APPLICATION OF MOLECULAR BIOLOGICAL TECHNIQUES TO THE IDENTIFICATION OF CYANOBACTERIA

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To my family

Declaration

I declare that while registed as a candidate for the University's research degree of Doctor of Philosophy, no material contained in this thesis has been used in any other submission for another academic award of Liverpool John Moores University or other academic or professional Institution.

Publications

Parts of this thesis have been published as follows:

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Abbreviations

A	adenine
Ap	ampicillin
bp	base pairs
C	cytosine
CCAP	Culture Collection Algae and Protozoa
DAF	DNA amplification fingerprinting
dATP	adenosine 5'-triphosphate
dCTP	cytosine 5'-triphosphate
dGTP	guanine 5'-triphosphate
DNA	deoxyribonucleic acid
dTTP	thymine 5'-triphosphate
dNTP	deoxynucleoside triphosphate
EDTA	ethylenediaminetetra-acetic acid
G	guanine
g	gram
G+C	guanine + cytosine
h	hour
ITS	internal transcribed spacer
Kb	Kilobase pairs
KDa	Kilodaltons
λ	lambda phage
LPP	*Lyngbya Plectonema Phormidium*
M	Molar
mA	milliamp
MD	megadalton
mg	milligram
min	minute

ml	millilitre
mm	millimeter
mM	milli-Molar
mRNA	messenger RNA
nm	nanometer
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PCC	Pasteur Culture Collection
PCR	polymerase chain reaction
RAPDs	Random Amplified Polymorphic DNAs
RFLP	restriction fragment length polymorphisim
RNA	ribonucleic acid
rRNA	ribosomal RNA
r.p.m	revolutions per minute
s	second
S	Svedberg unit - sedimentation coefficient
SD	standard deviation
SDS	sodium dodecylsulphate
STRR	short tandemly repeated repetitive
T	thymine
Tris	tris (hydroxymethyl) aminoethane
tRNA	transfer RNA
$tRNA^{ala}$	alanine tRNA
$tRNA^{ile}$	isoleucine tRNA
µg	microgram
µl	microlitre
µm	micrometer
v/v	volume for volume
w/v	weight for volume

Abstract

In order to identify cyanobacteria responsible for blooms in aquatic environments, methods are required that allow the rapid and reliable discrimination of specific cyanobacterial strains. Molecular methods have been applied to the identification and phylogenetic analysis of cyanobacteria.

A number of mini-methods for extraction of total genomic and plasmid DNA have been compared by using laboratory strains. The plasmid profile was not useful for establishing classification schemes because of limitations use in determining relationships between strains that contained plasmids. Polymerase chain reaction (PCR) based techniques involving random amplified polymorphic DNAs (RAPDs) and amplification of the 16S-23S ribosomal DNA (rDNA) spacer were optimised for selected cyanobacteria. RAPD profiles could be used to identify cyanobacteria to the strain level. Size heterogeneity and restriction fragment length polymorphisms (RFLPs) of the amplified rDNA spacer were useful in discriminating between different genera and species of cyanobacteria. Choice of primers proved critical to the use of spacer variation for species identification.

Variation was found between the rDNA spacer sequences of different genera and species. From sequence alignment two short (about 20 bp) high homology sequences were present, in addition to the transfer RNA genes of high sequence homology in the spacer region. Two primers designed from these high homology sequences were found to be cyano-specific and showed potential for use in the amplification of cyanobacterial DNA directly from environmental samples.

Cyanobacteria were isolated from water samples and identified, both on the basis of molecular characteristics (using the methods developed here) and morphological characteristics. Phylogenetic trees constructed on the basis of RAPDs, and of RFLPs and DNA sequences of the rDNA spacer separated the cyanobacteria into a number of clusters. The implications of these results for the phylogeny of the cyanobacteria are discussed.

Chapter 1

General Introduction

1.1 Cyanobacteria

The cyanobacteria are one of the largest sub-groups of Gram-negative prokaryotes. They are photosynthetic prokaryotes which possess the ability to synthesise chlorophyll *a* and at least one phycobilin pigment, with water acting as the electron donor during photosynthesis and leading to the release of oxygen. Cyanobacteria are by far the largest group of photosynthetic prokaryotes, as judged by their widespread occurrence, frequent abundance, and morphological diversity. They are not only currently represented in most types of illuminated environment, except for those at low pH values, but they have one of the longest geological records (Schopf and Walter, 1982). Their fossil record is fragmentary and biased towards the formations where preservation of the morphology was possible. The earliest unicellular and filamentous forms attributed to the cyanobacteria were found in sedimentary rocks formed from the early Precambrian period, over three billion years old. At that time, much of the Earth's original atmospheric oxygen was probably formed by organisms quite similar to modern cyanobacteria (Schopf and Walter 1982, 1983; Knoll, 1985) and they are still responsible for a considerable proportion of photosynthetic oxygen evolution in the oceans. Thus cyanobacteria were probably responsible for a major evolutionary transformation leading to the development of aerobic metabolism and to the subsequent rise of higher plant and animal forms.

Cyanobacteria occupy a wide range of illuminated niches in freshwater, marine, terrestrial and hypersaline environments, where they often occur in such abundance that they are readily visible by eye. The colour of these forms may vary from green, blue-green, or olive-green to various shades of red to purple, or even

black depending on pigments and light quality. In size the cyanobacteria range from sub-spherical cells, less than 1μm in diameter, to trichomes well over 100 μm in diameter. The cells of the latter are always much shorter than wide, but nevertheless cell volume ranges over more than five orders of magnitude. Some species are capable of differentiating several distinct types of cell, and forms which combine these various cell types with various patterns of branching are morphologically the most complex prokaryotes known (Fay, 1983; Whitton, 1992).

Cyanobacteria are of considerable importance in the natural environment as initial colonisers of arid land and as primary producers of organic matter (Fay, 1983). Perhaps of greater importance is their role in the fundamental process of biological nitrogen fixation. Many of the free-living cyanobacteria and those engaged in symbiotic associations with plants and animals contribute significantly to the nitrogen fertility of aquatic and terrestrial habits, including cultivated lands, particularly in the tropics. Their usually high content of protein makes them prospective candidates for new, unconventional sources of animal and human food. Indeed, in the central African state of Chad dried cakes of *Spirulina,* which grows abundantly in soda lakes, constitute an important part of the diet of local people (Fay,1983; Rippka, 1988). In the recent search for new sources of energy, some researchers consider cyanobacteria as the most promising agents for the development of a biological solar energy conversion system, based on the concurrent light-driven generation of elemental O_2 and H_2 through the action of cyanobacteria.

The increase in planktonic cyanobacterial populations has sometimes been dramatic and has brought with it considerable practical problems. Dense

populations present great difficulties to the water-supply industry, interfering with treatment processes and imparting tastes and odours to the water. The production of neurotoxins and hepatotoxins or the removal of oxygen by the respiration of living cells or by decomposition of dead cells can have serious consequences, causing human illness, death amongst fish, birds and occasionally cattle (Gibson and Smith, 1982; Carmichael, 1989, 1992). Cyanobacterial toxins that cause death in animals, birds and fish include the hepatotoxic heptapeptide microcystins and the pentapeptide nodularin, and neurotoxins including alkaloids and organophosphorus compounds (Lawton and Codd, 1991). Bathing in waters densely populated with *Aphanizomenon* can cause an unpleasant condition of the skin know as 'swimmers itch' (Schwimmer, 1964). Even without these unpleasant consequences, when the hitherto clear waters of lakes become clouded with plankton their aesthetic appeal is felt to diminish and the occurrence of surface scum (water-blooms) is still more objectionable.

1.2 Taxonomic classification

The first scientific descriptions of particular kinds of cyanobacteria did not appear until the early nineteenth century. The earliest description of the genus *Rivularia* was by Roth between 1797 and 1806, and Vaucher in 1803 described *Oscillatoria* and *Nostoc.* Vaucher placed these cyanobacteria in the animal kingdom, but nevertheless their subsequent study was undertaken mainly by botanists (Fogg, 1973). For about 150 years cyanobacteria were considered a special group of algae, the blue-green algae. According to the provisions of the International Code of Botanical Nomenclature, the name of a taxon above the family level is not subject to restrictions of priority. Consequently many names have been

proposed for this group of microorganisms, as listed in Table 1.1 (Fogg, 1973; Rippka,1988).

Table 1.1 Major names of cyanobacteria according to the International Code of Botanical Nomenclature.*

Name	Year	Authors
Myxophyceae	1833	Wallroth
	1860	Stitzenberger
		Rabenhorst
Phycochromophyceae	1863	Rabenhorst
Cyanophyceae	1874	Sachs
Schizophyceae	1879	Cohn
Cyanophyta	1914	Pascher
	1938	Smith

* Taken from Fogg 1973, Rippka 1988

The name Cyanophyceae has survived the longest, and Cyanophyceae and Cyanophyta are still widely used by phycologists and botanists today. Bacteriologists believe that the name "Cyanobacteria", first proposed by Stanier (1978), is more appropriate than "Cyanophyceae", in view of the prokaryotic cellular properties of these phototrophs. Many names have been in circulation including Myxophyceae, Myxophyta, Cyanophyceae, Blue-green algae, Cyanophyta and Cyanobacteria. The term cyanobacteria will be used throughout this report.

The cyanobacteria have traditionally been treated as algae. They could be defined by broad morphological, cytological and less ecophysiological characters and separated into distinct division, classes, orders, and lower categories on the basis of morphological distinctions (Geitler, 1932; Anagnostidis and Komarek, 1985; Hoffmann, 1988). The complexity of simple multicellular and branched filaments and even multiseriate and thalliform "macrophytes" among the cyanobacteria would also lead most casual observers to the conclusion that these were algae, not bacteria, that were studied by botanists and phycologists. In nature, the cyanobacteria usually behave like algae. They possess chlorophyll a and phycobilins and perform oxygenic photosynthesis that is extremely similar to that of the red algae (division *Rhodophyta*). The morphological characteristics of the cyanobacteria and their ecological niches were so similar to those of many micro-algae that few investigators doubted their place as the base or a branch of a true algae phylogenus (Fritsch, 1945). As early as 1853, Cohn indicated that these organisms were more related to bacteria and placed them in the kingdom prokaryota, division *Schizophyta* (cited in Castenholz and Waterbury, 1989). From early nineteenth century to the middle of the present century, cytological data was limited to impressions from light microscopy and only relatively crude chemical data was available. There were reasons to connect the blue-green algae with bacteria. However the evidence was not overwhelming, until electron microscopy and biochemical analyses showed convincingly that blue-green algae were unique and lacked the traditional nucleus and chloroplasts of the green and other algae. Recent nucleotide base sequence data from 16S and 5S rRNA place cyanobacteria within the Bacteria (Eubacteria), a group or domain distinct and apart from the Archaea (Archaeobacteria) and Eucarya (Eucaryotes) (Woese, 1987, 1994). For this

reason they should now be given extended treatment in the Bergey's Manual of Systematic Bacteriology. There are, however, considerable obstacles in the way of describing this group in the same manner as most other prokaryotes.

Cyanobacterial taxonomy has been approached in different ways, all with one major goal: defining those phenotypic properties that are taxonomically useful. Because the phenotype is the product of genetic and environmental factors, the stability of certain characteristics is a function of the properties of the environment. The development of an effective taxonomic system for cyanobacteria imposes problems for which there is no exact parallel with any other group of organisms since they have been treated under both the International Code of Botanical Nomenclature (as Blue-green algae) and the International Code of Bacteriological Nomenclature (as Cyanobacteria).

The building of the current morphology-based taxonomy of blue-green algae began in earnest with Bornet, Flahault and Gomont late last century (cited in Whitton, 1992). Under the International Code of Botanical Nomenclature, the starting point for the valid publication of names of heterocystous blue-green algae (family *Nostocaceae)* is with Bornet and Flahault, and that for the non-heterocystous, filamentous forms (family *Oscillatoriaceae*) is with Gomont (cited in Castenholz and Waterbury, 1989). The gathering of genera and species was continued, culminating with Geitler (1932), who produced a comprehensive treatise which recognized approximately 1300 species, 145 genera, 20 families, and 3 orders. This treatise, although regional in intent, in practice is worldwide, and gained the most widespread popularity among phycologists and bacteriologists. However, freshwater forms are more completely treated than marine forms, because

few marine forms had been studied. Other major gatherings of "blue-green" species on a regional basis are those by Tilden (1910), Desikachary (1959) and Starmach (1966), who reorganized the freshwater blue-greens at the familial and generic level. All followed a classification similar to that of Geitler (1932); this has become known as the "Geitlerian" system. In recent years, most "blue-green" specialists and nonspecialists have followed this organization. However, beginning with Drouet and Daily (1956), a major revision and consolidation of "blue-green" names was initiated (Drouet, 1968, 1973, 1978, 1981). The "Drouet" system, although gradually modified during the course of his work, resulted in an enormous reduction in the profusion of recognized generic and species names. Over 2000 species in over 140 genera were eventually reduced to 62 species in 24 genera. This system was not accepted by most "blue-green" taxonomists. However, several "blue-green" biochemists and physiologists have used the Drouet system because of its simplicity and the relative ease with which a binomial could be allocated. A series of papers provides detailed accounts of the *Chroococcales* (Komárek and Anagnostidis, 1986), *Oscillatoriales* (Anagnostidis and Komárek, 1988), *Nostocales* (Komárek and Anagnostidis, 1989), and *Stigonematales* (Anagnostidis and Komárek, 1990). The generic limits adopted tend to be quite narrow, leading to smaller and more homogeneous genera than in most other botanical floras. These papers provide the most detailed guides available to the morphological and ecological literature, considerable new insight into morphology, and accounts not only of well-known forms, but ones which are little known, though often widespread.

These and other more recent morphology-based systems have shown many improvements with time, but suffer to varying degrees from several problems. A large number of species and genera had been described over the years on the basis

of a single difference, such as trichome width, presence or nature of sheath, number of trichomes per sheath, colour, and other features which later have been shown to have a great flexibility within clonal populations of some species, particularly when environmental conditions vary (Pearson and Kingsbury, 1966; Evans *et al.,* 1976; Doers and Parker, 1988). However, some cyanobacterial clones in culture are quite conservative morphologically (Stam, 1978; Stulp, 1982; Stulp and Stam, 1982). Drouet (1981) presumed that relatively few cyanobacterial genotypes existed but that there were many phenotypes expressed in different environments (ecophenes). His decisions, however, were based mainly on his appreciation of the group through the microscopic examination of an extremely large number of collected specimens. No matter how much material is examined, decisions of this type are, of necessity, more arbitrary and less likely to reflect true relationship than decisions based on manipulations of clonal strains in culture. No clonal cultures were established by Drouet (1981); no physiological, biochemical or genetic information was included. The incorrectness of several decisions by Drouet (1981) has now been established by various means, including DNA/DNA hybridisation and DNA base composition comparisons, by Stam and associates (Stam and Holleman, 1975, 1979; Stam and Venema, 1977; Stam, 1980).

A second taxonomic approach was introduced by Stanier and his associates in the early 1970s (Stanier, 1970; cited in Rippka, 1988; cited in Whitton, 1992). This involved the long-term strategy of dealing with cyanobacteria in a similar way to other major groups of prokaryotes. The system is based on axenic, clonal cultures and the use of characters from a range of disciplines, rather than morphology alone; characters employed included: pigment composition, fatty acid analysis, chemoheterotrophic and photoheterotrophic capability and substrate

sepecificity, nitrogenase activity, DNA base composition, and genome length (Kenyon, 1972; Herdman, 1979a,b; Rippka, 1979). Because the cyanobacteria are photoautotrophic organisms, physiological studies did not furnish many useful taxonomic characters. The basis of the bacteriological taxonomy of the cyanobacteria was published by Rippka *et al.* (1979). This taxonomic system, which still relies largely on morphology, allows the identification of the strains of the Pasteur Culture Collection at the generic level. The five major sections recognised by Rippka (1979) coincide broadly with orders of other classifications, as illustrated in Table 1.2. A modified version of this system is given by Castenholz, Waterbury and Rippka (1989). This treatment is more global and includes taxonomic information on well-known taxa which are not in the Pasteur Culture Collection, as well as ecological features.

