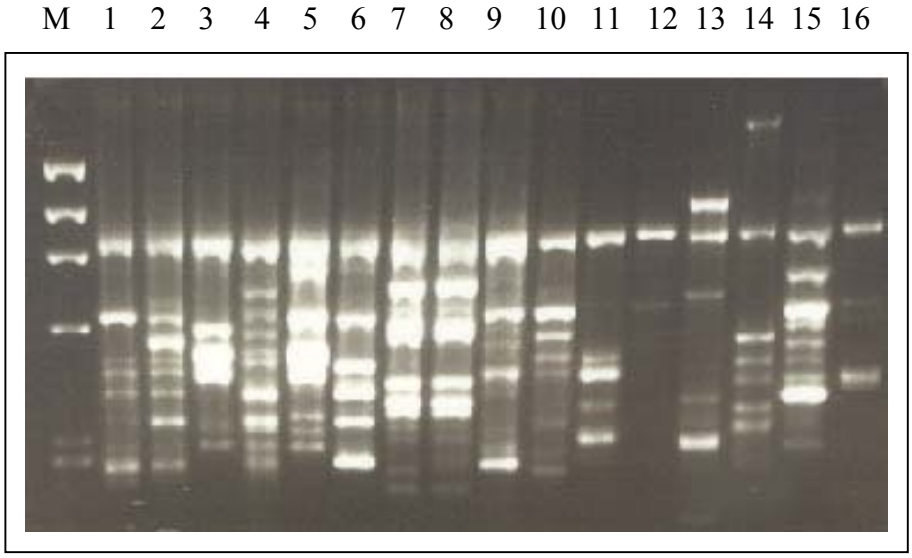


Figure 4.3 Hybridization pattern of PCR products amplified with primer $(AG)_8$YC detected by 900 bp band eluted from *T. chui* 8/6 lane (Fig 4.2, lane1)

Lane 1-16 are *T. chui* 8/6, *T. chui* 66/21B, *T. striata* 66/5, *T. striata* 66/16, *T. apiculata* 66/15, *T. verrucosa* 163/3, *T. verrucosa* 66/18B, *T. verrucosa* 66/6, *T. verrucosa* 66/46, *T. gracilis* 66/13, *Tetraselmis* sps. Kochi isolate, *S. dubia*, *Pedinomonas minor*1965/3B, *C. moewusii* 11/11, *C. proteus* 11/21, *C. plethora* 11/86B.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

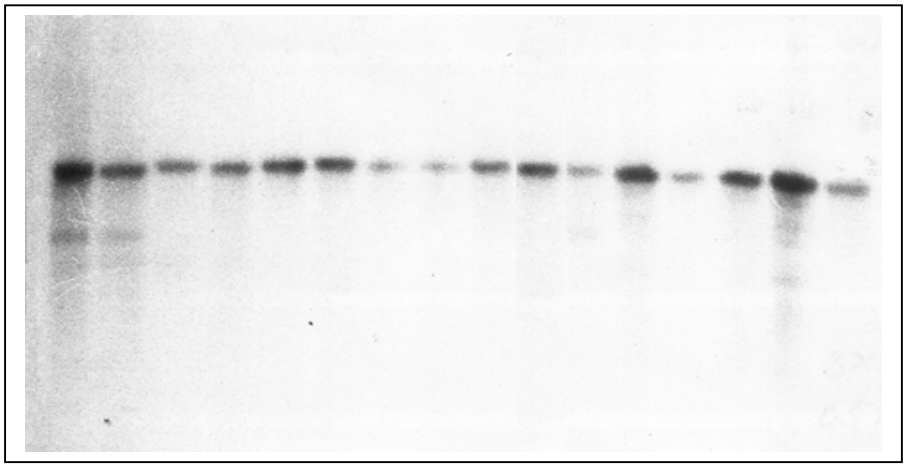


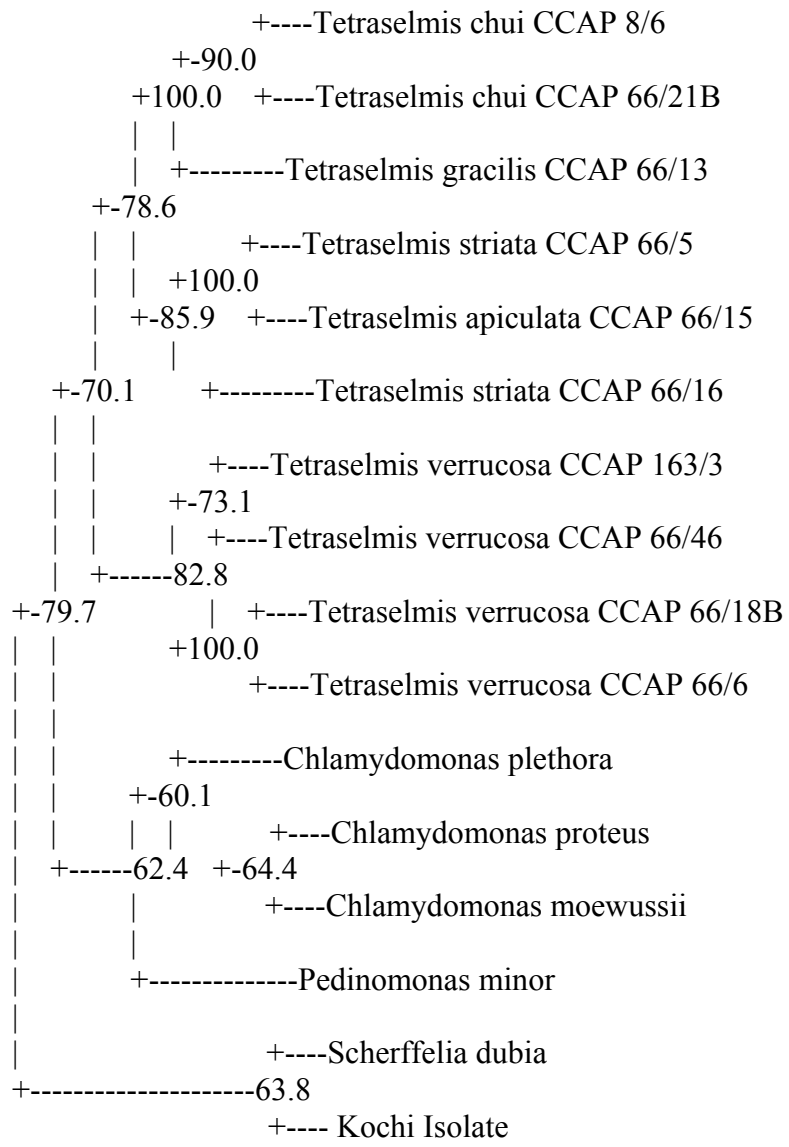
Figure 4.4 Sequence of 900 bp band from *T. chui* CCAP 8/6. The EMBL Accession number is AJ438040

```
AACCNGAGAGAGAGAGCCAAGCTTGATGTGTGGATGAGTAAACCCAAT
CTTGATCGCCACATCAGTGGTATGCTCGCCTGCATGCACGTCGGATCATC
GCACGTATCTCGTACATTTCAGGTGCTTGCNATTTTTTNGTCAGATTGTC
TTTATCGCAGGGTGGCNGCAAGCGCGCGCTATTCTTCATCAAGCATCATC
CGCCGCGCACGGCGCAGCGTGTTACACGCATCCACGTGAAGAACTCCTTG
CAATAGCTGATAATATCGAAGGCTTCAGACATGACGATGAATCCCTCTCA
CTCATGCATGCATGCATGCATGCATGGCACGCTGCAGCACCCCATGCAGG
CACACAGCAGCACCATGGGGGCGGGGGTGCCCGCGCAGCAACAGCGAACA
CAACAGCTGCAACAGCGCCCTAGACTCAACGGNCACCTGGCCGGAAGGAC
TCGGNCCCAGCGGCAGGGGCCTGTGGGTGTGCCTCCAGCCGCTCCNTACC
ACGCCATCGTCCTCGCAGCTCCCCTCAGCCACTCTTCTGGACCGCCTGGC
TCCGCCTAGCTCCGGCATCAAGGTCTCACCCTCCCTCACAGCGTCCCTGA
CCACATCTGCGGGCGCAGGCCTGATTGCCAAAGCTCCCATTGCCGAAGGT
TGGATCGTAGACATTGCTTCTCAACCAGCCTCCCCTGATATAGCACACAT
GTTGGGAACCAGCCTTTCCTGTTTTAGCACACATGTCCCCNAANNCAACG
CTTCTTGAACTTGACTTTCTCCCCACTGAGACCTGCGAGTGGTTTAGTTT
AGTTTGCGAGTCCNGCCCCCTTTATCCTTGCTACCAGTGCAAGATATACA
CACTGCTGCCAATAAATGCCACTCATCGCAAGCGACTCTCTCTCTCTCTC
TAA
```

Table 4.2: Similarity matrix generated using WINDIST program

	TC1	TC2	TS1	TS2	TA	TV1	TV2	TV3	TV4	TG	KI	SD	PM	CM	CP1	CP2
TC1	1.00															
TC2	0.63	1.00														
TS1	0.23	0.24	1.00													
TS2	0.22	0.23	0.32	1.00												
TA	0.24	0.27	0.55	0.29	1.0											
TV1	0.21	0.19	0.21	0.17	0.21	1.0										
TV2	0.17	0.14	0.15	0.15	0.17	0.26	1.00									
TV3	0.22	0.18	0.17	0.20	0.21	0.27	0.77	1.00								
TV4	0.22	0.20	0.20	0.17	0.17	0.36	0.27	0.38	1.00							
TG	0.56	0.48	0.22	0.21	0.23	0.24	0.13	0.19	0.32	1.00						
KI	0.17	0.15	0.11	0.19	0.08	0.11	0.09	0.14	0.12	0.15	1.00					
SD	0.10	0.08	0.09	0.08	0.12	0.08	0.10	0.12	0.16	0.13	0.28	1.00				
PM	0.20	0.20	0.21	0.24	0.23	0.10	0.25	0.24	0.16	0.18	0.23	0.11	1.00			
CM	0.15	0.14	0.19	0.21	0.17	0.16	0.13	0.17	0.15	0.16	0.08	0.09	0.19	1.00		
CP1	0.14	0.16	0.18	0.20	0.16	0.13	0.13	0.18	0.14	0.15	0.11	0.19	0.19	0.31	1.00	
CP2	0.17	0.17	0.20	0.20	0.24	0.19	0.20	0.15	0.21	0.17	0.13	0.12	0.26	0.24	0.29	1.00

Figure 4.5: Dendrogram, obtained using unweighted pair group method with arithmetic average (UPGMA). The numbers at the forks indicate the confidence limits for the grouping of those species, which are to the right of that fork.