The morphological and cytological data used by Stanier's group to distinguish genera were based on characteristics observable with light microscopy, except in the case of the presence or absence of thylakoids for which electron microscopy is required. The main characteristics include: the type of cellular division and the plane of subsequent divisions; the formation of baeocytes ("endospores" of the order blue-green algae terminology); the formation and structure of hormogonia; the presence and type of sheath or glycocalyx; the shape and dimensions of cells and the presence of constrictions between adjacent cells of trichomes; the nature of trichomes (helical or straight, unbranched, false branched or true branched, tapered or not); the presence and location of heterocysts and resting spores (akinetes); and the presence and location of gas vacuoles.

Table 1.2 Major diagnostic features of cyanobacteria in the Sections described by Rippka (1988), with the broadly equivalent orders following the botanical taxonomic system of Geitler (1925, 1932), the revision by Anagnostidis and Komarek (1986, 1988, 1989, 1990), and Bergey's Manual of Systematic Bacteriology (1989)

Unicellular; cells single or forming colonial aggregates held together by additional outer cell wall layers	Reproduction by binary fission or by budding		Section I of Rippka *et al.* (1988) Orders *Chroococcales* + *Chamaesiphonales* sensu Geitler (1932) Orders *Chroococcales* sensu Waterbury and Rippka (1989)
Order *Chroococcales* sensu Komarek and Anagnostidis (1986)	Reproduction by multiple fission giving rise to small daughter cells (baeocytes), or by both multiple and binary fission		Section II of Rippka *et al.* (1988) Order *Pleurocapsales* sensu Geitler (1925), Waterbury (1989)
Filamentous; cells forming a multicellular unit, the trichome	Trichome always composed only of vegetative cells; reproduction only by intercalary division, followed by random trichome breakage	Division in only one plane.	Section III of Rippka *et al.* (1988) Order *Oscillatoriales* sensu Geitler (1932), Anagnostidis and Komarek (1988), Castenholz (1989)
	In the absence of combined nitrogen trichome contains both vegetative cells and heterocysts; reproduction occurs by intercalary binary fission and random trichome breakage; some members also reproduce by formation of hormogonia or by germination of akinetes	Division in only one plane	Section IV of Rippka *et al.* (1988) Order *Nostocales* sensu Geitler (1925), Komarek and Anagnostidis (1989), Castenholz (1989)
		Division in more than one plane	Section V of Rippka *et al.* (1988) Order *Stigonematales* sensu Geitler (1925), Anagnostidis and Komarek (1990) Castenholz (1989)

In view of the fact that the cyanobacteria have been researched by supporters of the "bacteriological" and "botanical" systems of classification, the problems for taxonomists are now largely practical ones. These two systems continue to use largely morphological criteria in description and distinction between genera. The main problem is the great array of existing genera and species that have been described solely on the basis of morphological characteristics obtained from field-collected samples, and the difficulty of relating these to living populations growing as clones in axenic culture. Some previous researchers already suspected that morphology may change depending on environmental conditions and the diversity of strains within a culture may be suppressed because of selective culturing conditions and period (Pearson and Kingsbury, 1966; Evans *et al,* 1976; Doers and Parker, 1988). Various approaches have been adopted to deal with these problems. There is a need for an extensive body of standardised information on field populations and laboratory strains, including morphological, cytological, ultra-structural, physiological and biochemical characters, which can be used for assessing similarity. The methodology employed in the bacteriological approach to cyanobacterial studies, however, opened the possibility to study cyanobacterial genotypes more directly. The new molecular genetics methodology has been successfully applied to cyanobacterial phylogenetic and taxonomic studies. Molecular methods such as DNA base composition (Herdman *et al.* 1979a), genome length (Herdman *et al.*, 1979b), DNA-DNA hybridisation (Stam and Venema, 1977; Stam, 1980; Stam and Stulp, 1988) and restriction fragment length polymorphisms (RFLP) (Douglas and Carr, 1988; Wood and Townsend, 1990) have been applied to problems at lower taxonomic levels. The technique of partial 16S rRNA sequencing (Lane *et al*, 1985; Wilmotte, 1994), RFLP-PCR portions of rRNA genes (Vaneechoutte *et al.,* 1992; Ward and Akrofi, 1994), and randomly amplified

polymorphic DNA (RAPD) markers (Williams *et al.*, 1990; Eskew *et al.*, 1993; Neilan, 1995) have also been applied to problems. These new applications provide the opportunity for a combined approach, using morphological criteria as a guide for molecular applications, in order to calibrate the molecular probes and optimise the selection of materials for study.

1.3 Molecular techniques used in taxonomy

The technology of genetic engineering has now become well established within the biological sciences. Molecular biology has provided new tools to decipher genetic information and can be used in attempts to reconstruct the evolution of organisms and determine their taxonomy. In the cyanobacteria, the use of molecular methods to study the genotypic relationships is underway, and initial results are promising (Wilmotte and Stam, 1984; Golden, *et al* 1989; Eskew, *et al.* 1993 and Wilmotte, 1994).

In its broadest sense the study of cyanobacterial taxonomy by molecular methods should include chemotaxonomic markers. Rapid methods for chemotaxonomy are generally available. However, data are relatively scarce and several studies are still preliminary (Wilmotte, 1994). Problems of consistency and variations, due to factors such as growth conditions, have not always been systematically investigated (Holton, 1981). Moreover, since the molecules employed are typically synthesised through complex pathways, their presence or absence can have different causes. The chemotaxonomic and macromolecular markers presented in Table 1.3 (concluded from Wilmotte, 1994) have been shown to be useful. However, genetic information obtained by using nucleic acid

Table 1.3 The major chemotaxonomic and macromolecular markers of cyanobacteria

Reference	Marker	Comments
Kenyon *et al.* (1972)	Lipid composition	A uniform fatty acid composition of 66 cyanobacterial strains was observed for the groups *Anabaena* and *Calothrix*
Sallar *et al.* (1990)	Lipid composition	A highly polar unknown glycolipid present only in the three heterocystous strains studied and alcohol glycosides are not restricted to nitrogen-fixing strains.
Merritt *et al.* (1991)	Lipid composition	Three marine, picoplanktonic *Synechococcuss* strains had a similar fatty acid composition to freshwater strain *Synechocystis sp.* strain PCC 6308
Caudales *et al.* (1992)	Lipid composition	The fatty acid compositions from free-living strains of the genera *Nostoc* and *Anabaena* and from symbionts of the water fern *Azolla* sp.
Hegewald and Knefal (1983)	Polyamines	Spermidine and sym-homospermidine were present in different strains assigned to the genera *Oscillatoria, Phormidium, Calothrix* and *Chroococcus* (Botanical taxonomy)
Hamana *et al.* (1983)	Polyamines	Higher concentrations of sym-homospermidine were present in nitrogen-fixing strains
Hamana and Matsuzaki (1992)	Polyamines	Polyamines as a chemotaxonomic marker in bacterial systematics
Hall (1982)	Aromatic amino acids	Biochemical diversity for biosynthesis of aromatic amino acids among the cyanobacteria
Healey (1968)	Carotenoids	Similar carotenoid patterns were observed for *Phormidium ectocarpi* strain PCC 7375 and *Phormidium persicinum* strain CCAP 1462/5

Table 1.3 contd

Reference	Marker	Comments
Hertzberg *et al.* (1971)	Carotenoids	Variations of carotenoid patterns were probably useful for species identification.
Aakermann *et al.* (1992)	Carotenoids	The carotenoid content and composition was different in red or green isolates of the same species.
Klein *et al.* (1973)	Isozyme patterns	Shared α-esterase isozyme bands allowed the recognition of four clusters of related strains among the thirteen *Oscillatoriaceae* strains tested.
Schenk *et al.* (1973)	Malate-dehydrogenase patterns	Malate-dehydrogenase electrophoretic patterns were used to characterize eight cyanobacterial strains and shared bands were observed within the genera *Anabaena* and *Nostoc*, but *Synechococcus* sp. strain PCC 6301 and *Synechococcus elongatus* strain CCAP 1497/1, had no bands in common.
Stulp and Stam (1984)	Enzyme patterns	The electrophoretic patterns for five enzymes in axenic *Anabaena* sp. strains showed intra- and interspecific heterogeneity of enzymes in the strains tested.
Zimmerman and Rosen (1992)	Enzyme patterns	Zymograms for six enzymes were identical for twelve cyanobacterial symbionts from cycad
Philip *et al.* (1985)	Restriction randonucleases	Three site-specific endonucleases, *Afl*I, *Afl*II and *Afl*III have been partially purified from the cyanobacterium *Anabaena flos-aquae* CCAP 1403/13f. *Afl*II and *Afl*III are new specificities.
Schenk and Kuhfitting (1983)	Phycobiliprotein	Phycobiliprotein patterns in twenty-one cyanobacterial species were studied. A conspicuous heterogeneity was observed, even between strains assigned to the same species.
Guglielmi and Cohen-Bazire (1984)	Phycobiliprotein	Electrophoretic patterns of the phycobiliprotein subunits in *Pseudanabaena* were determined.

Table 1.3 contd

Reference	Marker	Comments
Bryant (1982)	Phycobiliprotein	Examined the entire Pasteur Culture Collection for strains capable of phycoerythrocyanin synthesis.
Ladha and Watanabe (1982)	Immunological proteins	Observed a high degree of antigenic similarity among cyanobacterial symbionts from different species of *Azolla*, but no crossreaction between the symbiots and free-living cultures.
Zilinskas and Howell (1987)	Immunological proteins	The antigenic determinants of two rod linkers polypeptide were very conserved in nine strains belonging to sections I, III, IV and V, whereas for a third polypeptide, only strains of section IV cross-reacted.
Bullerjahn *et al.* (1990)	Immunological proteins	The chlorophyll a/b-binding protein from *Prochlorothrix hollandica* was very similar to its counterpart in *Prochloron* sp., but not to light-harvesting chlorophyll a/b proteins of maize.

hybridisation, Polymerase Chain Reaction (PCR) - based techniques and rRNA or rDNA sequencing may provide the better tools to infer phylogenetic relationships. The genetic makers presented below have been shown to be worthy of further study.

DNA base composition is one of the few molecular characters that has been determined for almost 200 cyanobacterial strains (Herdman *et al.*, 1979a). It is however, a character marker. Large differences in DNA base composition indicate that the strains are not closely related, whereas similar Guanine + Cytosine (G+C) percentages give no clue concerning genotypic relationships (Stam and Venema, 1977; Wilmotte, 1994).

The method of DNA/DNA hybridisation allows the determination of sequence similarity between genomes of different organisms without the determination of exact nucleotide sequences. Generally DNA/DNA hybridisation work well for genomic comparisons at the species level (Stam and Venema, 1977; Stam, 1980; Stam and Stulp, 1988). It is based on the feature that denatured, single-stranded DNA will renature under suitable conditions. The extent of renaturation is proportional to the complementarity of the DNA strands involved. Furthermore, the stability of the renatured hybrid molecule is proportional to the accuracy of matching between the two strands. The thermal stability of a radioactively-labelled DNA hybrid can be established by cumulative plotting of the radioactivity eluted at each temperature against the elution temperature. The temperature at which 50% of the final eluted radioactivity is eluted is the thermal elution midpoint (Tm) for a hybrid. The difference between the Tm values of a homoduplex and a heteroduplex yields the ΔTm. Low ΔTm values indicate little mismatch in the heteroduplex, high ΔTm values indicate much mismatch. For higher taxa the renaturation level rapidly

drops down to background levels; for lower taxons the method is insufficiently sensitive. The method has become particularly popular for genetic studies of prokaryotes, owing to the rather uncomplicated nature of prokaryotic genomes and the subsequent straight forwardness of the method (Stam and Venema, 1977; Stam, 1980; Stam and Stulp, 1988; Wilmotte, 1994). Several cyanobacterial genera have been studied by the use of this technique. DNA/DNA hybridisation results showing high relative hybridisation rates and low ΔTm values, indicate that the cyanobacterial strains involved are genotypically closely related.

Wayne and colleagues (1987) proposed the use of DNA/DNA hybridisation as a criterion for the definition of eubacterial species. The phylogenetic definition of a species generally would include strains with approximately 70% or greater DNA-DNA relatedness and with 5 °C or less ΔTm. The use of this method for cyanobacteria was pioneered by Stam (see Stam and Venema, 1977; Stam, 1980), who used a filter hybridisation technique. The genotypic relationships among twenty-nine filamentous strains which belonged to the LPP (*Lyngbya, Phormidium* and *Plectonema*) group (Rippka *et al.*, 1979) were determined. The results showed the genotypic homogeneity of a group of eighteen strains centred around the freshwater strain *Plectonema boryanum*. Other freshwater and marine LPP strains showed conspicuous genotypic differences. Gugielmi and Cohen-Bazire (1984) used DNA/DNA hybridisations to elucidate the relationships of nine strains belonging to the genus *Pseudanabaena* and the LPP group (Rippka *et al.* 1979).

Lachance (1981) investigated by DNA/DNA hybridisation the genotypic relationships among 45 strains of heterocyst-forming cyanobacteria assigned to eight genera by Rippka *et al.* (1979). The genera *Nodularia, Cylindrospermum,*

Chlorogloeopsis and *Fischerella* appeared to form tight genotypic groupings. On the other hand, the genera *Anabaena, Nostoc* and *Calothrix* contained strains or clusters of strains which showed little genotypic similarity. Four strains previously assigned to *Anabaena* appeared to belong to *Nostoc.* Stulp and Stam (1984) performed DNA/DNA hybridisation studies with twenty-one *Anabaena* sp. strains. Strains of the same species showed very high hybridisation percentages (about 100%). On the other hand, strains from different species showed intermediate hybridisation values (below 60%).

Wilmotte and Stam (1984) demonstrated by DNA/DNA hybridisation that *Synechococcus* PCC 7502 and PCC 6307 probably belong to mutually different genera. Strain 6312 is a member of the same genus as strain 6301 and two *Anacystis nidulans* strains PCC 7942 and PCC 7943 show high RB (relative binding) values and low ΔTm values. This suggested that these strains belong to the same species as *Synechococcus* sp. strain PCC 6301. The result was later confirmed by restriction fragment length polymorphism (RFLP) analysis of the genomes of strains PCC 6301 and PCC 7942. The genomes of the two strains seemed identical, except for a rearrangement (Golden *et al.*, 1989; Wood and Townsend, 1990).

DNA/DNA reassociation studies have also been used to investigate the genotypic diversity of *Prochloron* sp. isolates from different didemnid species and locations. The fourteen *Prochloron* sp. isolates appeared to belong to a single species (Stam *et al.*, 1985; Holton *et al.*, 1990).

The RFLP technique for studying genomes is generally useful for identification and classification of organisms at the population or species level

(Douglas and Carr, 1988; Wilmotte, 1994). By judicious choice of the restriction endonucleases used to cleave the DNA in a reproducible, site-specific manner, and of the hybridisation probes used to identify specific genes in the fragmented DNA, it is possible to detect fine structure differences in the genomes of organisms, differences which may pass undetected in bulk DNA-DNA hybridisation studies. This method has been productively used to study genomes of marine *Synechococcus* sp. strains and cyanobacterial symbionts.