5.8S and ITS rDNA sequences

The ITS1-5.8S-ITS2 region of the nuclear ribosomal DNA of *Tetraselmis* species listed in Table 2.1 (Chapter 2) was 608 to 643 bp and that of *Chlamydomonas* species was 640 to 733 bp except for *C. moewusii* which was 1200 bp. Sequence alignment for *Tetraselmis-Scherffelia* and *Chlamydomonas-Pedinomonas* groups was done separately and is given in Figures 4.6 and 4.7 respectively. The genetic distances were calculated separately for 5.8S and ITS regions and these are given Table 4.3 and 4.4 respectively.

The 5.8S region of the *Tetraselmis* species was 152 bp and that of the *Chlamydomonas* species was 165 bp. The 5.8S region showed very high sequence similarity within *Tetraselmis* with only a single nucleotide variation at position 149 where, four strains of *T. verrucosa* have 'G' and the rest of the *Tetraselmis* species have 'A' nucleotide. The results showed that the 5.8S gene of *Tetraselmis* is highly conserved and can be used as a genus specific marker.

Scherffelia dubia, a relative of *Tetraselmis*, separated out from other *Tetraselmis* and varied in 16 nucleotides from *T. verrucosa* species and in 17 nucleotides from the rest of the *Tetraselmis* species (Fig. 4.6). 18S ribosomal DNA of *Scherffelia dubia* showed very high sequence similarity with that of *Tetraselmis* sequences and in 18S phylogenetic analysis also it showed high affinity with *Tetraselmis* species. However from the nucleotide differences in 5.8S region it was possible to separate *Scherffelia* and *Tetraselmis* genera.

The 5.8S sequences of the three *Chlamydomonas* species listed in Table 2.1 and two other *Chlamydomonas* sequences retrieved from databases showed high sequence variation (Table 4.3) within the genus. The variation ranged from 3 nucleotides between *Chlamydomonas* sp and *C. moewusii* to 57 nucleotides between *C. reinhardtii and C. plethora* supporting an earlier view that the genus *Chlamydomonas* is highly diverse. (Buchheim *et al*., 1990; Kim *et al*., 1994 and Buchheim *et al*., 1995).

As given in Table 4.3 the genetic distance between *Tetraselmis* and *Chlamydomonas* ranged between 0.2 to 0.6. From the data it is possible to

state with confidence that the *Tetraselmis* species can be separated using 5.8S rDNA sequences from other green algae. *P. minor* was almost equidistant between *Tetraselmis* and *Chlamydomonas*.

ITS1 region of *Tetraselmis* was 220 bp and ITS 2 region was 280 bp. As shown in figure 4.6 ITS1 and ITS2 regions of *Tetraselmis* were highly conserved within strains of same species. For example there was only a single nucleotide variation between two strains of *T. striata* and *T. apiculata* in ITS1 and a two nucleotide difference in ITS2 region. Two strains of *T. chui* and one strain of *T. gracilis* differed by a single nucleotide in ITS1 and by a three nucleotide in ITS2. The sequence variation for four *T. verrucosa* ranged between 1-9 nucleotides in ITS1 and 6-13 in ITS2.

A one or two nucleotide variation in sequences may occur during PCR amplification or sequencing. This error was eliminated by both strands sequencing as well as for each sample two clones was sequenced. The sequencing yielded consistent results for both clones.

In case of yeast, strains with more than 99% sequence similarity in ITS region are considered as same species (Chen *et al*., 2000 & 2001; Fell *et al*., 2000 and Kurtzman 2000). In the present analysis we found that there was 99.6% sequence similarity between strains of *T. chui* and *T. gracilis* and 99.3% sequence similarity between *T. striata* and *T. apiculata*. In this respect *T. chui* (Butcher 1959) and *T. gracilis* (Kylin 1935) may be considered as the same species. Similarly, *T. striata* and *T. apiculata* may be considered same species. The genetic similarity between these two species group has been suggested by the ISSR fingerprinting results. Furthermore the morphological closeness of these species has been reported by Butcher (1959).

The genetic distances between *Tetraselmis* species ranged between 0.2 to 0.5 (Table 4.4 A and B). The highest sequence similarity of 83 % was observed in ITS1 region of *T. chui-T. gracilis* and *T. striata-T. apiculata*. The sequence similarity between *T. verrucosa* and *T. chui* or *T. striata* ranged between 65-75% in ITS1 and 60-70% in ITS2. The Kochi isolate was almost equidistant from *T. chui-T. gracilis*, *T. striata-T. apiculata* and *T. verrucosa* groups. The isolate showed 68-75% sequence similarity in ITS1 and 60-70%

sequence similarity in ITS2 with that of other *Tetraselmis* species. This sequence similarity was comparable with that of sequence similarity between *T. verrucosa* and *T. chui* or *T. striata*. This result supports the conclusion drawn from the ISSR dendrogram where the Kochi isolated was separated out from *T. chui-T. gracilis*, *T. striata-T. apiculata* and *T. verrucosa* groups at equal distances. *S. dubia* showed sequence similarity of 47.3 to 59.1% in ITS 1 and 42.86 to 33% in ITS 2 with *Tetraselmis* species which is less as compared to that of between *Tetraselmis* species. Theses results indicate that ITS sequences are highly useful in separating closely related species as well as genera.

ITS sequence of *C. moewusii* was 1200 bp and those of the other *Chlamydomonas* studied here was 635 to 733 bp, hence *C. moewusii* ITS sequence could not be aligned with other *Chlamydomonas* sequences, and this sequence was removed from the analysis. The distance matrix for three *Chlamydomonas* species and *Pedinomonas minor* is given in Table 4.5. As reported by Coleman and Mai (1997) the genetic distance between 12 *Chlamydomonas* species ranged between 0.07 to 0.5 in ITS1 and 0.08 to 0.5 in ITS2. In the present analysis the genetic distance between three *Chlamydomonas* species ranged between 0.3 to 0.5 in ITS1 (Table 4.5A) and 0.4 to 0.48 in ITS-2 (Table 4.5B) which is within the reported range.

Phylogenetic analyses were conducted using Neighbor-Joining method in MEGA software, to evaluate relationships between *Tetraselmis* species (Fig 4.8). In the resulting tree, 10 strains of *Tetraselmis*, divided into three subclusters, the isolate from the Kochi separated out early from *T. chui-T gracilis*, *T. striata -T. apiculata* and *T. verrucosa* groups**.** *Scherffelia dubia* also separated out from all these *Tetraselmis* species.

The phylogenetic tree derived from the ITS1-5.8S-ITS2 rDNA sequences of *Tetraselmis* strains (Fig 4.8) showed that, *T. gracilis* CCAP 66/13 always clustered with two *T. chui* strains CCAP 8/6 and 66/21B which confirms that these two *T. chui* strains are close to *T. gracilis* CCAP 66/13. Thus the results support the ISSR analysis.

T. apiculata CCAP 66/15 always grouped with two *T. striata* strains indicated that these species are closely related. This indicated that these two *T. striata* and *T apiculata.* belonged to same species

The four *T. verrucosa* strains formed a third subcluster where the subgrouping of strains was similar as described in ISSR analysis.

T. species NCIM 7001 clustered neither with *Tetraselmis* species nor with S*cherffelia dubia*.

ITS sequence results were comparable with ISSR analysis. Both the analyses resolved same grouping of species and strains. It is possible to separate *Tetraselmis* species using ITS sequences.

From the ITS sequence results and ISSR analyses it is clear that in genus *Tetraselmis*, there is high sequence homogeneity. The morphological and ultrastructural heterogeneity observed in the genus by earlier worker (Norris *et al*., 1980; Hori *et al*., 1982; and Marin *et al*., 1993 have pointed out that the genus is highly heterogeneous and shows high variation in morphological characters between species. From the results presented here it appears that the observed heterogeneity in the genus might be as a result of variation in these characters due to environmental factors or age of the culture.

able 4.3: Genetic distances between 5.8S rDNA sequences calculated for pairwise species comparison using Kimura 2-parameter 1-*C.* Sp, 2-*C. proteus*, 3-*C. moewusii*, 4-Tv 163/3, 5-Tv 66/18B, 6-Tv 66/6, 7-Tv 66/46, 8-Ta 66/15, 9-Ts 66/16, 10-Tc 66/21B, 11-Tc 8/6, 12-Tg-66/13, 13-Ts-66/5, 14-kochi Isolate, 15-*S. dubia,* 16-*P. minor*, 17-*C. reinhardtii*, 18-*C. Plethora*

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
2	0.20																
3	0.47	0.05															
4	0.12	0.13	0.10														
5	0.12	0.13	0.10	0.00													
6	0.12	0.13	0.10	0.00	0.00												
7	0.12	0.13	0.10	0.00	0.00	0.00											
8	0.12	0.13	0.10	0.01	0.01	0.01	0.01										
9	0.12	0.13	0.10	0.01	0.01	0.01	0.01	0.00									
10	0.12	0.13	0.10	0.01	0.01	0.01	0.01	0.00	0.00								
11	0.12	0.13	0.10	0.01	0.01	0.01	0.01	0.00	0.00	0.00							
12	0.12	0.13	0.10	0.01	0.01	0.01	0.01	0.00	0.00	0.00	0.00						
13	0.12	0.13	0.10	0.01	0.01	0.01	0.01	0.00	0.00	0.00	0.00	0.00					
14	0.12	0.13	0.10	0.01	0.01	0.01	0.01	0.00	0.00	0.00	0.00	0.00	0.00				
15	0.18	0.20	0.16	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	.012			
16	0.20	0.21	0.16	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.22		
17	0.14	0.15	0.13	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.11	0.19	0.19	
18	0.59	0.56	0.55	0.58	0.58	0.58	0.57	0.57	0.57	0.57	0.57	0.57	0.57	0.57	0.65	0.55	0.53

Table 4.4: Genetic distances calculated for pairwise species comparison using Kimura 2-parameter A- between ITS1-rDNA sequences, B- between ITS2-rDNA sequences.

TV1- *T. verrucosa* 163/3, TV4 -*T. verrucosa* 66/46, TV2- *T. verrucosa* 66/18B , TV3- *T. verrucosa* 66/6, TG- *T. gracilis* 66/13, TC1-*T. chui* 8/6, TC2-*T. chui* 66/21B, TA- *T. apiculata* 66/15, TS1-*T. striata* 66/5, TS2-*T. striata* 66/16 KI- Kochi isolate, SD-*S. dubia,*

A

Taxa	TV1	TV4	TV2	TV3	TG	TC1	TC2	TA	TS1	TS2	KI
TV1											
TV4	0.006										
TV2	0.028	0.034									
TV3	0.045	0.051	0.040								
TG	0.485	0.474	0.454	0.487							
TC1	0.485	0.474	0.454	0.487	0.005						
TC2	0.485	0.474	0.454	0.487	0.005	0.000					
TA	0.469	0.469	0.438	0.480	0.207	0.201	0.201				
TS1	0.458	0.458	0.428	0.470	0.201	0.195	0.195	0.004			
TS2	0.469	0.469	0.438	0.480	0.207	0.201	0.201	0.000	0.004		
KI	0.359	0.359	0.324	0.350	0.456	0.456	0.456	0.479	0.470	0.479	
SD	0.924	0.924	0.917	0.984	1.157	1.157	1.157	1.302	1.318	1.302	0.845

B

Taxa	TV1	TV4	TV2	TV3	TG	TC1	TC2	TA	TS1	TS2	KI
TV1											
TV4	0.022										
TV2	0.050	0.034									
TV3	0.050	0.038	0.023								
TG	0.577	0.600	0.608	0.559							
TC1	0.555	0.578	0.585	0.537	0.013						
TC2	0.552	0.575	0.582	0.534	0.013	0.009					
TA	0.674	0.698	0.717	0.704	0.191	0.180	0.177				
TS1	0.675	0.698	0.709	0.704	0.196	0.185	0.181	0.008			
TS2	0.658	0.681	0.717	0.687	0.191	0.180	0.176	0.008	0.004		
KI	0.537	0.574	0.797	0.563	0.483	0.475	0.476	0.517	0.520	0.496	
SD	2.185	2.197	2.408	2.201	2.123	2.012	2.074	2.576	2.318	2.434	2.061

Table 4.6: Genetic distances calculated for pairwise species comparison using Kimura 2-parameter A. between ITS2-rDNA sequences B. between ITS2-rDNA sequences

A

Taxa	*C.* sp.	*C. plethora*	*C. proteus*
C. sp.			
C. plethora	0.349		
C. proteus	0.489	0.509	
P. minor	0.661	0.822	1.000

B

Taxa	*C.* sp.	*C. plethora*	*C. proteus*
C. sp.			
C. plethora	0.394		
C. proteus	0.484	0.479	
Pedinomonas	0.755	0.684	0.77