The taxonomy of small, planktonic, phycoerythrin-containing *Synechococcus* sp. strains is problematic. Wood and Townsend (1990) tested the genetic homogeneity of a group of eight strains by using RFLP that showed cross-reactivity to the antiserum directed against *Synechococcus* sp. strain WH 7803. In the tree topology obtained, the eight strains were distributed into four different branches. Moreover, the genetic distances between terminal taxa on different branches were as large within the serogroup as the distances found between members of the serogroup and two freshwater *Synechococcus* sp. strains that showed no cross-reactivity to the antiserum. This conclusion was confirmed by an analysis of the previously published results of Douglas and Carr (1988), no marine *Synechococcus* isolate showed any similarity to the freshwater *Synechococcus* strain PCC 6301.

A filamentous, nitrogen-fixing symbiont lives in the leaf cavities of the water fern *Azolla* sp. and is generally identified as *Anabaena azollae*, although it is not clear whether one or more taxa are involved. The symbiont and its host are associated during all the life cycle. In total, the symbioses involving seven *Azolla* sp., belonging to the Sections Euazolla and Rhizosperma, have been studied. The probes were derived from heterologous genes (e.g. *nif*K, *nif*D, *nif*H, *nif*DH, 1.8kb

*pAn*207.3) from *Anabaena* sp. strain PCC 7120 (Franche and Cohen-Bazire, 1987; Meeks *et al.*, 1988) or from genomic and plasmid DNA clones of freshly isolated symbionts (Plazinski *et al.*, 1990, 1991). The results have shown that (i) the leaf cavities of *Azolla* sp. probably harboured one major symbiont, accompanied by minor species; (ii) there is a good correlation between the genotypic relationships among the freshly isolated symbionts and the classification of their hosts. The division into the Sections Euazolla and Rhizosperma was confirmed, except for the symbiont from *Azolla nilotica* which showed genotypic differences with the symbionts from both Sections; (iii) Only the minor constituents of the symbiosis could be established as free-living cultures.

Plazinski and colleagues (1990) found two DNA probes (pAm3 and pAc51) of which were in fact derived from plasmid sequences (Plazinski *et al.*, 1991), are strain-specific and hybridised only to *A. azollae* strains isolated from *Azolla microphylla* or *Azolla caroliniana*. In addition, three probes (pAc34, pAc50, and pAc60) could discriminate among symbionts from the same *Azolla* sp. collected in different geographical areas. The free-living *Anabaena* sp. strain PCC 7120 shared little similarity with the studied symbionts, but *Nostoc cycas* strain PCC 7422 seemed closely related to them.

The taxonomic affiliation of the symbionts to the genera *Anabaena* and *Nostoc* was also investigated. Using as taxonomic marker the presence of two conserved restriction enzyme sites in the nitrogen-fixing (*nif*) genes of four *Nostoc* sp. strains and their absence in *Anabaena* sp. strain PCC 7120 and *Anabaena variabilis* strain ATCC 29413 (PCC 7937), Meeks and colleagues(1988) proposed that the symbionts belonged to the genus *Nostoc*, rather than *Anabaena* sensu

Rippka *et al.* (1979). However, the delimitation of the two genera was not clear and the two *Anabaena* strains used as reference by the authors were later assigned to *Nostoc* (Caudales and Wells, 1992). Thus, the data from Meeks *et al.* (1988) only served to illustrate the genotypic variability existing within the genus *Nostoc,* and did not allow classification of the symbionts.

Three short tandemly repeated repetitive (STRR) sequences from the genome of heterocystous cyanobacteria *Calothrix* sp. strain PCC 7601 have been used to distinguish between cyanobacterial strains. Mazel and colleagues (1990) suggested that the banding patterns could be used to distinguish strains at the species and genus level. The three different STRR sequences were found as repetitive genomic DNA components specific to the heterocystous strains tested, and the hybridisation patterns also indicated that the strain '*Anabaena*' PCC 7120 belonged to the genus *Nostoc* on the basis of hybridisation patterns with highly repetitive DNA sequences (Mazel *et al.*, 1990). It is noteworthy that repetitive elements have also been used to generate species- and strain-specific fingerprints in eubacteria (Versalovic *et al.*, 1991). The taxonomic value of two insertion elements (IS*701* and IS*702*) from *Calothrix* sp. strain PCC 7601 was investigated. The elements appeared to be restricted to the identification of identical strains (Mazel *et al.*, 1991).

Genetic analysis using random amplified polymorphic DNA markers (RAPDs) described by Williams *et al.* (1990), has permitted genetic mapping and diagnostics, and taxonomic studies. The RAPD technique is based on random DNA amplification by PCR with single, short primers of arbitrary nucleotide sequence. The assay is non-radioactive, requires only nanogram quantities of DNA, and is applicable to a broad range of species. Using genomic DNA, a set of DNA

fragments is generated that can be resolved by electrophoresis to form a DNA banding pattern (DNA fingerprint). In studies with cyanobacteria, Eskew and colleagues (1993) used this method to study the cyanobacterial symbionts of *Azolla ferna*. DNA fingerprints (DAF) were generated that were unique to the symbionts from three different *Azolla* sp. and that showed the maternal transmission of one symbiont. The method is very promising for strain identification. Neilan (1995) demonstrated by using multiplex (more than one primer per PCR-amplification) randomly amplified polymorphic DNA markers (MRAPDs) that there is a clear distinction between the genera *Anabaena* and *Microcystis*. It was possible to discriminate among all toxigenic cyanobacteria studied to three taxonomic levels of genus, species and strain using MRAPD markers.

PCR technology has also been used in several recent studies as a rapid means of amplifying the 16S-23S spacer regions of ribosomal DNA (rDNA) for the specific purpose of detecting heterogeneity between and within species (Barry *et al.*, 1991; Jensen *et al.*, 1993). The bacterial rRNA operon is transcribed into one pre-rRNA transcript that contains the following components in the order (5' to 3'): 16S, spacer, tRNA, spacer, 23S, spacer and 5S rRNA sequences (Watson *et al.*,1987; see Fig 1.1). Variability in both the number and the length of the spacers raises the possibility that this methodology has broad applicability as a rapid, possibly automatable, general method for bacterial identification and typing (Gurtler and Stanisich, 1996).

For a rapid taxonomic survey, PCR-amplified portions of rRNA genes may be digested by restriction enzymes. This kind of RFLP analysis has been used with success for eubacterial taxonomy (Vaneechoutte *et al.*, 1992). Ward and Akrofi

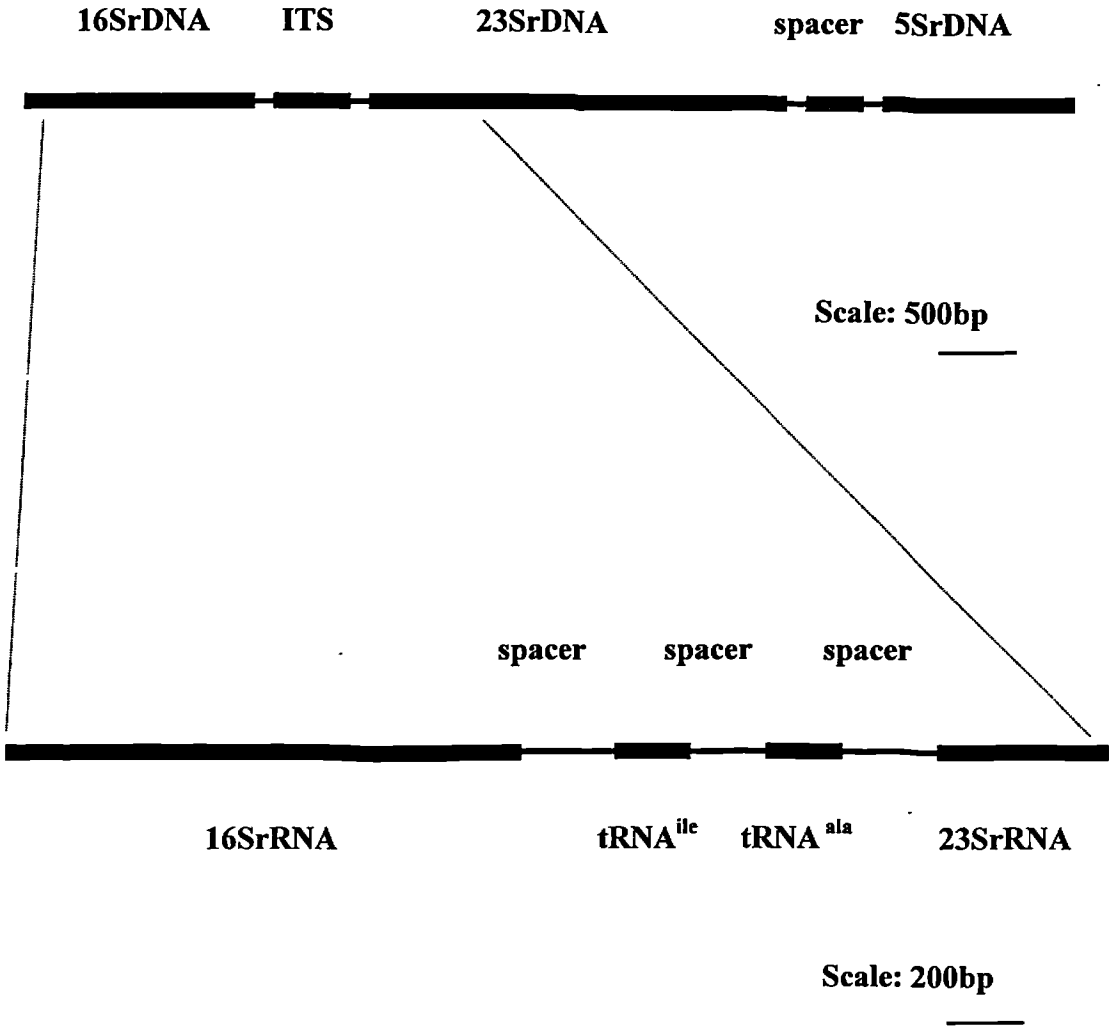


Figure 1.1. Schematic diagram of ribosomal DNA repeat showing internal transcribed spacer region (ITS). Transcription of the operon is from left to right. Some cyanobacteria have two tRNA genes as shown; others just have $tRNA^{ile}$.

(1994) used this method to identify the fungi in the *Gaeumannomyces-Phialophora* complex. In studies with cyanobacteria, Neilan and colleagues (1995) used RFLP analysis of the PCR-amplified phycocyanin locus to study toxigenic cyanobacteria. The assay was appropriate for the rapid identification of strains in freshwater samples and clearly discriminated among the eight genera (40 strains) tested. Bolch and co-workers (1996) investigated the genetic characterisation of the β- and α-phycocyanin subunits (*cpc* BA) intergenic spacer and flanking regions among 19 strains of three morphospecies of cyanobacteria by using PCR-RFLP. The presence of genetic variation between strains within these three divergent morphospecies, which span two orders of cyanobacteria (Chroococcales and Nostocales), may provide a versatile tool for discriminating cyanobacteria at a variety of taxonomic levels.

Information on the relatedness of phylogenetically more distant taxa can also be obtained by nucleotide sequencing of RNA and DNA. rRNA genes are at present the most useful molecular chronometers. They show a high degree of functional constancy, which assures relatively good clock-like behaviour (Woese,1982). They occur in all organisms, and different positions in their sequences change at very different rates, allowing most phylogenetic relationships (including the most distant) to be measured, which makes their range all-encompassing. Their sizes are large and they consist of many domains. There are about 50 helical stalks in the 16S rRNA secondary structure and roughly twice that number in the 23S rRNA (Noller, 1984; Gutell *et al.*, 1985), which makes them accurate chronometers on two counts. rRNAs can also be sequenced directly (Lane *et al.*, 1985). A technique of partial 16S rRNA sequencing was devised (Lane *et al*,1985), and applied to more than forty cyanobacterial strains (Tomioka and Sugiura, 1983; Giovannoni *et al.*, 1988, 1990;

Turner *et al.*, 1989; Britschgi and Giovannon, 1991; Ligon *et al.*, 1991; Weller *et al.*, 1991, 1992; Nelissen *et al.*, 1992; Urbach *et al.*, 1992; Wilmotte *et al.*, 1992,1993; Neilan, 1994).

The rRNA gene sequence analyses and the phylogenetic reconstructions based on these analyses have proved useful in assessing a wide range of phylogenetic relationships including those between higher taxonomic categories. The 16S rRNA has been shown to be an adequate macromolecule to study the evolution of the eubacteria. According to Woese (1987), the 16S rRNA is a good 'molecular chronometer' measuring the overall rate of evolutionary change in a line of descent. In studies with cyanobacteria, more than 50 strains have been studied and grouped into eight branches (Wilmotte, 1994). On the other hand, 16S rRNA gene sequences are probably too conserved to investigate intraspecies variability (Ward, *et al.*, 1992). The sequence of the internal transcribed spacer (ITS), situated between the 16S rRNA and the 23S rRNA gene (Fig. 1.1), may be an adequate marker. Species-specific probes inferred from the ITS sequence have been used for eubacterial identification (e.g Rossau *et al.*,1992).

1.4 Aims

The cyanobacteria are a diverse and widespread group of micro-organisms that are responsible for substantial productivity in both open ocean and freshwater courses (Fogg *et al.*, 1973). Problems with toxic blooms in British freshwater lakes (Carmichael, 1989) have given increasing impetus to studying the organisms involved. Rapid and reliable methods for identification of cyanobacteria are a necessary prerequisite for such studies. This project proposes to develop rapid

molecular technology for detection and identification of cyanobacteria. Rapid molecular methods based on plasmid typing, RAPD-PCR, PCR-amplification and RFLP analysis of the rDNA spacer, and rDNA sequencing will be developed and evaluated. Free living cyanobacteria will be isolated from water courses in Merseyside, Wales, Cumbria and China where cyanobacterial growth has been a problem in recent years. The organisms will be grouped by classical taxonomic procedures, such as those described by Rippka *et al.* (1979) and will be identified by the molecular techniques developed here. Comparisons will be made of molecular and traditional methods for identifying and classifying cyanobacteria.

The aims of this work are:

1. The use of morphological criteria for identification of free-living strains, which includes the isolation of other new organisms from local water courses and the determination of their status by morphological taxonomic methods.

2. To develop and select methods for recovery and characterisation of plasmids and total genomic DNA from cyanobacteria.

3. To develop and optimise rapid PCR-based methods: RAPD-PCR, RFLP-PCR and rDNA sequence to identify and classify different cyanobacteria.