Figure 4.6 Alignment ITS1-5.8S-ITS2 rDNA sequences of
TV1- *T. verrucosa* 163/3, TV4 -*T. verrucosa* 66/46, TV2- *T. verrucosa* 66/18B , TV3- *T. verrucosa* 66/6, TG- *T. gracilis* 66/13, TC1-*T. chui* 8/6, TC2-*T. chui* 66/21B, TA- *T. apiculata* 66/15, TS1-*T. striata* 66/5, TS2-*T. striata* 66/16 KI- Kochi isolate, SD-*S. dubia,*

```
TV1     AAGGATCATTGAATC-TATCAAA-CCACCCAGCGAACCTAAATGTCCGCGTCCTAGACCA 58
TV4     AAGGATCATTGAATC-TATCAAA-CCACCCAGCGAACCTAAATGTCCGCGTCCTAGACCA 58
TV2     AAGGATCATTGAATC-TATCAAA-CCACCCAGCGAACCTAAATGTCTGCGTCCTAGACCA 58
TV3     AAGGATCATTGAATC-TATCAAA-CCACCCAGCGAACCTAAATGTCTGCGTCCTAGACCA 58
TS1     AAGGATCATTGAATC-GATCAAATCCACCATGTGAACCGTTCTGTCTCCCTCCCGGGACC 59
TS2     AAGGATCATTGAATC-GATCAAATCCACCATGTGAACCGTTCTGTCTCCCACCCGGGACC 59
TA      AAGGATCATTGAATC-GATCAAATCCACCATGTGAACCGTTCTGTCTCCCACCCGGGACC 59
TG      AAGGATCATTGAATC-GATCAAATCCACACTGTGAACTGTT-TGTCTCCCTCTCGGGGCC 58
TC1     AAGGATCATTGAATC-GATCAAATCCACACTGTGAACTGTT-TGTCTCCCTCTCGGGGCC 58
TC2     AAGGATCATTGAATC-GATCAAATCCACACTGTGAACTGTT-TGTCTCCCTCTCGGGGCC 58
TK      AAGGATCATTGAACC-GATCAAAACCACAC-GCGAACCTTT-TGTCTGCGTCCTTGAGCT 57
SD      AAGGATCATTGAACCTGATCAAAACCACAC-GCGAACAGT--TGGCAGGTAAGCTGACC- 56

TV1     GCCCCTCCAACA--------GGCT---TAGGCTTTTGGGGGACCAGCTGGCT-------- 99
TV4     GCCCCTCCAACA--------GGCT---TAGGCTTTTGGGGGACCAGCTGGCT-------- 99
TV2     GCCCCCCCAACA--------GGCC---TAGGCTTTTGGGGGACCAGCTGGCT-------- 99
TV3     GCCCCTCCAACA--------GGCC---TAGGCTTTTGGGGGACCAGCTGGCT-------- 99
TS1     -CTCGCGGGGCC-TTGGTGCGGCT-GGGATGCGTTCCC-AGTCGGGCTCACCGCCCTCAA 115
TS2     -CTCGCGGGGCC-TTGGTGCGGCT-GGGATGCGTTCCC-AGTCGGGCTCACCGCCCTCAA 115
TA      -CTCGCGGGGCC-TTGGTGCGGCT-GGGATGCGTTCCC-AGTCGGGCTCACCGCCCTCAA 115
TG      GCTCGCGCGGCCGCTTGAGCGGCTAGGGATGCGTTCCCTAGTCGGGCCTACCCCC----- 113
TC1     GCTCGCGCGGCCGCTTGAGCGGCTAGGGATGCGTTCCCTAGTCGGGCCTACCCCC----- 113
TC2     GCTCGCGCGGCCGCTTGAGCGGCTAGGGATGCGTTCCCTAGTCGGGCCTACCCCC----- 113
TK      GCCCTCCAGGCCGGAAATATCGCGTCGGATACGAGCCTTGAACAGGGTTGGACAAGGCA- 116
SD      -CCCTGGCTACT----ATGGGGTTCCGAGAGGAGCCTAGATCTGGGTGTGGCAAAAACC- 110

TV1     ----------------------------------AGGCGCCAAAC-CAA--------ATTC 117
TV4     ----------------------------------AGGCGCCAAAC-CAA--------ATTC 117
TV2     ----------------------------------AGGCGCCAAAC-CAA--------AATC 117
TV3     ----------------------------------AGGCGCCAAAC-CAA--------ATTC 117
TS1     ACACGGGGCGCTCCTATTAACTTAGGCGTCTCGGGCGGCTGGGC-TGGCGTTATTTAAAC 174
TS2     ACACGGGGCGCTCCTATTAACTTAGGCGTCTCGGGCGGCTGGGC-TGGCGTTATTTAAAC 174
TA      ACACGGGGCGCTCCTATTAACTTAGGCGTCTCGGGCGGCTGGGC-TGGCGTTATTTAAAC 174
TG      -------GCGCCCCAGC----------------GGGGAATAGGT-CGGCGCT-CTTAAAC 148
TC1     -------GCGCCTCAGC----------------GGGGAATAGGT-CGGCGCT-CTTAAAC 148
TC2     -------GCGCCTCAGC----------------GGGGAATAGGT-CGGCGCT-CTTAAAC 148
TK      -------------------------------CCAGGAGTTTAGGC-AGGCAAACTTACATC 145
SD      ----------------------------------ATATGTTATACACAACAAACCTCTCGT 137

TV1     ACTCAACACAAAACCAA-GTCTGAAGCTATTTCTG-ATTGACCCAGTCGAT-CAGTCTAA 174
TV4     ACTCAACACAAAACCAA-GTCTGAAGCTATTTCTG-ATTGACCCAGCCGAT-CAGTCTAA 174
TV2     ACTCAACACAAAAACAA-GTCTGAAGCTATTTCTG-ATTGACCCAGTCGAT-CAGTCTAA 174
TV3     ACTCAACACAAAAACAA-GTCTGAAGCTATTTCTG-ATTGATTTTTTCGAT-CAGTCTAA 174
TS1     ACTCCACACCAAAACAACGTCTAAAGCTATGTGCGTATTGCCCCGTGCGATACCGCCTAA 234
TS2     ACTCCACACCAAAACAACGTCTAAAGCTATGTGCGTATTGCCCCGTGCGATACCGCCTAA 234
TA      ACTCCACACCAAAACAACGTCTAAAGCTATGTGCGTATTGCCCCGTGCGATACCGCCTAA 234
TG      AACCCACACAAAAACAACGTCTAAAGCTATGTGTATGTTGGCCTTGCCGACTGCATCTAA 208
TC1     AACCCACACAAAAACAACGTCTAAAGCTATGTGTATGTTGGCCTTGCCGACTGCATCTAA 208
TC2     AACCCACACAAAAACAACGTCTAAAGCTATGTGTATGTTGGCCTTGCCGACTGCATCTAA 208
```

```
TK      ATGCTACACGAAAACAATCTCTCAAGCTATGTGGG--TTGACCATAGCGATGACTTCTAA 203
SD      AGATACTAAGTGATTATCGTTCGGGGTCGCAAGGG--CTG--CAAAGCTCTTCCCTCGGA 193

TV1     CCTAAGACAACTCTCAACAACGGATATCTTGGCTCCTACAACGATGAAGAACGCAGCGAA 234
TV4     CCTAAGACAACTCTCAACAACGGATATCTTGGCTCCTACAACGATGAAGAACGCAGCGAA 234
TV2     CCTAAGACAACTCTCAACAACGGATATCTTGGCTCCTACAACGATGAAGAACGCAGCGAA 234
TV3     CCTAAGACAACTCTCAACAACGGATATCTTGGCTCCTACAACGATGAAGAACGCAGCGAA 234
TS1     CCAAAGACAACTCTCAACAACGGATATCTTGGCTCTTACAACGATGAAGAACGCAGCGAA 294
TS2     CCAAAGACAACTCTCAACAACGGATATCTTGGCTCTTACAACGATGAAGAACGCAGCGAA 294
TA      CCAAAGACAACTCTCAACAACGGATATCTTGGCTCTTACAACGATGAAGAACGCAGCGAA 294
TG      CCAAAGACAACTCTCAACAACGGATATCTTGGCTCTTACAACGATGAAGAACGCAGCGAA 268
TC1     CCAAAGACAACTCTCAACAACGGATATCTTGGCTCTTACAACGATGAAGAACGCAGCGAA 268
TC2     CCAAAGACAACTCTCAACAACGGATATCTTGGCTCTTACAACGATGAAGAACGCAGCGAA 268
TK      CCAAAGACAACTCTCAACAACGGATATCTTGGCTCTTACAACGATGAAGAACGCAGCGAA 263
SD      GCATGTAAAACTCTCAGCAATGGATATCTTGGCTCTTGCAACGATGAAGAACGCAGCAAA 253

TV1     ATGCGATACGTAGTGTGAATTGCAGAATTCCGTGAACCATCGAATCTTTGAACGCATATT 294
TV4     ATGCGATACGTAGTGTGAATTGCAGAATTCCGTGAACCGTCGAATCTTTGAACGCATATT 294
TV2     ATGCGATACGTAGTGTGAATTGCAGAATTCCGTGAACCATCGAATCTTTGAACGCATATT 294
TV3     ATGCGATACGTAGTGTGAATTGCAGAATTCCGTGAACCATCGAATCTTTGAACGCATATT 294
TS1     ATGCGATACGTAGTGTGAATTGCAGAATTCCGTGAACCATCGAATCTTTGAACGCATATT 354
TS2     ATGCGATACGTAGTGTGAATTGCAGAATTCCGTGAACCATCGAATCTTTGAACGCATATT 354
TA      ATGCGATACGTAGTGTGAATTGCAGAATTCCGTGAACCATCGAATCTTTGAACGCATATT 354
TG      ATGCGATACGTAGTGTGAATTGCAGAATTCCGTGAACCATCGAATCTTTGAACGCATATT 328
TC1     ATGCGATACGTAGTGTGAATTGCAGAATTCCGTGAACCATCGAATCTTTGAACGCATATT 328
TC2     ATGCGATACGTAGTGTGAATTGCAGAATTCCGTGAACCATCGAATCTTTGAACGCATATT 328
TK      ATGCGATACGTAGTGTGAATTGCAGAATTCCGTGAACCATCGAATCTTTGAACGCATATT 323
SD      ATGCGATACCTAATGCGAATTGCAGAATTCCGTGAGTCATTGACACTTTGAATGCACATT 313

TV1     GCGCTCGAGGCCTCGGCCAAGAGCACGCCTGCCTCAGGGTCAT--GATTACCTCACCCCT 352
TV4     GCGCTCGAGGCCTCGGCCAAGAGCACGCCTGCCTCAGGGTCAT--GATTACCTCACCCCT 352
TV2     GCGCTCGAGGCCTCGGCCAAGAGCACGCCTGCCTCAGGGTCAT--GATTACCTCACCCCT 352
TV3     GCGCTCGAGGCCTCGGCCAAGAGCACGCCTGCCTCAGGGTCAT--GATTACCTCACCCCT 352
TS1     GCGCTCGAGGCCTCGGCCAAGAGCACGCCTGCCTCAGAGTC----GGTTTTCCC-CCTCA 409
TS2     GCGCTCGAGGCCTCGGCCAAGAGCACGCCTGCCTCAGAGTC----GGTTTTCCC-CCTCA 409
TA      GCGCTCGAGGCCTCGGCCAAGAGCACGCCTGCCTCAGAGTC----GGTTTTCCC-CCTCA 409
TG      GCGCTCGAGGCCTCGGCCAAGAGCACGCCTGCCTCAGAGTC----GGTTTTCCC-CCTCA 383
TC1     GCGCTCGAGGCCTCGGCCAAGAGCACGCCTGCCTCAGAGTC----GGTTTTCCC-CCTCA 383
TC2     GCGCTCGAGGCCTCGGCCAAGAGCACGCCTGCCTCAGAGTC----GGTTTTCCC-CCTCA 383
TK      GCGCTCGAGGCCTCGGCCAAGAGCACGCCTGCCTCAGAGTCTTAGGGTGGTTATACCTCC 383
SD      GCGCTCGAGGCTTCGGCCAAGAGCACGTCTGCCTCAGGGTCCTT-GATAACTGGTATCGG 372

TV1     ACCTACCTAGGTACCGGGTGGACCTGGCCTCCTCACCTGCAAG----GGTTGG--GCTGG 406
TV4     ACCTACCTAGGTACCGGGTGGACCTGGCCTCCTCACCTGCAAG----GGTGGG--CTGGC 406
TV2     ACCTACCTGGGTATCGGGTGGACCTGGCCTCCTCACCCGCAAG----GGTGGG--CTGG- 405
TV3     ACCTACCTAGGTATTGGGTGGACCTGGCCTCCTCACCCGCAAG----GGTGGG--CTGG- 405
TS1     ACCCCCCAGC----------------CCCCCCCTTCACCGGG----GGCGGGCGGCGGA 448
TS2     ACCCCCCAAC----------------CCCCCC-TTCACCGGG----GGCGGGCGGCGGA 447
TA      ACCCCCCACC----------------CCCCCCCTTCACCGGG----GGCGGGCGGCGGA 448
TG      ACCCCCCTAC----------------CCCTT----------A----GGTAGA--GCCGG 410
TC1     ACCCCCCTAC----------------CCCTT----------A----GGTAGA--GCCGG 410
TC2     ACCCCCCTAC----------------CCCTT----------G----GGTAGA--GCCGG 410
TK      ACCAACCGACTCAAACGGAGGCGCCTGCGGCCGGACGTCGCAG----GCGCGTTGTCTGG 439
SD      GGAGTGGTGCTTAC--GATCCAGGTCACTTACGGGAAATCGGGATATGCTCCACCGCGGC 430
```

```
TV1     CTGAAGT-CAAGAGAT-CGAACCACTGCCATAT-CTCGGGCCCCCTG-GGGATGCCTCGG 462
TV4     CTGAAGTGCAAGAGATTCGAACCACTGCCATATTCTCGGGCCCCCTG-GGGATGCCTCGG 465
TV2     CTGAAGTGCA-GAGAT-CGAACCACTGCCATAT-CTCGGGCCCCCTG-GAG-TGCCTCGG 460
TV3     CTGAAGTGCA-GAGAT-CGAACCACTGCCATAT-CTTGGGCCCCCTG-GAG-TGCCTCGG 460
TS1     TTGGAC--CTGGCAGTCTCATTGGCAGCAATGCGCATGGGTCTGCTG-AAG-TGCA--GA 502
TS2     TTGGAC--CTGGCAGTCTCATTGGCAGCAATGCGCATGGGTCTGCTG-AAG-TGCA--GA 501
TA      TTGGAC--CTGGCAGTCTCATTGGCAGCAATGCGCATGGGTCTGCTG-AAG-TGCA--GA 502
TG      TTGGAC--CTGGCAGTCTCAGAGCTTTCATTAGCGCTGGGTCTGCTG-AAG-TGCA--GA 464
TC1     TTGGAC--CTGGCAGTCTCAGAGCTTTCATTAACGCTGGGTCTGCTG-AAG-TGCA--GA 464
TC2     TTGGAC--CTGGCAGTCTCAGAGCTTTCATTAGCGCTGGGTCTGCTG-AAG-TGCA--GA 464
TK      CCGGAGTCTCATTGATTAAGAGGATAAGGGGAGCTTCGGACTCGTAGCGAAACGGGTCGG 499
SD      TTCTAACTGCGGAACTGGCGATCTCTACCTGAGCTTCATGCTCTCGTAGCGTTTCCG-AT 489

TV1     CACCCAGGTGGGC-----TTGGGGGCGAGCACCGGGTAGGTAGCCC-AAGGGGTTATTTC 516
TV4     CACCCAGGTGGGC-----TTGGGGGCGAGCACCGGGTAGGTAGCCCCAAGGGGTTATTTC 520
TV2     CACCCAGGTGGGC-----TTGGGGGCGAGCACCGG-TAGGTAGCCC--AAGGGTTATT-C 511
TV3     CACCCAGGTGGGC-----TTGGGGGCGAGCACCGG-TAGGTAGCCT--AAGGGTTATT-C 511
TS1     GATCCAGACAGGACCCTATTATGGGCAAACACTAGGTAGGTAGCCT--TCGGGTTATTCC 560
TS2     GATCCAGACAGGACCCTATTATGGGCAAACACTAGGTAGGTAGCCT--TCGGGTTATTCC 559
TA      GATCCAGACAGGACCCTATTAAGGGCAAACACTAGGTAGGTAGCCT--TCGGGTTATTCC 560
TG      GATTTAACCGGGACCC-GCTAAGGGCAAACACTAGGTAGGTAGCCT--TCGGGTTATTCC 521
TC1     GATTTAACCGGGACCC-GCTAAGGGCAAACACTAGGTAGGTAGCCT--TCGGGTTATTCC 521
TC2     GATTTAACCGGGACCC-GCTAAGGGCAAACACTAGGTAGGTAGCCT--TCGGGTTATTCC 521
TK      GACCCAGGTATTG------TTGGGGCTAGCACGCGGTAGGTAGCCT--TGGGGTTATTGG 551
SD      GCTCTCGACATAAGCA---TGGGGTTGTGTTCCTCGCAT---GCCTTGCTGGGGCTTCGT 543

TV1     CGTGCGCGCTGGTGGCCGCTTGGACTGAT-AAGCAGGTATTTTGCTTCCGGGTAAATAAA 575
TV4     CGTGCGCGCTGGTGGCCGCTTGGACTGATCAAGCAGGTATTTTGCTTCGGGGTAAATAAA 580
TV2     CGTGCGCGCTGGTGGCCGCTGGGACTGATCAAGCAGGTATTT-GCCTCTGGGTAAATA-- 568
TV3     CGTGCGCGCTGGTGGCCGCTGGGACTGATCAAGCAGGTATTT-GCCTCCGGGTAAATA-- 568
TS1     TGTGCGTGTCCAAGGCCT----GGCCCGT-GATCAG------------CAGGAAAACAAC 603
TS2     TGTGCGTGTCCAAGGCCT----GGCC-GT-GATCAG------------CAGGAAAACAAC 601
TA      TGTGCGTGTCCAAGGCCT----GGCC-GT-GATCAG------------CAGGAAAACAAC 602
TG      TGTGTGTGTCTAAGGCCT----GGCT-AT-AATCAA------------CAGGAAAACACC 563
TC1     TGTGTGTGTCTAAGGCCT----GGCT-AT-AATCAG------------CAGGAAAACACC 563
TC2     TGTGTGTGTCTAAGGCCT----GGCT-AT-AATCAG------------CAGGAAAACACC 563
TK      TGTGTGCGCTGGAAGCCT----GCCTAGTGAAGCTTTCCCTG----GACAGGAAAATAGT 603
SD      CGCTGGTATCATAGACAG---GCACTAGTGGGGCTAATCGCT------AATGCCGTTGAC 594

TV1     ATCAACATTT-GACCTGAGTTCAGAC-GAGACTACCCGCCGA 615
TV4     ATCAACATTTTGACCTGAGTTCAGACCGAGACTACCCGCCGA 622
TV2     ATCAACATTT-GACCTGAGTTCAGAC-GAGGCTACCCGCCGA 608
TV3     ATCAACATTT-GACCTGAGTTCAGAC-GAGACTACCCGCCGA 608
TS1     TTAAACCTTCGA-CCTGAGTTCAGAC-GAGACTACCCGCCGA 643
TS2     TTAAACCTTCGA-CCTGAGTTCAGAC-GAGACTACCCGCCGA 641
TA      TTAAACCTTCGA-CCTGAGTTCAGAC-GAGACTACCCGCCGA 642
TG      TTAGCCATTCGA-CCTGAGTTCAGAC-GAGACTACCCGCCGA 603
TC1     TTAACCATTCGA-CCTGAGTTCAGAC-GAGACTACCCGCCGA 603
TC2     TTAACCATTCGAACCTGAGTTCAGAC-GAGACTACCCGCCGA 604
TK      GTAAACTTTCGA-CCTGAGTTCAGAC-GAGACTACCCGCCGA 643
SD      ATGAGTGCCGGA-CAAGAGTTTAGAC-GAGGATACCCGCCGA 634
```

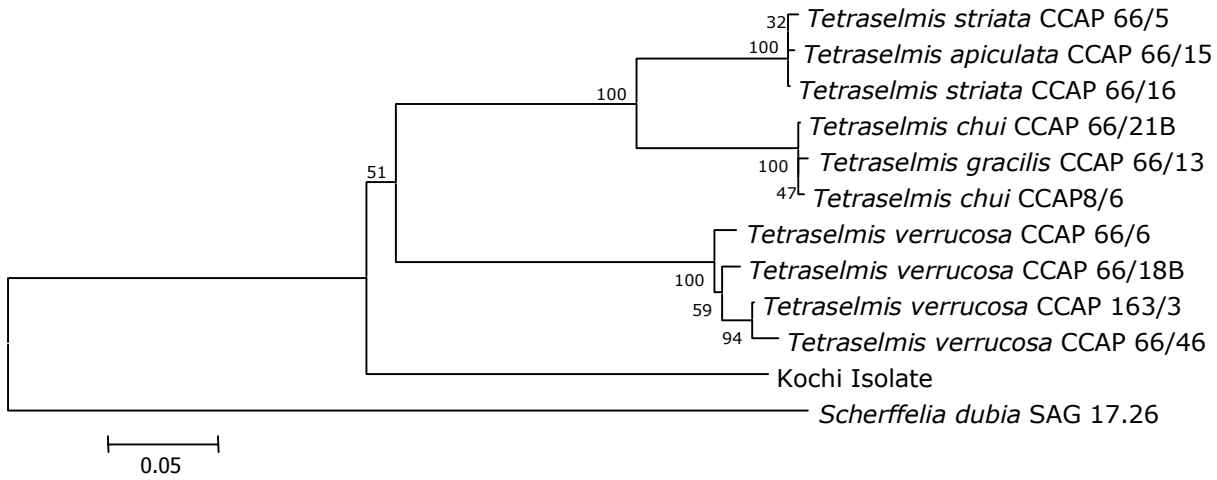
Figure 4.7 Alignment ITS1-5.8S-ITS2 rDNA sequences of *Chlamydomonas* and *Pedinomonas minor*.