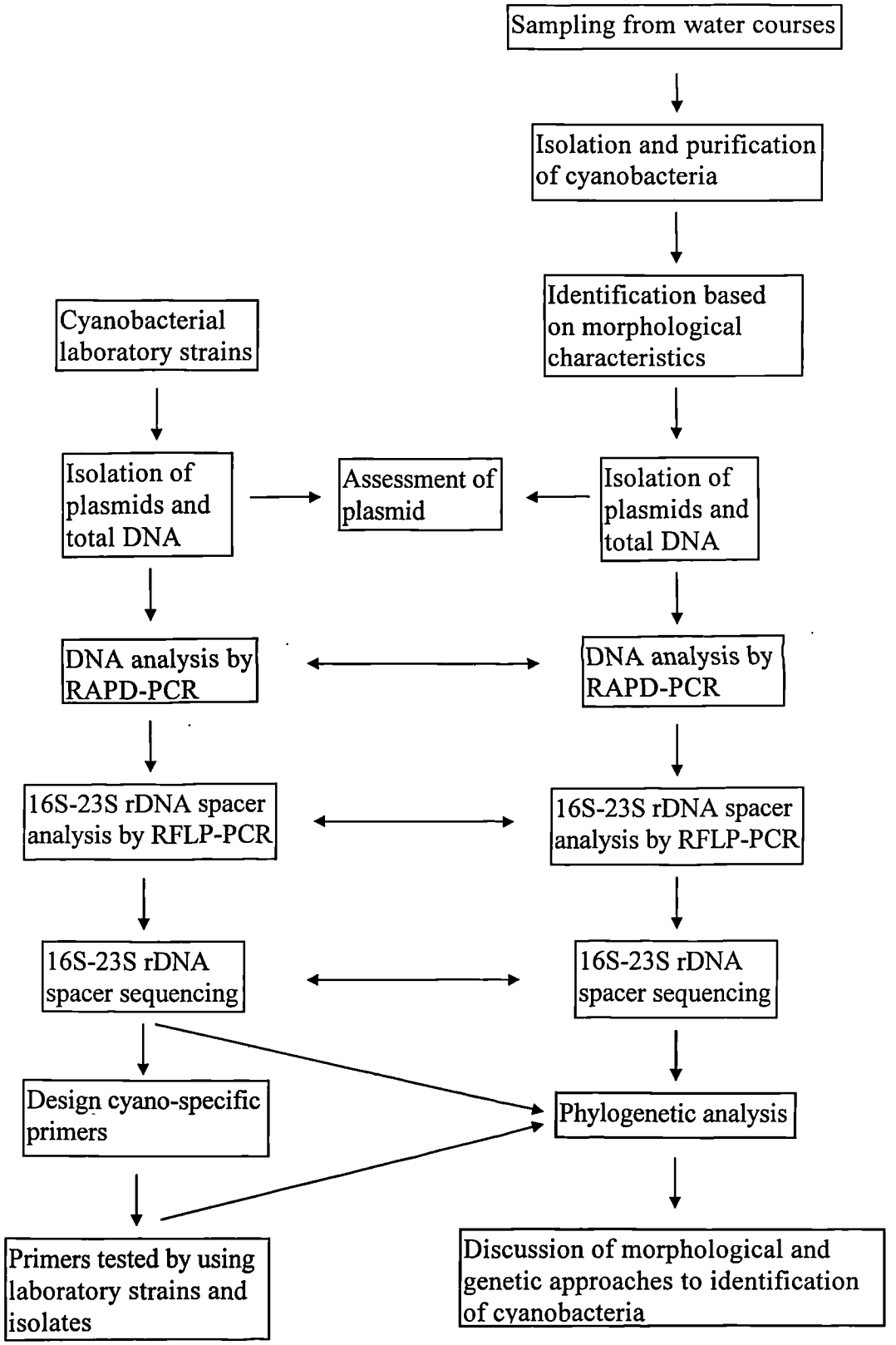
4. To store and catalogue data from RAPDs, RFLP-PCR and rDNA sequencing and to analyse by using appropriate computer software. Dendograms will be constructed to indicate relationships among the laboratory strains and cyanobacterial isolates.

5. To compare molecular and traditional methods for identifying cyanobacteria and consider taxonomic implications.

6. To assess the possibility of designing cyano-specific PCR primer/probes derived from aligned 16S-23S rDNA spacer region sequences with a view to direct identification of cyanobacteria from water samples.

A flow diagram summarising the schedule of work is shown in Fig. 1.2

Figure 1.2. Flow diagram summarising the schedule of work:



Chapter 2

Materials and Methods

2.1 Materials

2.1.1. Chemicals

Unless otherwise stated, the chemicals used were obtained from either British Drug Houses Chemicals Ltd. (BDH, Poole, Dorset, U.K.) or the Sigma Chemical Company (Poole, Dorset, U.K.). The chemicals were of the highest analytical grade available. *Taq* DNA Polymerase was obtained from PE Applied Biosystems (Warrington, U.K.), Advanced Biotechnologies Ltd. (Surrey, U.K.), Promega Ltd. (Southampton, U.K.), Bioline (London, U.K.) and MBI Fermentas Molecular Biology (U.K.). DynaZyme II polymerase was obtained from Finnzymes OY (Espoo, Finland). Oligonucleotide primers for conserved rRNA sequences were obtained from PE Applied Biosystems (Warrington, U.K.), random primers were obtained from Advanced Biotechnologies Ltd (Kit1 1-20 and Kit A 1-20); dATP, dCTP, dGTP and dTTP were obtained from Bioline (U.K.). Restriction enzymes were obtain from Sigma (UK) or Boehringer Mannheim (Lewes,UK). Wizard PCR preps DNA and minipreps DNA purification system, and pGEM-T vector were obtained from Promega (UK). PCR II vector was obtained from Invitrogen (Oxford, UK). Sequenase 2.0 sequencing kit (USB) was obtained from Amersham (Little Chalfont, UK). ABI PRISM dye terminator cycle sequencing kit was obtained from PE Applied Biosystems (U.K.). Sequagels was obtained from National Diagnostics (U.K.). Molecular Markers were obtain from Advanced Biotechnologies Ltd. (1kb ladder -- 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 8.0 and 10.0 Kb) or Sigma (123 bp ladder -- 123, 246, 369, 492, 612, ...bp) or Boehringer Mannheim (Lambda Hind III -- 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, 0.6, 0.1 kb; pBR32 -- 4.36 kb).

2.1.2. Cyanobacterial Strains

Table 2.1 describes the cyanobacterial strains used.

2.1.3. Media

The composition of media used for growth of cyanobacteria is given in Table 2.2. Preparation of media is described in Appendix A.

The composition and preparation of buffers used for this study are given in Appendix B.

2.2. Methods

2.2.1. Growth and Maintenance of Cyanobacteria

Strains were cultured on agar medium (15ml medium per plate) or liquid medium (50, 125, 500ml medium in 100, 250, 1000ml flask, respectively), and incubated with shaking at 100 rpm in a GALLENKAMP rotary illuminated incubator (U.K.) which was continuously bubbled with CO_2, at 20-30^{o}C (standard condition) autocycle under continuous light with two warm-white fluorescent (OSRAML, 30W, Germany) lights. Cultures were stored in 25% (v/v) glycerol appropriate liquid medium at -80^{o}C for long term maintenance. For routine use they were maintained on BG11 (Table 2.2) slopes at 10 ^{o}C-15 ^{o}C in the dark.

Table 2.1. Cyanobacterial strains used

Strain	Source[a]	Site	Reference	Growth condition	Medium[b]
Unicellular					
1. *Chroococcidiopsis* JM013	LJMU	Tai lake, Jiangsu, China	Lu (this study)	100μmol photon m^{-2} s^{-1}, 30°C	BG11, C
2. *Chroococcus prescottii*	BSM			100μmol photon m^{-2} s^{-1}, 30°C	BG11
3. *Gloeocapsa* sp.	BSM			60 μmol photon m^{-2} s^{-1}, 25°C	BG11
4. *Microcystis aeruginosa*	BSM			60 μmol photon m^{-2} s^{-1}, 25°C	BG11
5. *Synechococcus* PCC 7942	LJMU	California, U.S.A (Floyd 1973)	Grigorieva and Shestakor 1976	100μmol photon m^{-2} s^{-1}, 36°C	BG11
6. *Synechococcus* CCAP1479/7	LU			100μmol photon m^{-2} s^{-1}, 25°C	BG11
7. *Synechococcus* WH7803	LU			100μmol photon m^{-2} s^{-1}, 25°C	MN
8. *Synechococcus* JM 002	LJMU	Raby pool, Wirral, England	Lu (this study)	60μmol photon m^{-2} s^{-1}, 25°C	BG11
9. *Synechococcus* JM003	LJMU	Raby pool, Wirral, England	Lu (this study)	60μmol photon m^{-2} s^{-1}, 25°C	BG11
10. *Synechococcus* JM004	LJMU	Raby pool, Wirral, England	Lu (this study)	60μmol photon m^{-2} s^{-1}, 25°C	BG11
11. *Synechococcus* JM020	LJMU	Windmere, Cubria, England	Lu (this study)	60μmol photon m^{-2} s^{-1}, 25°C	BG11
12. *Synechocystis* PCC6803	UW	California, U.S.A	Stanier (1971)	100μmol photon m^{-2} s^{-1}, 30°C	BG11
Filamentous non heterocystous					
13. *Leptolyngbya* JM012	LJMU	Tai lake, Jiangsu, China	Lu (this study)	100μmol photon m^{-2} s^{-1}, 30°C	BG11, C
14. *Leptolyngbya* JM014	LJMU	Tai lake, Jiangsu, China	Lu (this study)	100μmol photon m^{-2} s^{-1}, 30°C	BG11
15. *Oscillatoria animalis*	BSM			100μmol photon m^{-2} s^{-1}, 30°C	BG11
16. *Phormidium autumnale*	BSM			100μmol photon m^{-2} s^{-1}, 30°C	BG11, C
17. *Phormidium* JM017	LJMU	Bala lake, North Wales	Lu (this study)	100μmol photon m^{-2} s^{-1}, 30°C	BG11
18.*Pseudanabaena* JM001	LJMU	Raby pool, Wirral, England	Lu (this study)	100μmol photon m^{-2} s^{-1}, 30°C	C, BG11
19.*Pseudanabaena* JM005	LJMU	Raby pool, Wirral, England	Lu (this study)	100μmol photon m^{-2} s^{-1}, 30°C	C, BG11
20.*Pseudanabaena* JM006	LJMU	Raby pool, Wirral, England	Lu (this study)	100μmol photon m^{-2} s^{-1}, 30°C	C, BG11
21.*Pseudanabaena* JM007	LJMU	Raby pool, Wirral, England	Lu (this study)	100μmol photon m^{-2} s^{-1}, 30°C	C, BG11
22.*Pseudanabaena* JM009	LJMU	Tai lake, Jiangsu, China	Lu (this study)	100μmol photon m^{-2} s^{-1}, 30°C	C, BG11

cont. Table 2.1

Strain	Source	Site	Reference	Growth condition*	Medium
23.*Pseudanabaena* JM011	LJMU	Tai lake, Jiangsu, China	Lu (this study)	100μmol photon m^{-2} s^{-1}, 30°C	BG11
24.Pseudanabaena JM016	LJMU	Bala lake, North Wales	Lu (this study)	100μmol photon m^{-2} s^{-1}, 30°C	BG11
25.Pseudanabaena JM019	LJMU	Windmere, Cubria, England	Lu (this study)	100μmol photon m^{-2} s^{-1}, 30°C	BG11
Filamentous heterocystous					
26. Anabaena PCC 7120	UW		Adolph & Haselkorn, 1971	100μmol photon m^{-2} s^{-1}, 30°C	BG11
27. Anabaena ATCC27892 (PCC7118)	LU		Kratz & Myers, 1955	100μmol photon m^{-2} s^{-1}, 30°C	BG11
28.Anabaena ATCC27892 R 3.2	LU			100μmol photon m^{-2} s^{-1}, 30°C	BG11
29. Anabaena cylindrica	UW	pond, Cambridge, England (Chu, 1939)	Fogg, 1942	100μmol photon m^{-2} s^{-1}, 30°C	BG11
30. Anabaena cylindrica AN 23	LJMU	pond, Cambridge, England	Lu (this study)	100μmol photon m^{-2} s^{-1}, 30°C	BG11
31. Anabaena flos-aquae D0848	UD		isolated by Walsby, 1991	60 μmol photon m^{-2} s^{-1}, 25°C	AD P(1.0)Fe(0.4)
32. Anabaena sp. D0697	UD	Aldabra cinq cases pool, Seychelles	Whitton (isolated), 1984	100μmol photon m^{-2} s^{-1}, 25°C	AD P(1.0)Fe(0.4), BG11
33. Anabaena sp. D0746	UD	Huntra dwr(pin gaew)field, Thailand	Catling (isolated), 1984	100μmol photon m^{-2} s^{-1}, 25°C	CHU10D-NBG11
34. Anabaena sp. D0752	UD	Huntra dwr(pin gaew)stem, Thailand	Catling (isolated), 1983	100μmol photon m^{-2} s^{-1}, 25°C	CHU10D-N, BG11
35. Aphanizomenon gracile	BSM			60 μmol photon m^{-2} s^{-1}, 25°C	BG11
36. Chlorogloeopsis fritschii	LJMU	Indian soil	Mitra and Pandey, 1966	100μmol photon m^{-2} s^{-1}, 30°C	C, BG11
37. Fischerella musicola	BSM			100μmol photon m^{-2} s^{-1}, 25°C	BG11
38. Gloeotrichia sp.	UW			100μmol photon m^{-2} s^{-1}, 30°C	BG11
39. Nostoc commune	BSM			100μmol photon m^{-2} s^{-1}, 25°C	BG11
40. Nostoc commune	UD	Scotland	isolated by Gibson	100μmol photon m^{-2} s^{-1}, 25°C	ACM, BG11
41. Nostoc ellipsospora	UW			100μmol photon m^{-2} s^{-1}, 30°C	BG11
42. Nostoc linckia D0734	UD	Huntra dwr(pin gaew)field, Thailand	Catling (isolated), 1983	100μmol photon m^{-2} s^{-1}, 25°C	CHU10D-N, BG11
43. Nostoc MAC PCC8009	UW		Bowyer & Skerman, 1968	100μmol photon m^{-2} s^{-1}, 25°C	BG11
44. Nostoc MAC R1	LU			100μmol photon m^{-2} s^{-1}, 25°C	BG11
45. Nostoc muscorum	BSM			100μmol photon m^{-2} s^{-1}, 25°C	BG11
46. Nostoc piscinale D0201	UD	Aldabra atoll, Seychelles	Donaldson (isolated), 1972	100μmol photon m^{-2} s^{-1}, 25°C	AD P(1.0)Fe(0.4), BG11
47. Nostoc sp. D0766	UD	Sonargaon dwr field, Bangladesh	Kelly (isolated), 1985	100μmol photon m^{-2} s^{-1}, 25°C	CHU 10D-N, BG11

cont. Table 2.1

Strain	Source	Site	Reference	Growth condition*	Medium
48. Nostoc planctonicum	BSM			100μmol photon m^{-2} s^{-1}, 30°C	BG11
49. Nostoc sp. D0740	UD	Huntra dwr(pin gaew)roots, Thailand	Catling (isolated), 1984	100μmol photon m^{-2} s^{-1}, 25°C	CHU 10 D-N, BG11
50. Nostoc pelaeo	UCL			100μmol photon m^{-2} s^{-1}, 36°C	BG11
51. Tolypothrix distorta	BSM			100μmol photon m^{-2} s^{-1}, 30°C	BG11

a LJMU, UW, BSM, LU, UD,UB,UCL, Liverpool John Moores University, University of Warwick, Biobred Sciento Manchester, Lancaster University, University of Durham, University of Bristol, University of Central Lancashire, respectively.

b Media are described in Table 2.2

Table 2.2. Media for growth of cyanobacteria

Component[a] (mM)	Medium[b]							
	ASM-1	BG11	BG11/10	C	CHU 10D	ACM	AD P(1) Fe(0.4)	MN
NaCl	---	---	---	---	---	0.393	3.936	---
$NaNO_3$	2.00	17.65	1.765	---	---	---	---	---
KCl	---	---	---	---	---	---	0.256	---
KNO_3	---	---	---	9.89	---	4.945	---	8.8
KH_2PO_4	---	---	---	---	0.057	---	---	---
K_2HPO_4	0.10	0.18	0.18	0.574	---	0.057	0.032	0.09
Na_2HPO_4	0.10	---	---	---	---	---	---	---
$MgSO_4$	0.20	0.30	0.30	1.01	0.101	0.985	0.811	0.15
$MgCl_2$	0.20	---	---	---	---	---	---	---

Component[a] (mM)	Medium[b]							
	ASM-1	BG11	BG11/10	C	CHU 10D	ACM	AD P(1) Fe(0.4)	MN
$CaCl_2$	0.20	0.25	0.25	---	---	0.135	0.45	0.12
$Ca(NO_3)_2$	---	---	---	0.11	0.244	---	---	---
$NaHCO_3$	---	---	---	---	0.189	---	---	---
Na_2CO_3	---	0.19	0.19	---	---	---	---	0.19
Disodium EDTA	0.020	---	---	---	0.009	0.0013	---	---
Disodium-magnesium EDTA	---	0.003	0.003	---	---	---	0.007	0.0013
Citric acid	---	0.029	0.029					0.014
Trisodium citrate	---	---	---	0.561	---	---	---	---
Ferric ammonium citrate	---	0.03	0.03	---	---	---	---	0.015
$FeCl_3$	0.004	---	---	---	0.009	0.0045	0.0072	---
$Fe_2(SO_4)_3$	---	---	---	0.008	---	---	---	---

Component[a] (mM)	Medium[b]							
	ASM-1	BG11	BG11/10	C	CHU 10D	ACM	AD P(1) Fe(0.4)	MN
Na_2SiO_3 $5H_2O$	---	---	---	---	0.013	---	---	---
H_3BO_3 (μM)	40.00	46.00	46.00	46.00	11.5	46.00	46.00	46.00
$MnCl_24H_2O$ (μM)	7.00	9.20	9.20	9.20	2.30	9.10	9.10	9.20
$ZnSO_47H_2O$ (μM)	3.20	0.77	0.77	0.77	0.2	0.77	0.77	0.77
$Na_2MoO_42H_2O$ (μM)	---	1.60	1.60	1.60	0.03	1.60	1.60	1.60
$CuSO_45H_2O$ (μM)	0.0008	0.32	0.32	0.32	0.08	.032	0.32	0.32
$Co(NO_3)_2$ (μM)	0.080	0.17	0.17	---	---	0.16	0.16	---
$CoSO_4$ $7H_2O$	---	---	---	---	0.04	---	---	---
$NiSO_4$ $7H_2O$	---	---	---	---	---	0.16	0.16	---
Sea water	---	---	---	---	---	---	---	75% v/v

a Components are given as mM unless otherwise stated.

b ASM-1 from Gorham, *et al.* (1964); CHU, MN, BG11 and BG11/10 from Rippka, *et al.* (1979); C from Carr (1969); AD and ACM from Whitton (personal communication).

2.2.2. Description of the sampling sites

In recent years, cyanobacterial growth has been a problem in water courses in the United Kingdom and Peoples' Republic of China. Freshwater lakes in Merseyside, North Wales and Lancashire in U.K., and Jiangshu Province of China were chosen as sampling regions for this study.

(I) United Kingdom

a) Merseyside

Raby pool; Wirral (Plate 1) 53°19' N, 03° W

Sampling date: 19th June 1994

Sampling was undertaken at a freshwater pool near Raby Hall (Fig 2.1, 2.2), Wirral, Northwest England. (Details of sampling, see section 2.2.3, Table 2.3).

b) North Wales

Bala Lake; Southern Snowdonia (Plate 2) 52°54' N, 03°36' W

Sampling date: 19th August 1996

Sampling was undertaken at a freshwater lake near Bala (Fig.2.1, 2.3), Southern Snowdonia, North Wales. (Details of sampling, see section 2.2.3, Table 2.3).

c) Cumbria

Bowness, Windermere Lake (Plate 3) 54° 23' N, 02 ° 55' W

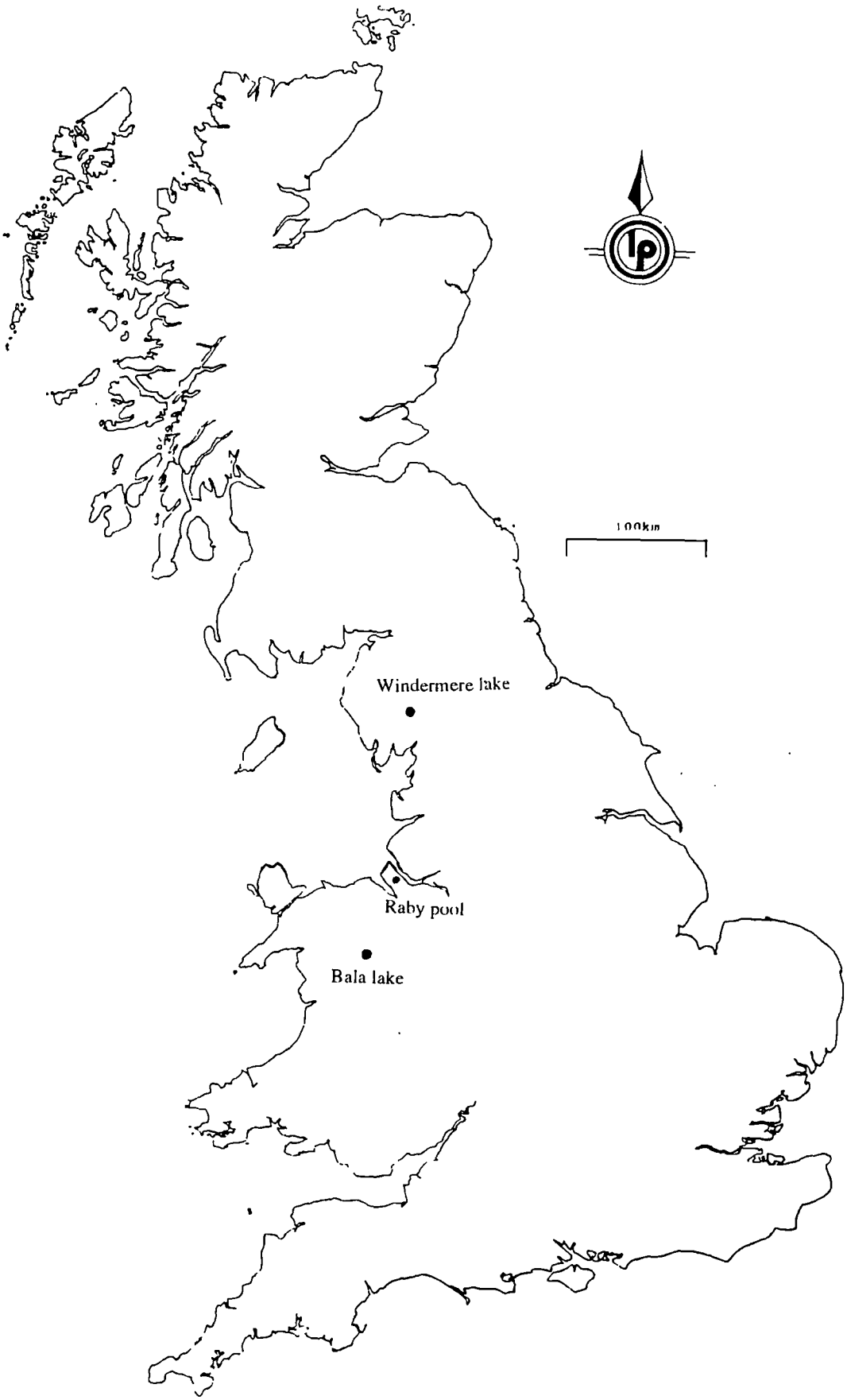


Figure 2.1. Map of the United Kingdom Showing collecting sites

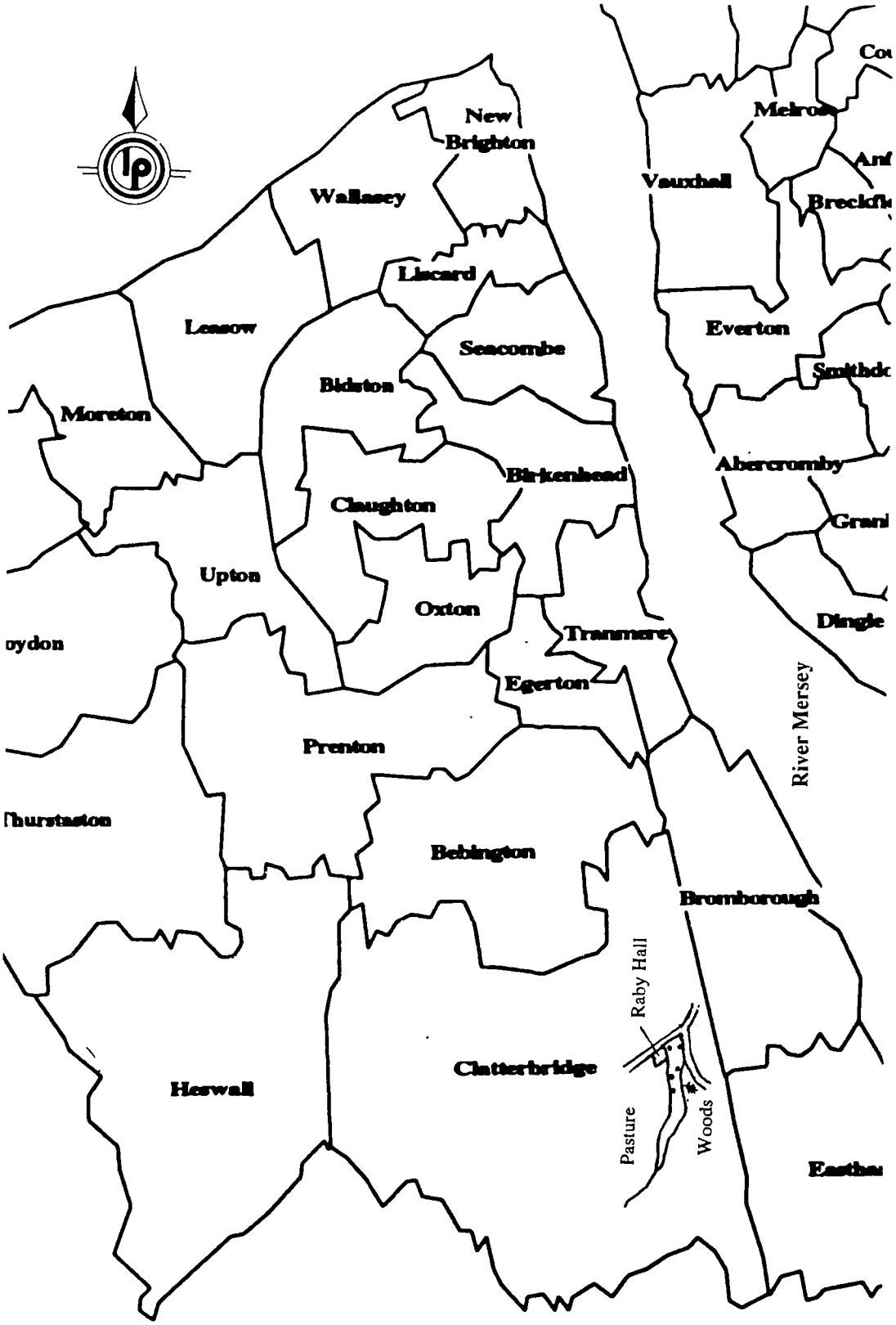


Figure 2.2. Map of Wirral showing sampling location

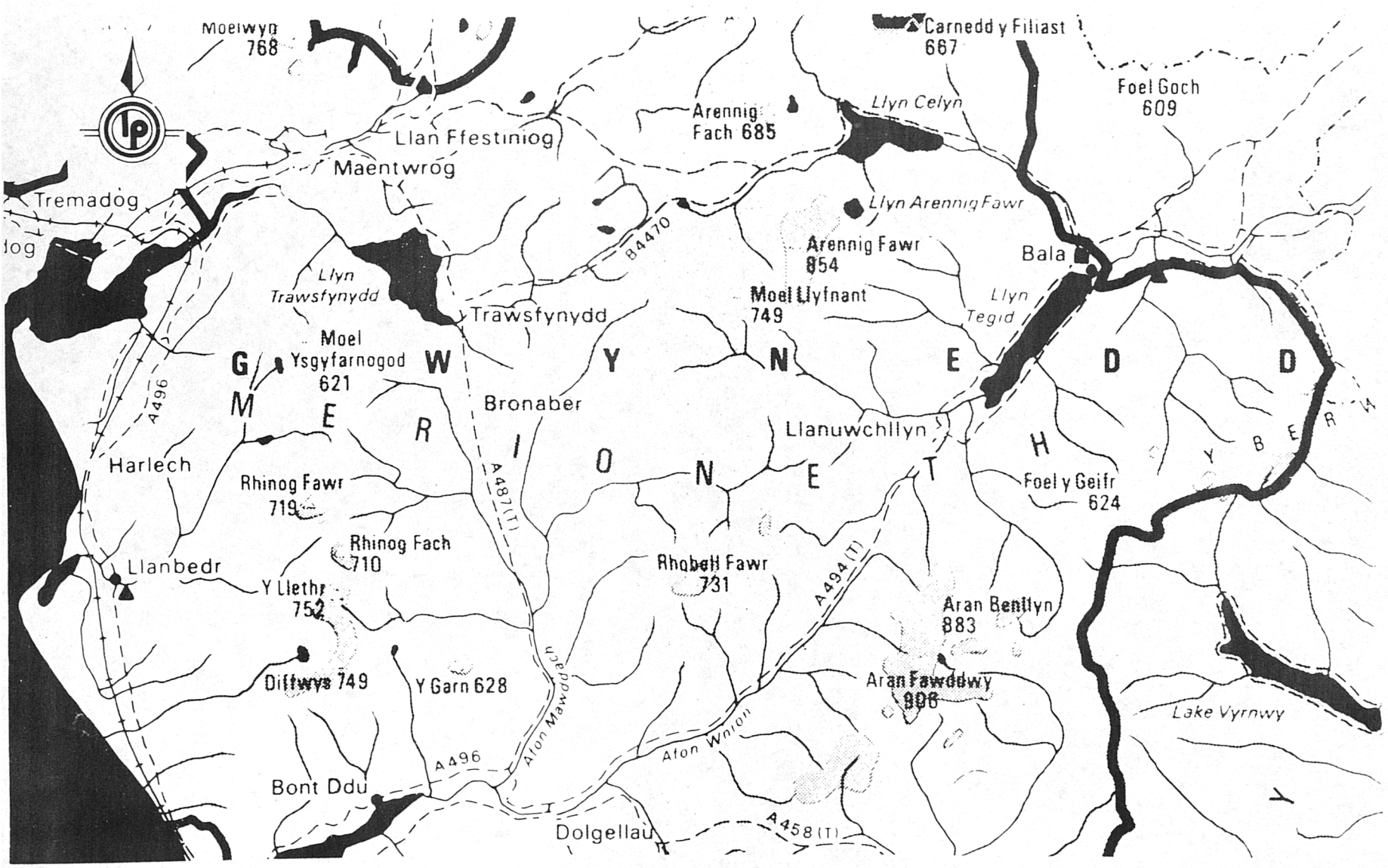


Figure 2.3. Map of Bala lake showing sampling location

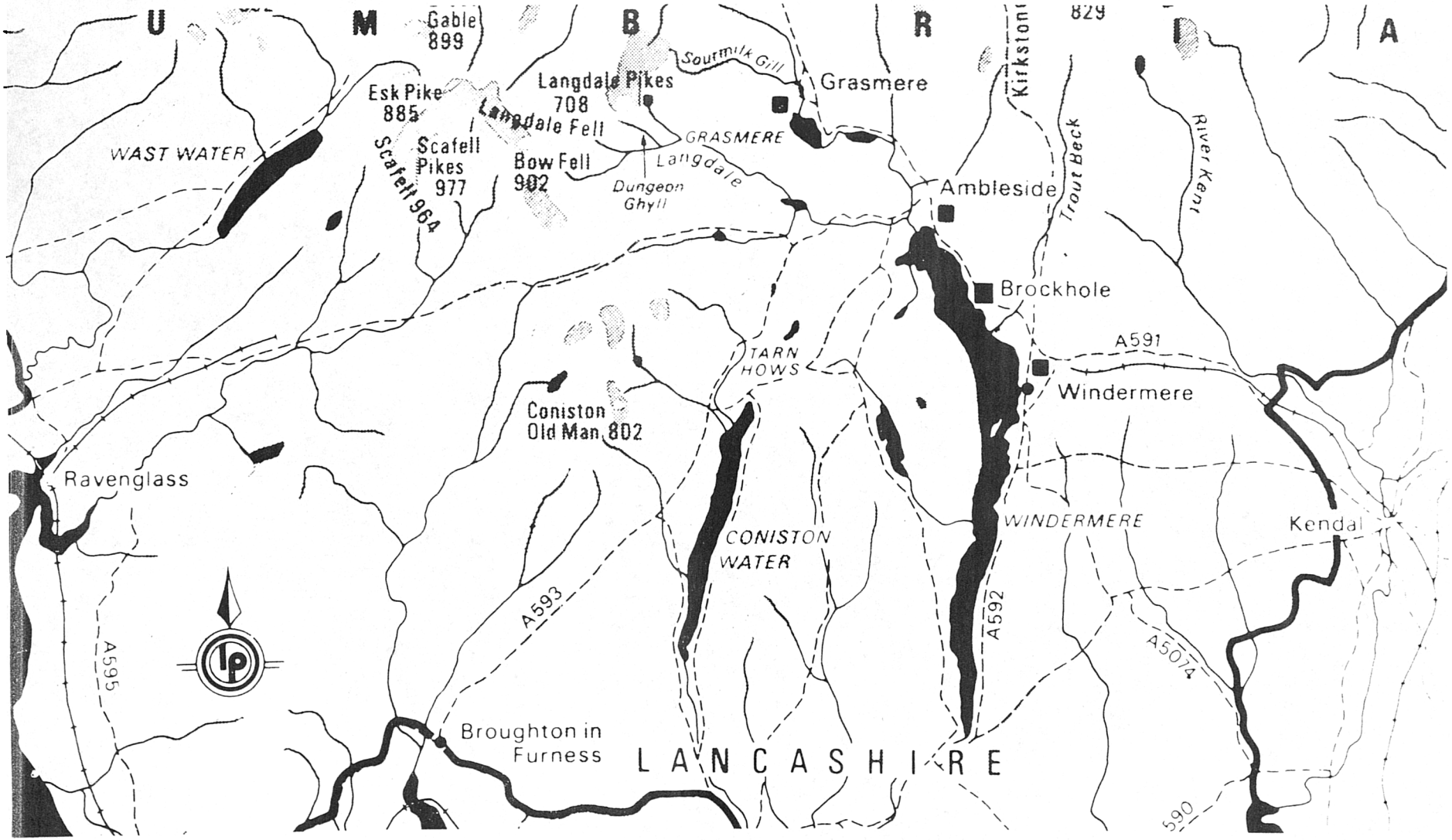


Figure 2.4. Map of Windermere lake showing sampling location

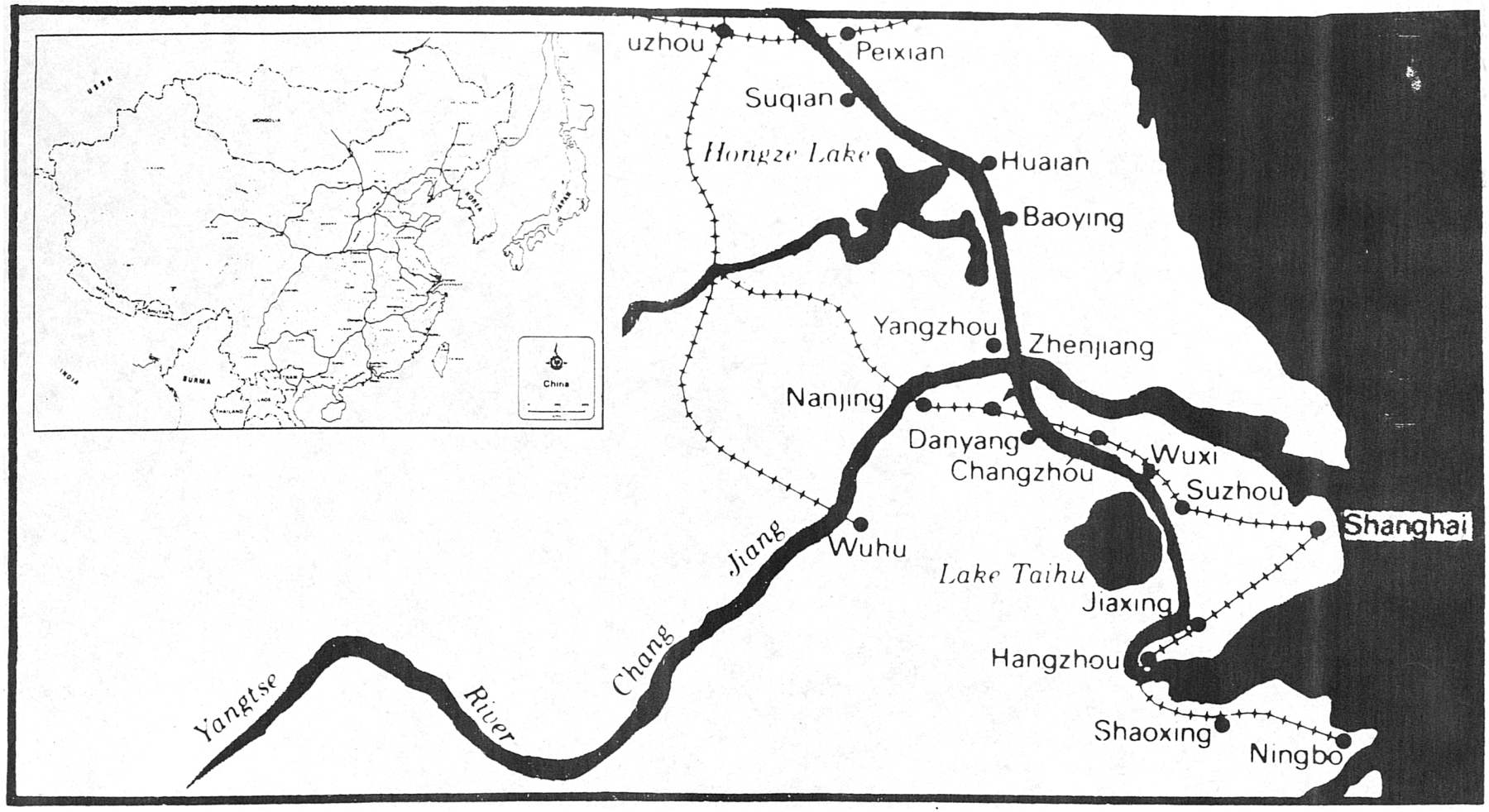


Figure 2.5. Map of China showing sampling site

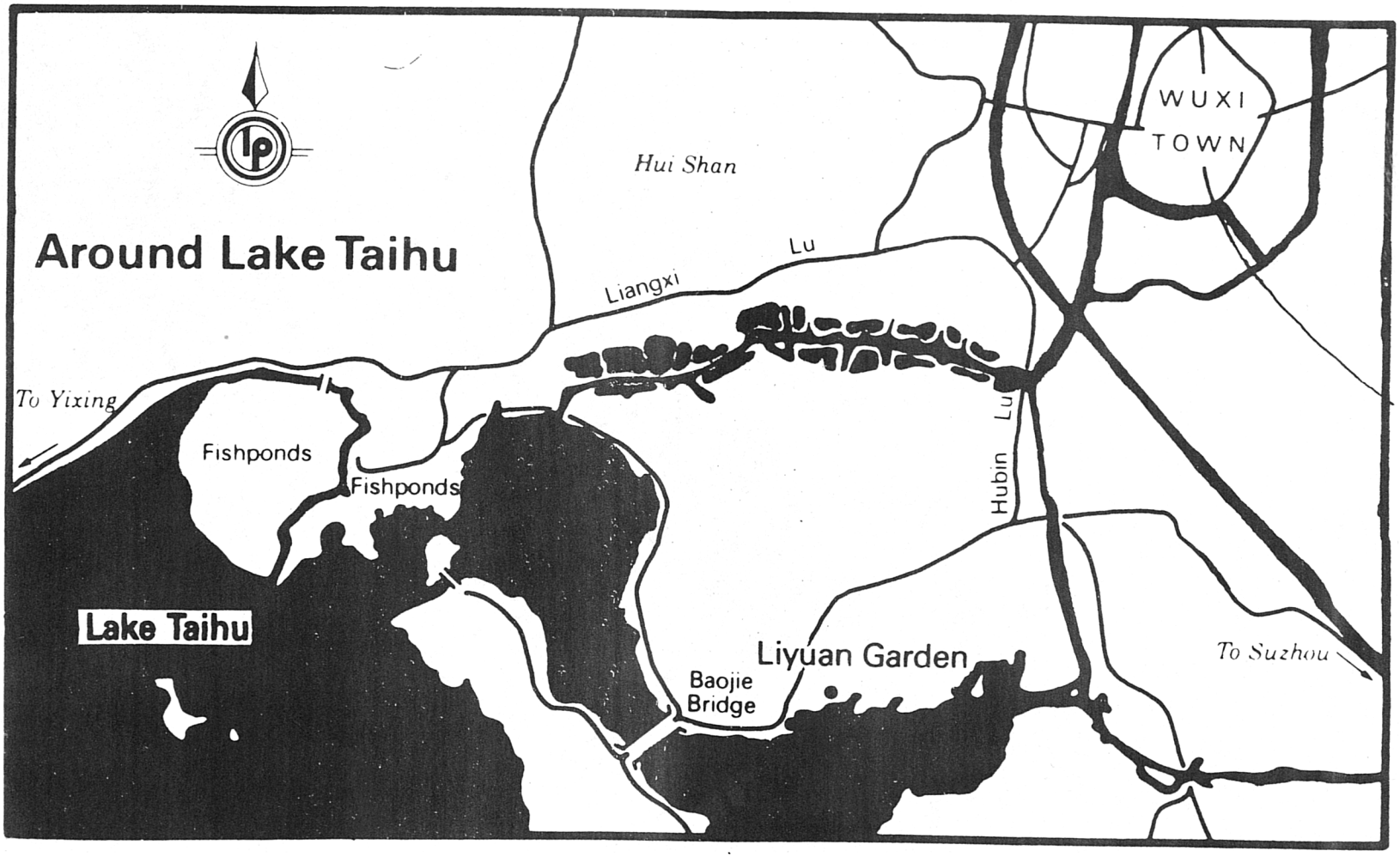


Figure 2.6. Map of Taihu lake showing sampling location