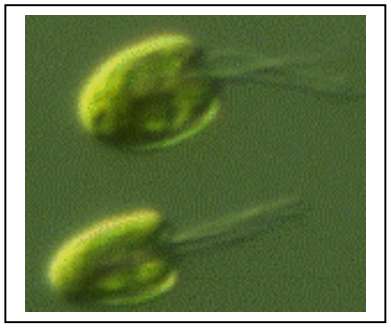
C sp = *Chlamydomonas* species (ACC no), C.PL= *C. plethora*, C. PR= *C. proteus*, PM= *Pedinomonas minor*

```
Csp      AAGGATCATTGAATCTATCAACAACCACACCTGGCGAACACTGATGAACGTCGGCCTCGA 60
C.PL     AAGGATCATTGAATCTATCAACAACCACACCTGGCGAAC-CTTATGAACGTCGGCCTCGA 59
C.PR     AAGGATCATTGAATCTATCAACAACCCCCCACCATGCGAACCTATCAATGTCGGTCTTTC 60
PM       AGGGATCATTGAATCGATCGA-ATCCAC------CGAGAACT-GTAATCGTGGACCCTGT 52

Csp    GAGTAC-----------------TGTTCTCTAATAATGGTGCGTTCGGTGTCGGATGCTG 103
C.PL   GCGTGTATGTATATGTGTGTGTGTCTTTTTTGATACTACTACACTTTGCGT-GTACGCTC 118
C.PR   GTGCAG----------------------CCTGGCAACGG----------GTTGTGCGTTG 88
PM     -CGTGG----------------------CCGCCGAGA-----------GGCGGACGC-G 76

Csp    CTGCTGATGGTGCTTATGTATTGTCATCAACATCA----TTCGACAGGCC-GTTCATGTT 158
C.PL   GGTGTCGTGGT-TTTCCTTCTTCTCATGAACAAGGAAACCACGGCAGGTCTGTTTTCGGC 177
C.PR   TGTCGAATGCTTCTTCATTGCGGCATTCGGCAGGG---TCTCGGCCAAT---TTTTTGGT 142
PM     CAGCAGCCGGAGTCTAAACTGGCTGATCCTCGTCTCCGCTTGCGGAGGC---------TT 127

C sp   AGAAA---TGACATGAGC-GGTCGGCCCCTT--CTTTCTTACCACAACACTCCAAACCAA 212
C.PL   AAAAGCTTTGCTGAAAACAGGTCGGCCCCTTTTCTTTTTCACCAACAAACTCCTAAACGA 237
C.PR   CGAGG---------------GTCGGCCCTTT---TCATTAACCAAC-CACTCCAAACTAA 183
PM     GGAAG---------------GTCGACCAAAC--CAACCTTATC----CAACCCAAACCAC 166

C sp   TTGACATCTGAAGCAAAACTCAGTGCAGCCGGCTTGGCCGTT-CTGCACGATCTAACCAA 271
C.PL   AACGAATCTGAAGTCAA--TCAGTGCAACCGGCTTGGCCGTTTCTGCACGTCTTAACCAA 295
C.PR   A-CAAATCTGAAGCAA----CAGTGTAACCGGCTTGGCCGTC-TTACAC-ATCTAACCAA 236
PM     A-GTTGTCTAAAGTGAG-TTCTTTCCGAC---TTCGGTCGAAAAGGCAAAACAAACCAAA 221

C sp   GACAACTCTCAACAACGGATATCTTGGCTCTCGCAACGATGAAGAACGCAGCGAAATGCG 331
C.PL   GACAACTCTCAACAACGGATATCTTGGCTCTCGCAACGATGAAGAACGCAGCGAAATGCG 355
C.PR   GACAACTCTCAACAACGGATATCTTGGCTCTCGCAACGATGAAGAACGCAGCGAAATGCG 296
PM     GACAACCCTCAACAACGGATAACTTGGCTCGTGCAACGATGAAGAACGCAGCGAAATGCG 281

Csp    ATACGTAGTGTGAATTGCAGAATTCCGTGAATCATCAAGTCTTTGAACGCATATTGCGGT 391
C.PL   ATACGTAGTGTGGATTGCAGAATTCCGTGAATCATCAAGTCTTTGAACGCATATTGCGGT 415
C.PR   ATACGTAGTGTGAATTGCAGAATTCCGTGAATCATCAAGTCTTTGAACGCATATTGCGCT 356
PM     ATACGTAGTGTGAATTGCAGAATCACGTGAACCATCGAATCTTTGAACGCAACTTGCGCT 341

Csp    GGAGGTTTCGGCCAACACCATCTCTGGTTCAGCGTCGATGTAACCCTCATCCA-CATAT- 449
C.PL   GAAGGCTTCGGCTAACACCATCTCTGGTTCAGCGTCGATGTATCCCTCATTCATCATATA 475
C.PR   GGAGGCTTCGGCTGACAGCATGTCTGGATCAGCGTCGATGTATCCCTCATCCCAAACAC- 415
PM     TGATCCTTCGGGAGAAAGCACGTCTGCCTGAGCGACGGCCT-TCCCTC-TCGACTCCCT- 398

Csp    --TATCTATATA----ATAATGATGGATGGATCTGGCCGTCTTGGTATGTATGCACATGC 503
C.PL   CACATCTTTGTTTTGTGTATATGTGGATGGACCTGGCCGTCCCGGTCA--ATGAATTGGC 533
C.PR   ---CCCTTTGGA----GTGCTTG-GTATGGATCTGACTGTCCCGGCGTTCA-GTCTTTGA 466
PM     ----TCCCTCCG---GGGAAACGAAGTCGGACCTGGCCCTCCCAGTTGCCTTTCTTCGGA 451

Csp    ATACATAGCGGGTCTGCTGAAGTGAAGAGGCACCTCAAACGGACCTTTTTTAACTATGAT 563
C.PL   --------CGGGTCTGCTGAAGAGCAGAGGCAACTCAAAGGGACCTCCAT--ATTTTG-- 581
C.PR   TTGAAAACCGGGTCAGTTGAAGCATAGAGGCTAACCAA--GGACCATTAT---------- 514
```

```
PM      AAACGCGCTGGGTTGGCTGAAGCGCAGAGGCCTGAGCAGGGAGCCCATAC---------- 501

Csp     GGTTAAAAAGGCCGCATCTAGGTAGTTTCCTTCCTTCTGTGGAATGTTTATTTAGATGTT 623
C.PL    KATGTATGGGGCCGCATCTGGGTAGAGGCTTCCCTTGCCTTGAATACT---TTGGATGTT 638
C.PR    --------GGGCCACATCTAGGTAGGCACGCATCA-CTGCTACATATT----TAGATGTT 561
PM      ----CAAAGGGGCACGGCAAGGTAGG---TTGTCTCACGACAGCTCCTTA-CCCGCCGCT 553

Csp     GGCTTTGGTCGCATTGAGCTACATGATTGCCTTTGTTCTGGAAACCACGTTTACTTTACT 683
C.PL    GGCGTGCGTTCTTTCGAGTT--TTTGTTGCCCAAAGCCAAAGGAACACCACACTCTTACT 696
C.PR    GGCTTGGACT---TTGGGT------GTGCCCTCAAACCAGGAAACCAC-----TTACATC 607
PM      GTCCCGGGATCCTATGCTT-------CGGGCCCAG--CAGGAAAGCAC------CTATTT 598

Csp     TTCGACCTGAGCTCAGGGGAGAACACCCGCTGAACTT 720
C.PL    TTCGACCTGAGCTCAGGGGAGAACACCCGTTGAACTT 733
C.PR    CTCGACCTGATCTCAGGCAAGAACACCCGCTGAACTT 644
PM      TTCGTCCTCAGTTCAGGCGAGATTACCCGCTGAACTT 635
```

Figure 4.8: Phylogenetic tree from ITS1-5.8S-ITS2 rDNA of *Tetraselmis* species and *Scherffelia* inferred by Neighbor joining method.





CHAPTER 5

GENERAL DISCUSSION

From the results of ultrastructure and 18S rDNA sequences, the Kochi isolate has been identified as *Tetraselmis* (Chlorodendrales, Prasinophyceae). ISSR fingerprinting and ITS sequence information suggested that the isolate was genetically distinct from the other *Tetraselmis* species studied here. As discussed in chapter 2 the Kochi isolate was separated from *T. cordiformis* Stein (Stein 1878; Melkonian 1979), as it required salt for growth and had a pyrenoid located immediately below the nucleus which was not surrounded by starch plates, and the cytoplasmic channels in the pyrenoid matrix contained electron dense material not surrounded by a membrane, the chloroplast was smooth with almost equal anterior lobes. The isolate differed from *T. contracta* Carter (Carter 1937, Butcher 1959) in that it had equal apical lobes, pyrenoid was central in position and the cyst did not have an apical depression although a papilla-like structure was occasionally observed on the cyst wall. The isolate has been deposited in the National Collection of Industrial Microorganisms (NCIM) as *Tetraselmis kochinensis* (?) NCIM 7001.

In the genus *Tetraselmis* species characterization has always been difficult. Recently Marin *et al*., (1996) described a new species *T. desikachary*, based on the characters such as cell symmetry and size, position of eyespot and structure of flagellar hairs. However their earlier descriptions of flagellar hair ultrastructure (Marin *et al.*, 1993) showed that it is sometimes difficult to identify organisms unambiguously, for example *T. striata* M 580 and M614 (page no 214, Table 1, Marin *et al.*, 1993) and two strains of *T. convolutae* CCMP 888 and CCMP 886 apparently belonged to different subtypes and similar result was seen for *T. tetrathele*. From this it appears that the structure of flagellar hairs is variable between isolates of any given species. This variation in flagellar hairs might be due either to age of the culture/cell or to disruption of structure during sample processing for electron microscopy. Thus even flagellar hairs may not be reliable characteristics for species identification and there appears to be no single

defined set of light or electron microscopical characters, which will allow unambiguous identification of a new isolate.

Hori *et al*., (1982) used ultrastructure of pyrenoid to divide the genus *Tetraselmis* into four subgenera. However it is interesting to note that *Chloromonas clathrata*, a unicellular biflagellate member of Chlamydomonadales, which is distinguished from *Chlamydomonas* by the absence of a pyrenoid (Ettl 1970 and 1980). However the phylogenetic analysis using 18S rDNA sequences showed that this snow alga *C. clathrata*, clustered with *Chlamydomonas augustae* which is another snow alga with 98 % bootstrap value (Buchheim *et al*., 1997). Thus indicate that the absence of pyrenoid is not a good indicator for species relationships whereas habitat correlates well with phylogenetic pattern (Buchheim *et al*., 1997).

18S rDNA phylogenetic analysis of 14 *Tetraselmis* strains comprising seven species and *Scherffelia dubia* separated from other prasinophyte taxa forming one group with 100% bootstrap value suggesting that the order Chlorodendrales is monophyletic. *Scherffelia* has been separated from *Tetraselmis* because it lacks pyrenoid otherwise in morphology *Scherffelia* is indistinguishable from *Tetraselmis* (Presig and Melkonian, 1986). In the 18S rDNA phylogeny *Scherffelia* did not separate from *Tetraselmis* and showed 96 % sequence similarity with other *Tetraselmis* species suggesting that the separation of these two genera on the basis of absence of pyrenoid might not be a taxonomically significant. However 5.8S and ITS rDNA sequences of *Tetraselmis* and *Scherffelia* were considerably variable, hence further study is needed to determine whether these two genera should be merged or not.

Within the present data set, ISSR analysis and ITS sequence analysis suggested that the 10 strains of the *Tetraselmis* formed three groups and that the Kochi isolate diverged from these. ISSR results compared well with ITS sequence results. However the fact that no global database exists for ISSR profiles, limits comparison of these results with other taxa. On the other hand ITS sequence databases are rapidly growing and the sequences can be simultaneously compared between various taxa.

ITS sequences within a species are known to show either high sequence homogeneity or high sequence variation. In the present analysis it was observed that the strains of same species showed more than 96% sequence similarity in ITS regions whereas the sequence similarity between species was 60-70%. ITS sequences of *Tetraselmis* were species specific and it was possible to separate each species from one another using ITS sequences.

In conclusion the analysis of multiple data sets based on morphological, ultrastructural and different molecular tools provides a robust set of characters for species identification.

Due to lack of reliable taxonomic markers for species identification, most of the *Tetraselmis* listed in various culture collections have not been identified to the species level. Therefore it would be important to study all the *Tetraselmis* strains listed under various culture collections using morphological and molecular tools to establish suitable and unambiguous identification markers for delimiting the *Tetraselmis* species.

References

Aken, M. E. and Pienaar, R. N. (1985) S. Afr. J. Bot. **51**: 408-416.

Alberto, F., Santos, R. and Leitao, J. M. (1997) J. of Phycol. **33**: 706-710.

Andersen, R. A., Barr, D. J. S., Lynn, D. H., Melkonian, M., Moestrup, Ø. and Sleigh, M. A. (1991) Protoplasma. **164**:1-8.

Barlow, S. B. and Cattolico, R. A. (1980) Br. Phycol. J. **15**: 321-333.

Beakes, G., Canter, H. M. and Jaworski, G. H. W. (1988). Can. J. Bot. **66**(6): 1054-1067.

Becker, D., Becker, B., Satir, P. and Melkonian M. (1990) Protoplasma. **156**: 103-112.

Bhattacharya, D. and Druehl, L. D. (1989) Mar. Biol.(Berl.) 102:15-23.

Bot, P. V. M., Holton, R. W., Stam, W. T. and van den Hoeck, C. (1989a) Mar. Biol. **102**:159-168.