Plate 1 A view of the Raby pool which was one of the freshwater sampling sites in this study.

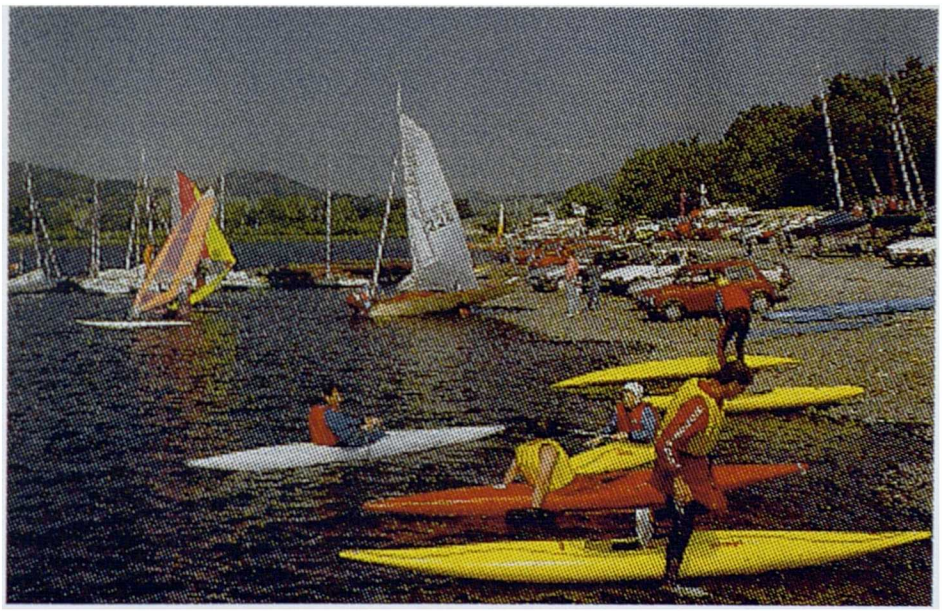


Plate 2 A view of the Bala lake which was selected for freshwater sampling in this study.

Plate 3 A view of the Bowness, Windermere Lake which was one of the freshwater sampling sites in this study.

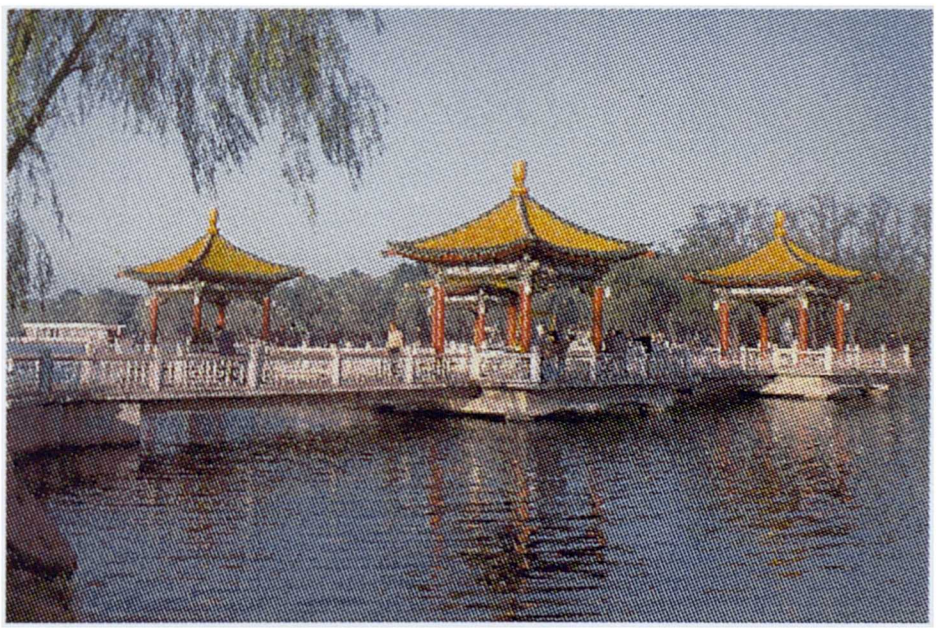


Plate 4 A view of the Liyuan Garden, Taihu lake which was selected for freshwater sampling in this study.

Sampling date: 24th July 1996

Sampling was undertaken at the freshwater lake Windermere (Fig. 2.1, 2.4), Lake District, Northwest England. (Details of sampling, see section 2.2.3, Table 2.3).

(II) Peoples' Republic of China

Taihu Lake was chosen as sampling site in Jiangshu province of P.R. China.

Liyuan Garden, Wuxi, Jiangshu (Plate 4) 31°22' N, 120°30' E

Sampling date 1st October 1995

The sampling was undertaken at the freshwater lake Taihu (Fig. 2.5, 2.6), Wuxi, Jiangshu province of China. (Details of sampling, see section 2.2.3, Table 2.3).

2.2.3. Sampling

Twelve fresh-water samples (approximately 50ml each) were collected at random from the surface layer of water (i.e. the top 2-6cm), around each site within an approximate $500m^2$ area. Figures 2.2, 2.3, 2.4 and 2.6 show the sampling location of the lakes and the pool respectively, and Table 2.3 shows the location and type of water samples. Samples were transported to the laboratory in sterile glass bottles in the dark and analysed immediately. Samples were maintained in the dark at 15-20^{o}C.