Bot, P. V. M., Boele-Bos, S. A., Stam, W. T., van den Hoeck, C. and van Delden, W. (1989b) Phycologia. **28**: 159-168.

Buchheim, M. A., Turmel M., Zimmer, E. A. and Chapman, R. L. (1990) J. Phycol. **26**: 689-699.

Buchheim, M. A., Buchheim, H. A., and Chapman, R. L (1995) J. Phycol. (Suppl.) **30**:17.

Butcher, R. W. (1959) Part I: Introduction and chlorophyceae. Fish. Invest. Minist. Of Agricult.,Fish and Food, Ser. IV **1**: 1-74.

Caron, D. A., Lim, E. L., Dennett, M. R. and Gast, R. J. (1999) J. Phycol. **35**: 824-837.

Carroll, M. A. (1989) J. Phycol. (Suppl) **25**: 24.

Carter, N. (1937) Arch. Protistenk. **90**: 1-68.

Chadefaud, M. (1941) La Rev. Scient. **79**: 113-114.

Chadefaud, M. (1960) Systematique **1**: 1-1018.

Chapman, R. L. and Buchheim, M. A. (1992) Biosystems **28**: 127-137.

Chapman, R. L., Buchheim, M. A., Delwiche, C. F., Friedl, T., Huss, V. A. R., Karol, K. G., Lewis, L. A., Manhart, J., McCourt, R. M., Olsen, J. L. and Waters, D. A. (1998) Molecular Systematics of Plants II. Kluwer Academic Publishers, Boston, PP 508-540.

Chen, Y. C., Eisner, J. D, Kattar, M. M., Rassolutian-Barrett S. L., LaFe, K., Bui, U., S. L., Limaye, A. P. and Cookson B. T. (2001) J. Clin. Microbiol. **39**: 4042-4051.

Chen, Y. C., Eisner J. D, Kattar, M. M., Rassolutian-Barrett S. L., LaFe, K., Yarfitz, S. L., Limaye, A. P. and Cookson B. T. (2000) J. Clin. Microbiol. **38**: 2889-2892.

Cheney, D. P. (1985) Handbook of Phycological Methods-Ecological Field Methods: Macroalgae. Cambridge Univ. Press Cambridge. 87-119.

Christensen, T. (1962) In :Systematisk Botanik, Vol.2, no. 2. (Eds Boscher, T. W., Lange, M. and Sorensen, T.) 178 pp.

Coleman. A. W. and Mai, J. C. (1997) J. Mol. Evol. **45**:168-177.

Coyer, J.A., Olsen, J. L. and Stam, W. T. (1997). J. phycol. **33**:561-568.

Cozzolino, S., Campo, I., Moretti, M. A. and Pollio, A. (1999) Algological Studies **95**: 31-42.

Diez. B., Pedros-Alico, C. and Massana, R. (2001) Appl. Environ. Microbiol. **67**: 2932-2941.

Domozych, D. S. (1984) J. Phycol. **20**:415-418.

Donaldson, S. L., Chopin, T. and Saunders, G. W. (1998) J. Appl. Phycol. **10**: 365-370.

Donaldson, S.L., Chopin, T. and Saunders GW (1998) J. Phycol. **34** (Suppl): 16.

Donaldson, S. L., Chopin, T. and Saunders, G. W. (2000) J. Appl. Phycol. **12**: 25-35.

Dutcher, J. A. and Kapraun, D. F. (1994) J. Appl. Phycol. **6**: 267-273.

Ettl, H. and Moestrup, Ø, (1980) Pl. Syst. Evol. **135:** 177-210.

Fain, S. R., Druehl, L. D. and Baillie, D. L. (1988) J. Phycol. 24: 292-302

Fama, P., Olsen J. L., Stam, W. T. and Procaccini, G. (2000) Eur. J. Phycol. **35**: 349-356.

Fawley, M.W. (1992). J. Phycol. **28**: 26-31.

Fell, J. W., Boekhout, T., Fonseca, A., Scorzetti, G. and Statzell-Tallman, A. (2000) Int. J. Syst. Evol. Microbiol. **50**: 1351-1371.

Felsenstein, J. (1985). Evolution. **39**: 783--91.

Fridel, T. and Zeltner, C. (1994) J. Phycol. **30**: 500-506.

Fridel, T. (1995) J. Phycol **31**: 632-639.

Gardes, M. and Bruns, T. D. (1993) Mol. Ecol. **2**: 113-118.

Gast, R. J., McDonnell, T. A. and Caron, D. A. (2000) J. Phycol. **36:** 172-177.

Goff, L. J. and Coleman, A. W. (1988) J. Phycol. **24**: 357-368.

Gopinathan, (1972) as in Chapter 6, Fish and Fisheries of India.

Guillard, R. L. and Ryther, J. H. (1962) Can J. Microbiol. **8**: 229-239.

Guillard, R. R. L., Keller, M. D., O'Kelly, C. J. and Floyd, G. L. (1991) J. Phycol. **27**: 39-47.

Gunderson, J. H., Elwood, H., Ingold, A., Kindle K., and Sogin M. L, (1987) Proc. Natl. Acad. Sci. USA, **84**: 5823-5827.

Hori, T., Inouye, I., Horiguchi, T. and Boalch, G. T. (1985) Bot. Mar. **28**: 529-537.

Hori, T., Norris, R. and Chihara, M. (1982) Bot. Mag, Tokyo. **95**: 49-61.

Hori, T., Norris, R. and Chihara, M. (1983) Bot. Mag. Tokyo. **96**: 385-392.

Hori, T., Norris, R. and Chihara, M. (1986) Bot. Mag. Tokyo. **99**: 123-135.

Hoz, De La., Sanchez, M. P., Davila, J. A., Loarce, Y. and Ferrer, E. (1996) Genome. **39**: 112-117.

Huber, M. E. and Lewin, R. A. (1986) Phycologia. **25**: 205-209.

Huss, V.A. R. and Sogin M. L., (1990) J. Mol. Evol. **31**: 432-442.

Huss, V. A. R., Franck, C., Hartmann, E. C., Hirmer, M., Kloboucek, A. Seidel, B. M., Wenzeler, P. and Kessler, E. (1999) J. Phycol. **35**: 587-598.

Huss, V. A. R., Ciniglia, C., Cennamo, P., Cozzolina, S., Pinto, G. and Pollio, A. (2002) BMC Evol. Biol. **26**:13

Inouye, I., Hori, T. and Chihara, M. (1990) J. Phycol. **26**: 329-344.

Inouye, I. and Hori, T. (1991) Protoplasma **164**: 54-69.

Inouye, I. and Pienaar, R. N. (1984) Nord J. Bot. **4**: 409-423.

Jeffrey, S. (1989) In: The Chromophyte Algae: problems and Perspectives (Eds. Green, J. C., Leadbeater, B. S. C., and Diver, W. L.,) pp. 13-36. Clarendon Press, Oxford.

Jeffreys, A. J., Wilson, V. and Thein, S. L. (1985) Nature **314**: 67-73.

Joshi, S. P., Gupta, V. S., Aggarwal, R. K., Ranjekar, P. K. and Brar, D. S. (2000) Theor. Appl. Genet. **100**:1311-1320.

Kantety, R. V., Zeng, X. P., Bennetzen, J. L. and Zehr, B. E. (1995) Mol. Breeding. **1**:365-373.

Kantz T. S., Theriot E. C., Zimmer E. A. and Chapman R. L. (1990) J. Phycol. **26**: 711-721.

Kim, Y.-S., Oyaizu, H., Matsumoto, S., Watanabe, M.M. and Nozaki, H. (1994) J. Phycol. **29**: 213-217.

Ko, K. S. and Jung, H. S. (2002) Mol. Phylo. Evol. **23**: 112-122.

Kooistra, W. H. C. F., Stam W. T., Olsen, J. L. and C. van Den Hoek (1992) J. Phycol. **28**: 660-668

Kurtzman, C. P. (2000) Int. J. Syst. Evol. Microbiol **50**: 395-404.

Kumar, S., Tamura, K., Jakobsen, I. B. & Nei, M. (2001) MEGA2: Molecular Evolutionary Genetics Analysis software, Arizona State University, Tempe, Arizona, USA.

Kimura, M. (1980) J. Mol. Evol. **16**: 111-120.

Kylin, H. (1935) Über Rhodomonas, Platymonas and Prasinocladus, Kgl. Fysioger. Sälsk, i Lund Forhandl. **5**: 1-13.

Lechtreck, K. F. and Melkonian, M. (1991) Protoplasma. **164**: 38-44.

Lee, S. B. and Taylor, J. W. (1991) Mol. Biol. Evol. **8**: 620-640.

Lewis, L. A., Wilcox, L. W., Fuerst, P. A. and Floyd G. L. (1992) J. Phycol. **28**: 375-380.

Maidak, B. L., Cole, J. R., Lilburn, T. G., Parker, C. T. Jr., Saxman, P. R., Farris, R. J., Garrity, G. M., Olsen, G. J., Schmidt, T. M. and Tiedje, J. M. (2001) Nucleic Acids Res. **29**: 173-174.

Manton, I. (1959) J. Mar. Biol. Ass. U. K. **38**: 319-333.

Manton, I. (1966) J. Cell. Sci. **1**: 429-438.

Manton, I. (1975). Arch. Protistenk. **117**: 358-368.

Manton, I. (1977) Phycologia. **16**: 427-438.

Manton, I. and Parke, M. (1965) J. Mar. Biol. Ass. U.K. **45**: 743-754.

Marchant, H. J., Buck, K. R., Garrison, D. L. and Thomsen, H. A. (1989) J. Phycol. **25**:167-174.

Marin, B. and Melkonian, M. (1999). Protist **150**: 399-417.

Marin, B., Hoef-Emden, K. and Melkonian, M. (1996) Nova Hedwigia. **112**: 461-475.

Marin, B., Matzke, C. and Melkonian, M. (1993) Phycologia. **32**: 213-222.