Table 2.3. Location and type of water sample

Sample Number	Location	Description of the sample
1	Raby pool, light-niche, stone-stair	Clear water, pH 6.8
2	Raby pool, dim-niche, wood-bottom	Clear water with rotten leaves, pH 6.9
3	Raby pool, dim-niche, wood-bottom	Clear water with rotten leaves, pH 6.9
4	Raby pool, light-niche, broken stone bottom	Clear water, pH 6.8
5	Raby pool, light-niche, broken stone bottom	Clear water, pH 6.8
6	Liyuan garden, light-niche, mud bottom, stone bank	Light green bloom water, organic pollution, pH 7.2
7	Liyuan garden, light-niche, mud bottom, stone bank	Light green bloom water, organic pollution, pH 7.2
8	Lake at Bowness on Windermere, dim-niche, mud bottom	Clear water, pH 6.8
9	Lake at Bowness on Windermere, dim-niche, mud bottom	Clear water, pH 6.8
10	Lake at Bowness on Windermere, light-niche, broken stone bottom	Clear water, pH 6.8
11	Lake at Bowness on Windermere, light-niche, broken stone bottom	Clear water, pH 6.8
12	Bala Lake near car park, light-niche, broken stone and mud bottom	Clear water, pH 7.0

2.2.4 Isolation of cyanobacteria

(a) *Direct isolation* 0.1ml water from each sample was spread with a sterile glass spreader on to the following agar medium: C, BG11 and ASM-1.

(b) *Indirect isolation* 1.0 ml water samples from each of the sampling bottles were inoculated in to 50ml of sterile mineral liquid medium (C, BG11 and ASM-1) in a 100ml flask and incubated until growth was visible. The culture was then streaked on to the appropriate agar plates (as per liquid medium).

All the plates and flasks were incubated aerobically under two conditions: (1) In the laboratory, at 15-20^{o}C, under a daylight light-dark cycle. (2) In the rotary light incubator under standard conditions (see section 2.2.1).

Putative cyanobacterial colonies growing on the surface of the agar plates of appropriate medium were transferred to appropriate fresh liquid medium, and incubated in GALLENKAMP illuminated incubator under standard conditions (see section 2.21).

2.2.5 Recognition of cyanobacterial isolates

Initial identification of the cyanobacterial isolates was on basis of their characteristic colour. The UV-vis absorption spectra of putative cyanobacteria liquid cultures (see section 2.2.4) were measured (against a blank which was strain free medium) from 400nm-720nm using a KONTRON UVIKON-930 spectrophotometer. Absorption peaks were detected for chlorophylls and

phycobilins. Samples were also examined under the light microscope (UNIL UX-11) for the absence of the nucleus. The cyanobacterial isolates were confirmed by light-induced O_2-evolution (see 2.2.6) and determination of pigment content (see 2.2.7). The cyanobacterial isolates were then transferred to appropriate agar medium, and culturing and maintenance were as described in section 2.2.1.

2.2.6 Measurement of O_2-evolution

A 2ml liquid culture of cyanobacteria, obtained as in section 2.2.5, was harvested by centrifugation at 6000g for 5min at 4°C in a MSE microcentrifuge. The cells were resuspended in 2ml (×1) distilled water and put in an O_2 evolution chamber in the dark (by covering with aluminium foil). After two minutes, the aluminium-foil was removed, the microscopy light switched on and O_2 evolution measured by using YS1 MODEL 5300 Biological OXYGEN MONITOR and SERUOGOR 120 channel recorder. Cyanobacteria and Prochloron evolve oxygen in the light during photosynthesis. Other bacteria will not evolve oxygen.

2.2.7 Determination of pigment content

Approximately 1g wet weight (see 2.2.13) of cyanobacterial cells were harvested by centrifugation at 6000g for 15 minutes at 4°C in a MSE Europa 24M centrifuge using 10×100ml rotor, washed with 5ml 5% (w/v) HEPES buffer (pH 7.8) and resuspended in 10ml 5% (w/v) HEPES buffer (pH 7.8). The cells were broken by sonication with ice chilled for 2 min. The sonicate was centrifuged at 35,000g for 60min at 10 °C in BECKMAN L-80 Ultracentrifuge using Ti70 rotor.

The uv-vis absorption spectrum of the supernatant was determined by using the KONTRON UVIKON-930 spectrophotometer. Absorbance peaks at 565nm and 620nm indicate the presence of phycoerythrin (PE) and phycocyanin (PC) respectively. The pellet was extracted with 5ml of methanol and twice with 5ml of methanol/diethyl ether (1:1,v/v) for 40 min. The combined extracts were centrifuged at 5,000g for 10min in a MSE Europa 24M centrifuge at 10 ^{o}C using 4×25ml rotor to remove unbroken cells and cell debris. The supernatant was examined spectrophotometrically by KONTRON UVIKON-930 spectrophotometer (380nm-720nm). The absorbance peaks at 380nm-470nm and 665nm indicate the presence of carotenoids and chlorophyll a (Danks *et al.*, 1983).

2.2.8 Purification of cyanobacteria

Single colonies of each cyanobacterial sample were sub-cultured repeatedly until pure cultures were obtained. Each colony isolated and restreaked in this way was given a reference number to facilitate comparison with the colonies obtained from either different isolation procedures or different water samples.

Purification of some filamentous cyanobacteria directly from the original growing plates proved difficult, accordingly Nystatin (0.02g/l) was added to BG11 medium to suppress the growth of competitive microorganisms during the purification (Jawad, 1982). The antibiotic treatment was carry out in BG11 medium which was supplemented with 0.05% casamino acids, 0.5% (w/v) glucose, and 1mg/ml ampicillin, and incubation in the dark for 16 h (Rippka, 1988).

2.2.9 Genus identification of cyanobacteria

Cyanobacterial isolates were identified to the generic level by using the method of Rippka *et al.* (1988) based on a bacteriological approach. Details of this method are given in Appendix C.

2.2.10 Determination of Nitrogenase Activity

A modification of the method of Rippka and Waterbury (1977) was employed for the measurement of nitrogen fixation by isolates as determined by nitrogenase activity. Mid-exponential phase cultures were harvested by centrifugation at 9000 g for 10 minutes at 15^{o}C in a MSE Europa 24M centrifuge using 10×100ml rotor, and resuspended in 50ml of nitrate-free BG11 medium in 100ml flasks. Cultures were incubated for 48 h in a GALLENKAMP illuminated incubator under standard conditions with three warm-white fluorescent light, after which time they started to show signs of nitrogen depletion, the culture turning to a yellow colour. Flasks were bubbled with a mixture of nitrogen and carbon dioxide (97% and 3% v/v respectively), and incubated under reduced light intensity with one warm-white fluorescent light, at 100 rpm. Nitrogenase positive cultures are green or light blue-green.

2.2.11. Determination of ability to grow photoheterotrophically

A method of Rippka (1972) was employed for determination of photoheterotrophy. Photoheterotrophy was demonstrated by growth of strains in the light in the BG11 medium with 10^{-5} M dichlorophenyldimethylurea (DCMU),

supplemented with each of the following organic substrates alone: 0.5% (w/v) glucose, 0.5% (w/v) fructose, 0.5% (w/v) sucrose, 0.5% (w/v) ribose and 0.1% (w/v) glycerol. Stock solutions of 5% (w/v) substrate solution and 0.1 M dichlorophenyldimethylurea were sterilised by passing through 0.2um filters (Whatman, UK), and stored at -20 ^{o}C.

2.2.12 Electron microscopic and light microscopic examination

Cyanobacterial cells were fixed by a modification of the method of Rippka *et al.*(1974). Cells were prefixed at room temperature with 0.5% (w/v) glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.2) for 30 min. Cells were then fixed in 3.5% (w/v) glutaraldehyde for 2 h at room temperature and 15 h at 4^{o}C, and then rinsed six times (15min for each rinse) in cacodylate buffer (pH 7.2). After fixation, the cells were enrobed in 3% (w/v) agar (in ×1 distilled water), which was cut into small pieces and washed four times in cacodylate buffer (pH 7.2). The cells were post-fixed in 1% (w/v) osmium tetroxide in cacodylate buffer for 1 h at room temperature and for another 4 h at 4 ^{o}C. Following three washes in cacodylate buffer (pH 7.2) and one in distilled water, the cells were stained in 1% (w/v) aqueous uranyl acetate for 2 h at room temperature, and washed three times with distilled water, dehydrated through an ethanol series (incubated with each 30% v/v, 50% v/v, 70% v/v, 90% v/v, 100% v/v ethanol for 10 minutes) and embedded in Taab spurr resin. Thin sections were obtained by using Ultra-cut microtomes. Glass knives were cut on an L.K.B. knifemaker type 7800. Sections exhibiting silver-gold interference colours were collected on formvar-coated copper grids of 200 mesh size. Thin sections were post-stained with both uranyl acetate (2% w/v aqueous) and

Reynolds' lead citrate, and examined and photographed in a JEOL JEM-1200EX electron microscope.

Light microscopy was used to examine cyanobacterial cell division using specimens mounted on agar-coated slides (Appendix A), and photographed using OLYMPUS BH-2 NOMARSKI illumination. Colonies or trichome forms were directly photographed from agar plates with using Leitz LABORLUX 12 phase contrast illumination.

2.2.13 Determination of growth rates of cyanobacterial cultures

Growth was determined as a function of dry weight from optical density (OD) measurements at 720nm. Different sized inocula (0.5ml, 1.0ml, 1.5 ml; OD_{720} 1.2-1.6) of cultures were transferred to 1 litre flasks containing 500ml of appropriate liquid medium, Cultures were incubated in a GALLENKAMP illuminated incubator which was continuously supplemented with CO_2, at 20-30^{o}C (30-37^{o}C for *Synechococcus* PCC 7942) autocycle under continuous light with two warm-white fluorescent lights, at 100 rpm as described in section 2.2.1. Culture samples (2ml) were removed at various time intervals and measured at 720nm using the UV-930 spectrophotometer against a blank of appropriate uninoculated medium. OD_{720} of 1.0 corresponds to approximately 1mg dry weight of cells per 1ml of culture (Lambert and Carr, 1982).

2.2.14 Preparation and purification of cyanobacterial DNA

A number of methods was used for the extraction of plasmid and genomic DNA from cyanobacteria.

2.2.14.1 Mini-methods for preparation of plasmid DNA

Method 1: Lysis by lysozyme, a modification of a method of Kado and Liu (1981) and Birnboim and Doly 1979. Approximately 1.5mg dry weight of cells were harvested by centrifugation at 6000g for 15 minutes at 4 ^{o}C in a MSE 24 M centrifuge using 4×25ml rotor and washed with 0.5 ml of TSE buffer (10mM Tris-HCl, 50mM NaCl, 50mM EDTA, pH 7.8). The pellet was resuspended in 0.5ml of Birnboim and Doly solution I (50mM glucose, 25mM Tris-HCl, 10mM EDTA, pH 8.0), and incubated with lysozyme (final concentration 5mg/ml) at 37^{o}C for 1 hour. RNase was then added to 20μg/ml and proteinase K to 100μg/ml (see Appendix B), and the mixture incubated at 55^{o}C for 2 hours. Two phenol-chloroform-isoamyl alcohol extractions, followed by two chloroform-isoamyl alcohol extractions were carried out (see 2.2.14.4). After extraction was complete, the plasmid DNA was recovered by ethanol precipitation (see 2.2.14.4) and the pellet resuspended in 50μl of TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) and stored at -20 ^{o}C.

Method 2: Lysis by Sodium Dodecyl Sulphate (SDS) based on the method of Van den Hondel *et al.* (1979). Approximately 1.5mg dry weight of cells were harvested by centrifugation, the pellet washed and resuspended in 0.5ml of sucrose buffer (50mM Tris-HCl, 50mM EDTA, 25% (w/v) sucrose, pH8.0), and incubated

with lysozyme (final concentration 5mg/ml) at 37°C for one hour. 75µl of 10% (w/v) SDS was then added and incubation continued for a further hour. 150µl of 5M NaCl was added with gentle mixing and the mixture incubated over-night on ice. RNase was then added to 20µg/ml and proteinase K to 100µg/ml, and incubated at 37°C for 30 min. Two phenol-chloroform- isoamyl alcohol and then two chloroform-isoamyl alcohol extractions were carried out (see 2.2.14.4). After extraction was complete, the cleared lysate was precipitated with ethanol (see 2.2.14.4), and the pellet resuspended in 50µl TE buffer and stored at -20 °C.

Method 3: Alkaline lysis based on a method of Birnboim and Doly (1979). Approximately 1.5mg dry weight of cells were harvested by centrifugation, washed, and the pellet resuspended in 200µl of Birnboim and Doly solution I. 400µl of alkaline mixture (0.2M NaOH, 2% (w/v) SDS) was added and the tube inverted. After cooling on ice for 5 min, 300µl 3M sodium acetate (pH 5.2) was added and the mixture incubated on ice for 10 min. Two phenol-chloroform-isoamyl alcohol and then two chloroform-isoamyl alcohol extractions were carried out (see 2.2.14.4). After extraction was complete, the cleared lysate was precipitated with ethanol (see 2.2.14.4), the pellet was resuspended in 50µl TE buffer and stored at -20 °C.

Method 4: Boiling method of Holmes and Quigley (1981). Approximately 1.5mg dry weight of cells were harvested by centrifugation, the pellet washed and resuspended in 350µl STET (50mM Tris-HCl, 50mM EDTA, 8% (w/v) sucrose, 5% (w/v) Triton X-100, pH8.0), and boiled with lysozyme (final concentration 2.5mg/ml) for 40 seconds. After holding on ice for 5 min, two phenol-chloroform

and two chloroform extractions were carried out (see 2.2.14.4). After centrifugation at 12,000g in a SANYO microcentrifuge for 5 min at room temperature, 40μl of 3M sodium acetate (pH 5.2) was added to the supernatant and the DNA precipitated with ethanol (see 2.2.14.4), The pellet was resuspended in 50μl TE buffer and stored at -20 ^{O}C.

Method 5: Lysis by heat-alkali co-denaturation based on the methods of Birnboim and Doly (1979) and Holmes and Quigley (1981). Approximately 1.5mg dry weight of cells were harvested by centrifuge, the pellet washed and resuspended in 0.75ml of TES (25 mM Tris-HCl, 50mM EDTA, 10% (w/v) sucrose, pH 8.0), and incubated with lysozyme (final concentration 5mg/ml) on ice for 20 min. 1.5 ml of the denaturation solution (0.15M NaOH, 1% (w/v) SDS) was added and mixed quickly. The mixture was incubated at 80^{O}C for 8 min with gently shaking. Then 1.5ml of the cold neutralising solution (3.5M KAc, pH5.8) was added with thorough mixing, and incubated on ice for 20 min. Two phenol-chloroform-isoamyl alcohol and then two chloroform-isoamyl alcohol extractions were carried out (see 2.2.14.4). After extraction was complete, a 0.4 volume of 2-isopropanol was added to the supernatant, and incubated at room temperature for 10 min. After centrifugation in a SANYO microcentrifuge at 12,000g, for 5 min at room temperature, the precipitate was resuspended in an appropriate volume of TE buffer and stored at -20 ^{O}C.

2.2.14.2 Large scale plasmid DNA preparation

Large scale plasmid preparation was carried out by caesium chloride-ethidium bromide (CsCl-EtBr) density gradient centrifugation (van den Hondel *et al.*, 1979). A 1L mid-exponential phase liquid culture (2×500ml) of cyanobacteria was harvested by centrifugation at 6000g for 15 min at 4°C in a MSE Europa 24M centrifuge using a 6×300ml rotor. The cells were washed and the pellet resuspended in 50ml of sucrose buffer (see 2.2.14.2 method 2), and incubated with lysozyme (final concentration 5mg/ml) at 37°C. After 30 min, 6.2ml of 10% (w/v) sodium dodecyl sulphate was added and mixed by inversion. After incubation at 37°C for a further hour, 15ml of 5M NaCl were added and gently mixed by inversion. The mixture was incubated on ice overnight and the centrifuged at 10,000g for 15 minutes at 4°C in a MSE 24 M centrifuge using 10×100ml rotor. The cleared lysate was precipitated with ethanol (see 2.2.14.4) and the precipitate was resuspended in a total of 16ml of TE buffer. Solid CsCl was then added to a final concentration of 1.05g/ml and 0.8ml of ethidium bromide solution (5mg/ml) was added. Plasmid DNA was separated from chromosomal DNA by caesium chloride-ethidium bromide density gradient centrifugation in a Beckman L-80 ultracentrifuge using a Ti-80 rotor at 65,000g for 40 hours at 18°C. The lower band of supercoiled plasmid DNA was collected through a #21 hypodermic needle inserted into the side of the tube. EtBr was extracted from the collected DNA fractions with an equal volume of 1-butanol saturated with 5M NaCl in TE buffer. The aqueous material was dialysed against TE buffer for several hours, with at least 3 changes of TE buffer, at room temperature. The sample was then ethanol precipitated, resuspended in an appropriate volume of TE buffer and stored at -20 °C.