Mattox, K. R. and Stewart, K. D. (1984) In Systematics of the Green Algae (Eds Irvine, D. E. G. and John, D. M.), Academic Press, Orlando pp 29-72.

McFadden, G. I., Hill, D. R. A. and Wetherbee, R. (1986) Nord. J. Bot. **6**: 209-234.

McLachlan, J. and Parke, M. (1967) J. Mar. Biol. Asso. U. K. **47**: 723-733.

Melkonian, M. (1977) Pl. Syst. Evol. **128**: 79-88.

Melkonian, M. (1979) Protoplasma **98**: 139-151.

Melkonian, M. (1984) In: Systematics of the Green Algae (Eds. Irvie, D. E. G. and John, D. M.) pp 73-120. Academic Press, Orlando.

Melkonian, M. (1989). Pl. Syst. Evol. **164**: 93-133.

Melkonian, M. (1990) Phylum Chlorophyta Class Prasinophyceae In: Handbook of Protoctista (Eds. Margulis, L., Corliss, J O., Melkonian, M. and Chapman, D. J.) 600-607. Jones and Bartlett Publishers, Boston.

Melkonian, M. and Preisig, R. H. (1986) Nor. J. Bot. **6**: 235-256.

Melkonian, M., Beech, P. L., Katsaros,C. and Schulze, D. (1992) In Algal Cell Motility (Ed. Melkonian, M.), Chapman and Hall, Inc., New York. pp. 179-221.

Melkonian, M. and Surek, B. (1995) Bull. Soc zool. Fr. **120**: 191-208.

Melkonian, M., Marin, B. and Surek, B. (1995). Algae. Biodiversity and Evolution (Eds. R Arai, M Kato and Y Doi) The National Science Museum Foundation, Tokyo. pp 153-176.

Melkonian, M., Schulze, D., McFadden, G. I. and Robenek, H. (1988) Protoplasma. **144**: 56-61.

Moestrup, Ø. (1982) Phycologia. **21**: 427-528.

Moestrup, Ø. (1984) Nord. J. Bot. **4**: 109-121.

Moestrup, Ø. (1990) Phycologia. **29**: 437-442.

Moestrup, Ø. and Ettl, H. (1979) Opera. Bot. **49**: 1-40.

Moestrup, Ø. and Hori, T. (1989) Protoplasma. **148**: 41-56.

Moestrup, Ø. and Throndsen, J. (1988) Can. J. Bot. **66**: 1415-1434.

Moore, L. J. and Coleman, A. W. (1987) International Botanical Congress,

Berlin, Abst. 178

Nakayama, T., Marin, B., Krantz, H. D., Surek, B., Huss V. A. R., Inouye I., and Melkonian, M. (1998) Protist. **149**: 367-380.

Norris, R. E. (1980) In: Phytoflagellates (Cox, E. R. ed.), pp. 85-145. Elsevier North Holland, Inc., New York.

Norris, R. E. and Pearson, B. R. (1975) Arch. Protistenk. Bd. **117**: 192-213.

Norris, R. E., Hori, T. and Chihara, M. (1980) Bot. Mag. Tokyo. **93**: 317-339.

Norton, T. A., Melkonian, M., and Andersen, R. A. (1996) Algal biodiversity, Phycologia. **35**: 308-326.

Odorico, D. M. and Miller, D. J. (1997) Mol. Biol. Evol. **14**: 465-473.

Ohta, T. and Dover, G. A. (1983) Proc. Natl. Acad. Sci. USA 80: 4079-4083.

O'Kelly, C. J. (1992) In: The Cytoskeleton of the Algae (Menzel, D. ed.), pp. 315-345. CRc Press Inc., Boca Raton, Florida.

Olsen, J. L., Valero, M., Meusnier, I., Boele-Bos, S. and Stam, W. T. (1998) J. Phycol. **3**: 850-856.

Oppen, M. J. H. van, Klerk, H., Graaf, M. de., Stam, W. T. and Olsen, J. L. (1996) J. Phycol. **32**: 433-444.

Parke, M. and Manton, I. (1967) J. Mar. Biol. Ass. U.K. **47**: 445-465.

Parke, M. (1966) In: Some Contemporary studies in Marine Science (Branes, H. Ed) pp 555-563. George Allen and Unwin Ltd. London.

Parke, M. and Den Hartog-Adams, I. (1965) J. Mar. Biol. Ass. U. K. **45**: 537-557.

Parke, M., Boalch, G. T., Jowett, R. and Harbour, D. S. (1978). J. Mar. Bio. Ass. U. K. **58**: 239-276.

Pennick, N. C. (1977) Arch Protistenk. **119**: 388-394.

Pillmann, A., Woolcott, G. W.,Olsen J. L., Stam, W. T. and King, R. J. (1997) Eur. J. Phycol., **32**: 379-386.

Ricketts, T. R. (1966) Phytochem. **5**: 223-229.

Ricketts, T. R. (1970) Phytochem. **9**: 1835-1842.

Rogers, S. O. and Bendich, A. J. (1988) Plant Molecular Biology Manual A6: 1 (Eds. Gelvin, S. B. and Schilperoort) Kluwer Academic Publishers, Dordrecht.

Rohlf, F.J. (1989) NTSYS-pc Numerical taxonomy and multivariate analysis system. Setauket, NY:Exeter Publishing Co, Ltd.

Salimath, S. S., de Oliveira, A. C., Godwin, I. D. and Bennetzen, J. L. (1995) Genome. **38**: 757-763.

Salisbury, J. L. and Floyd, G. L. (1978) Science **202**: 975-977.

Salisbury, J. L., Swamson, J. A., Floyd, G. L., Hall, R. and Maihle, N. J. (1981). Protoplasma **107**: 1-11.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular cloning: a Laboratory manual (2nd edn). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Schnepf, E. and Maiwald, M. (1970) Experientia. **26**: 1343-1344

Scolfield, S. C., Gacesa, P., Price, J. H., Russell, S. J. and Bhoday, R. (1991) J. Appl. Phycol. **3**: 329-334.

Serrão, E., Alice, L. A. and Brawley, S. H. (1999) J. Phycol. **35**: 382-394.

Smith, G. J. and Alberte, R. S. (1991). J. Phycol. **27**: 92-101.

Smith, J. J., Scott-Craig, J. S., Leadbetter, J. R., Bush, G. L., Roberts, D. L. and Fulbright, D. W. (1994). Mol. Phylog. Evol. **3**:135-145.

Sogin, M. L. 1991. In Warren, L. and Coprowski, H. (eds), New Perspectives on evolutuon- Proceedings of a Multidisciplinary Symposium Designed to Interrelated Recent Discoveries and New Insights in the Field of Evolution, Wiley-Liss, New York, NY pp. 175-188.

Sosa, P. A. and Garcia-Reina, G. (1992) Mar. Biol. **113**: 679-688.

Sosa, P. A. and Garcia-Reina, G. (1993) J. Phycol. **29**: 118-124.

Stam, W. T. and Venema, G. (1977) Acta Bot. Neerl. **26**: 327-242.

Stam, W. T., Bot, P. V. M., Boele-Bos, S. A., van Rooij, J, M. and van den Hoeck, C. (1988) Helgoländer Meeresunter **42**: 251-267.

Staay, S. Y. Moon-van der, Wachter, R. de and Vaulot, D. (2001) Nature **409:** 607-610.

Stein, F. R. von (1878) Abt. III 1.-Engelmann.

Steinkötter, J., Bhattacharya, D., Semmelroth, I., Bibeau, C. and Melkonian, M. (1994) J. Phycol. **30**: 340-345.

Stewart, K. D., Mattox, K. R. and Chandler, C. D. (1974) J. Phycol. **10**: 65-79.

Surek, B., Beemelmanns, U., Melkonian, M and Bhattacharya, D. (1994) Pl. syst. Evol. **191**: 171-181.

Sym, S. D. (1992). Ph. D. Thesis University of the Witwatersrand, Johannesburg, South Africa. 331 pp.

Sym, S. D. and Pienaar, R. N. (1991) J. Phycol. **27**: 277-290.

Sym, S. D. and Pienaar, R. N. (1993) Progress in Phycological Research **9**: 281-376.

Thompson, J. D., Higgins, D. G. and Gibson, T. J. (1994) Nucl. Acids. Res **22**: 4673-4680.

van Oppen, M. J. H., Dieckmann, O. E., Wiencke, C., Stam, W. T. and Olsen J. L. (1994) J. Phycol. **30**: 67-80.

Vis, M. L. (1999) Phycol. **38**: 70-73.

West, G. S. (1916) J. Bot. London **54**: 1-10.

Wilcox, L.W. Fuerst, P. A. and Floyd G. L. (1993) Am. J. Bot. **80**: 1028-1033.

Wilcox, L.W. Lewis, L. A., Fuerst, P. A. and Floyd, G. L. (1992) J. Phycol. **28**: 381-386.

White, T. J., Bruns, T., Lee, S. and Taylor, J. W. (1990) In: PCR Protocols: A Guide to Methods and Applications, Pp. 315-322. (eds. Innis, M. A., Gelfand, D. H., Sninsky, J. J. and T. J. White. Academic Press, Inc., New York

Woese, C. R. (1987) Microbiol. Rev. **52**: 221-271.

Wright, S. W. and Jeffrey, S. W. (1997) In Phytoplankton pigments in Oceanopgraphy: guidelines to modern methods (Eds. Jeffrey, S. W., Mantoura, R. F. C. and Wright, S. W.) pp. 327-341

Yap, I. V. and Nelson, R. J. (1996) WinBoot: A program for performing bootstrap analysis for binary data to determine the confidence limit of UPGMA based dendrograms. International Rice Research Institute discussion paper series number 14.

Zechman, F. W., Theriot, E. C., Zimmer, E. A. and Chapman R. L. (1990) J. Phycol. **26**: 700-710.

Zhou, Z. H., Miwa, M. and Hogetsu, T. (1999) New Phytol. **144**: 55-63.

Zietkiewicz, E., Rafalski, A. & Labuda, D. (1994) Genomics. **20**: 176-183.