2.2.14.3 Preparation of total genomic DNA

Method 1: Total genomic DNA was prepared by the method of Porter (1988). Approximately 1.5 mg dry weight of cells (mid-exponential phase) were harvested by centrifugation, the pellet washed and resuspended in 0.5ml of sucrose buffer (10-25% (w/v) sucrose, 50mM Tris-HCl, 100mM EDTA, pH 8.0) and incubated with lysozyme (final concentration 5mg/ml) at 37^{o}C. After 30 minutes, SDS to1% (w/v) and proteinase K to 100μg/ml were added. The mixture was incubated at 50^{o}C for at least 2 h followed by two phenol-chloroform-isoamyl alcohol and then two chloroform-isoamyl alcohol extractions (see 2.2,14.4). 1/3 volume of 10.5 M ammonium acetate and 2 volumes of 2-propanol were added to the aqueous phase. The sample was incubated at -20^{o}C for at least 15 min and then centrifuged for 5 min at room temperature in a microfuge at 13000g. The pellet was washed twice with 70% (v/v) ethanol. The pellet was dried under reduced pressure in a desiccator, resuspended in an appropriate volume of TE buffer and stored at -20 ^{o}C.

Method 2: Total genomic DNA was prepared by the method of Neilan (1995). Approximately 1.5 mg dry weight of cells (mid-exponential phase) were harvested by centrifugation, the medium was decanted, and the pellet washed and resuspended in 0.5ml of 50mM Tris-HCl, 50mM EDTA, 50mM NaCl, pH 8.0. Lysozyme was added to a final concentration 1mg/ml, and the solution was incubated at 55^{o}C for 30 min. After addition of 20 μl 1% (w/v) SDS and 10 μl of proteinase K (10mg/ml), the mixture was incubated at 55^{o}C, until the solution cleared. The solution was chilled on ice and extracted twice with an equal volume of phenol-chloroform-isoamyl alcohol and then with chloroform-isoamyl alcohol

(see 2.2.14.4). An equal volume of 4 M ammonium acetate and 2 volumes of 2-propanol were added to the aqueous phase. The sample was incubated at -20^{o}C for at least 15 min and then centrifuged for 5 min at room temperature in a SANYO microfuge at 13000g. The pellet was washed twice with 70% (v/v) ethanol. The pellet was dried under reduced pressure in a desiccator and resuspended in an appropriate volume of TE buffer stored at -20 ^{o}C. Both methods provided template DNA for PCR (see 2.2.19 and 2.2.20).

Total genomic DNA as template for PCR was also obtained from cells directly. A 1ml aliquot of mid to late exponential phase culture was pelleted by centrifugation for 2 min at room temperature in a SANYO microfuge at 6000g, the cells were washed, resuspended in 0.1 ml of TE buffer or sterilise ×1 distilled water and used directly for PCR (section 2.2.20).

2.2.14.4 Phenol-chloroform extraction and ethanol precipitation of DNA

Proteins were routinely removed from DNA solutions by phenol-chloroform extraction (Sambrook *et al.,* 1989). DNA solutions were extracted with the same volume of phenol (saturated with 0.1M Tris-HCl, pH 8.0) : chloroform : isoamyl alcohol (25:24:1 (v/v)) (see Appendix B) by vortexing the two phases and centrifuging for 5 min at 12,000g at room temperature in a SANYO microfuge. The upper aqueous layer was then transferred to a fresh sterile microfuge-tube, followed by extraction with chloroform:isoamyl alcohol (24:1 (v/v)) twice. Two phases were separated by centrifuging for 5 min at 12,000g at room temperature in a SANYO microfuge.

Ethanol precipitation was used to remove traces of organic solvent or to concentrate the DNA solution. DNA was precipitated at -20°C for 1 h after the addition of 1/10 volume 3M sodium acetate, pH4.8 and 2.2 volumes of cold (-20°C) 100% (v/v) ethanol. The precipitated DNA was collected by centrifugation for 5 min at 12,000g at room temperature in a SANYO microfuge. The DNA pellet was washed with 70% (v/v) ethanol, dried at room temperature, resuspended in an appropriate volume of TE (10mM Tris-HCl and 1mM EDTA, pH 8.0) buffer or sterile ×1 distilled water and stored at -20°C.

2.2.15 Agarose gel electrophoresis

Horizontal, 0.6% (w/v) to 1.5% (w/v) agarose gels (Boehringer Mannheim GmbH, multi purpose agarose (MP)) and 3% gels (2% (w/v) agarose NA (nucleic acids) + 1% (w/v) agarose MP, see section 2.2.21) in 1×TAE buffer (40mM Tris-Acetate, 1mM EDTA, pH 7.6) were cast in horizontal gel tanks (BRL, Horizon 58 and H5 or Flowgen). DNA (up to 0.5μg) in loading buffer (100mM EDTA, 1% (w/v) SDS, 0.1% (w/v) bromophenol blue, 50% (v/v) glycerol) was applied to the gel slots together with appropriate molecular mass standard and electrophoresis was performed at 50-120 Volts by using a LKB BROMMA 2197 power pack, until the bromophenol blue tracking dye front reached the end of the gel. The DNA was stained by immersing the gel in ethidium bromide solution (0.5μg/ml in ×1 distilled water) for 1 h at 4 °C in the dark. The gel was destained in distilled water for 20 min at room temperature in the dark. DNA was visualized by mid-range UV induced fluorescence on a transilluminator (LKB-2011 Macrovue Hi switch UV, Sweden)

and photographed with a Polaroid MP4 land camera equipped with an orange filter (Kodak Wratten 22,USA) using Polaroid 665 film (exposure 15 s).

2.2.16 Recovery of DNA from agarose gels

The DNA to be isolated was separated on a 1% (w/v) agarose gel. The band of interest was located on the gel using a long-wavelength UV lamp (LKB-2011 Macrovue Lo switch UV, Sweden) to minimise damage to the DNA. Using a sharp scalpel, a slice of agarose containing the band was cut out and placed in the fluid-filled (0.5 ×TAE buffer) pre-treated dialysis bag (see Appendix B). The gel slice was allowed to sink to the bottom of the bag, and most of the buffer removed, leaving just enough fluid to keep the gel slice in constant contact with the electrophoresis buffer. The bag was tied just above the gel slice, avoiding trapping air bubbles. The bag was immersed in a shallow layer of 0.5 × TAE buffer in a horizontal electrophoresis tank (BRL, Horizon 58). Electric current was passed through the bag (usually 100V for 2-3 h) then the polarity of the current reversed for 2 min to release the DNA from the wall of the dialysis bag. The dialysis bag was opened and carefully all the buffer surrounding the gel slice was recovered. Two phenol-chloroform-isoamyl alcohol and then two chloroform-isoamyl alcohol extractions were carried out (see 2.2.14.4). After extraction was complete, the cleared lysate was precipitated with ethanol (see 2.2.14.4), the pellet was resuspended in 50µl TE buffer and stored at -20 ^{o}C.

2.2.17 Measurement of DNA concentration

The concentration and purity of DNA samples were determined by UV-spectrophotometry (at 260 nm and 280 nm) using 5µl samples of DNA and 995µl TE buffer (Sambrook *et al.*, 1989) using a blank of TE buffer. DNA concentration may be calculated as follows:

$$\text{concentration of DNA sample (µg/ml)} = 50 \times (1000/5) \times A_{260}$$

(where A_{260} = absorbance at 260nm)

The ratio of the absorbance readings at 260 nm and 280 nm (A_{260}/A_{280}) provided an estimate of the purity of the nucleic acid. Pure preparations of DNA have A_{260}/A_{280} values of 1.8 and above. If there was contamination with protein or phenol, the A_{260}/A_{280} was significantly less than this value, If there was contamination with RNA, the A_{260}/A_{280} was significantly more than this value, and accurate quantification of the amount of DNA was not possible.

Sometimes there was more than one plasmid DNA species in the preparation, or the DNA was heavily contaminated with other substances that absorb ultraviolet light and therefore impeded accurate analysis. 5µl of such samples of DNA, mixed with 1µl of gel-loading buffer, were subject to electrophoresis on an 0.8% (w/v) agarose horizontal gel (see 2.2.15) together with an appropriate DNA standard (e.g. pBR 322). Electrophoresis was carried out until the bromophenol blue had migrated 2 cm. The intensity of fluorescence (using the transilluminator in section 2.2.15) of

the unknown DNA samples was compared with that of DNA standards of known amount and the quantity of DNA in the sample was estimated.

2.2.18 DNA restriction

To 13µl of DNA samples (0.5-2.0µg), was added 1.5µl of 10× restriction buffer (as specified by manufacturers) and 5-8 units of appropriate restriction enzyme (Boehringer Mannheim GmbH) in a total reaction mixture of 15µl and mixtures were normally incubated at 37°C for 2 h. Incubations were carried out in a heated block (Techne, Dri-Block OB-1, UK). Reactions were terminated by incubation at 65°C for 10 min. Restriction fragments were resolved by agarose gel electrophoresis using appropriate DNA standards (section 2.2.15).

2.2.19 Random Amplified Polymorphic DNA - Polymerase Chain Reaction (RAPD-PCR)

The polymerase chain reaction (PCR) using random primers was carried out essentially as described by Williams *et al.* (1990). The primers used were 10-base single oligonucleotides of arbitrary DNA sequence (see Table 2.4). Each PCR mix contained in a total reaction volume of 50µl: 1×PCR amplification buffer, 2.5mM $MgCl_2$, 150µM each of dATP, dCTP, dGTP and dTTP, 0.2µM primer, 0.1µg of total DNA, and 1 unit of AmpliTaq polymerase, overlaid with 1 drop of mineral oil (Cat. No. M3516; Sigma). Amplifications were performed in a Perkin-Elmer Cetus DNA thermal cycler (UK) programmed for 35 cycles:

Programme 1 (Williams *et al.*, 1990):

Denaturing, 1 min at 94^{o}C;

Annealing, 1 min at 34^{o}C;

Extension, 2 min at 72^{o}C,

using the fastest available transitions between each temperature.

Programme 2 (Bielawski *et al.*, 1995):

Denaturing, 30 s at 94^{o}C;

Annealing, 30 s at 34^{o}C;

Extension, 2 min at 72^{o}C,

using the fastest available transitions between each temperature.

Programme 3 (Yu and Pauls 1992):

Denaturing, 5s at 94^{o}C;

Annealing, 30s at 34^{o}C;

Extension, 60s at 72^{o}C,

using the fastest available transitions between each temperature.

Programme 4 (this study):

Denaturing, 30 s at 94^{o}C;

Annealing, 30 s at 34^{o}C;

Extension, 2 min at 72^{o}C,

using the transition interval between each temperature, the rate of change of temperature being 0.45 ^{o}C/s.

Programme 5 (this study):

Denaturing, 1 s at 94^{o}C;

Table 2.4 10-base single random primers used in RAPD-PCR[a].

Code	Sequence	Code	Sequence
AB1-01	5'-GTTTCGCTCC-3'	AB1-11	5'-GTAGACCCGT-3'
AB1-02	5'-TGATCCCTGG-3'	AB1-12	5'-CCTTGACGCA-3'
AB1-03	5'-CATCCCCCTG-3'	AB1-13	5'-TTCCCCCGCT-3'
AB1-04	5'-GGACTGGAGT-3'	AB1-14	5'-TCCGCTCTGG-3'
AB1-05	5'-TGCGCCCTTC-3'	AB1-15	5'-GGAGGGTGTT-3'
AB1-06	5'-TGCTCTGCCC-3'	AB1-16	5'-TTTGCCCGGA-3'
AB1-07	5'-GGTGACGCAG-3'	AB1-17	5'-AGGGAACGAG-3'
AB1-08	5'-GTCCACACGG-3'	AB1-18	5'-CCACAGCAGT-3'
AB1-09	5'-TGGGGGACTC-3'	AB1-19	5'-ACCCCCGAAG-3'
AB1-10	5'-CTGCTGGGAC-3'	AB1-20	5'-GGACCCTTAC-3'
ABA-01	5'-CAGGCCCTTC-3'	ABA-11	5'-CAATCGCCGT-3'
ABA-02	5'-TGCCGAGCTG-3'	ABA-12	5'-TCGGCGATAG-3'
ABA-03	5'-AGTCAGCCAC-3'	ABA-13	5'-CAGCACCCAC-3'
ABA-04	5'-AATCGGGCTG-3'	ABA-14	5'-TCTGTGCTGG-3'
ABA-05	5'-AGGGGTCTTG-3'	ABA-15	5'-TTCCGAACCC-3'
ABA-06	5'-GGTCCCTGAC-3'	ABA-16	5'-AGCCAGCGAA-3'
ABA-07	5'-GAAACGGGTG-3'	ABA-17	5'-GACCGCTTGT-3'
ABA-08	5'-GTGACGTAGG-3'	ABA-18	5'-AGGTGACCGT-3'
ABA-09	5'-GGGTAACGCC-3'	ABA-19	5'-CAAACGTCGG-3'
ABA-10	5'-GTGATCGCAG-3'	ABA-20	5'-GTTGCGATCC-3'

a, Primers were obtained from Advanced Biotechnologies, Surrey, UK

Annealing, 1 s at 34^{o}C;

Extension, 1 s at 72^{o}C,

using the transition interval between each temperature, the rate of change of temperature being 0.244 ^{o}C/s from 72 ^{o}C to 94 ^{o}C, 0.400 ^{o}C/s from 94 ^{o}C to 34 ^{o}C and 0.211 ^{o}C/s from 34 ^{o}C to 72 ^{o}C.

DNA negative controls consisted of the standard PCR reaction mixture, but with the DNA template replaced by an equal volume of sterrile × 1 distilled water.

Amplification products were analysed by electrophoresis in 1.5% (w/v) agarose gels and detected by staining with ethidium bromide (see section 2.2.15), using appropriate molecular weight markers (see section 2.1.1).

2.2.20 PCR amplification of rDNA spacer

The PCR was used to amplify the internal transcribed spacer of rDNA (see Fig 1.1). Each 100 µl reaction mixture for PCR contained 40 pmol of each primer, 2.5 units of *Taq* polymerase (Bioline) or DyNAZyme II (Finnzymes Oy), 0.2 mM deoxyribonucleoside triphosphates (dATP, dCTP, dGTP and dTTP), 2.5mM $MgCl_2$ and 0.1µg cyanobacterial DNA or 1µl TE buffered culture (see 2.2.14.3). The reaction mixture was overlaid with mineral oil, and thermal cycling was achieved in a Perkin-Elmer Cetus Thermal Cycler according to the following programme: initial denaturation at 94 ^{o}C for 3 min, 35 cycles of 94 ^{o}C for 1 min, 55^{o}C for 1min and 72 ^{o}C for 3min. Primers 1, 14, 16, 17, 18 as described by Wilmotte, *et al.*(1993) (see Table 2.5), were

used. DNA negative controls consisted of the standard PCR reaction mixture, but with the DNA template replaced by an equal volume of sterile ×1 distilled water.

Table 2.5 Primers used for PCR and sequence determination of rDNA regions

Primer	Sequence	Strand [a]	Position [b]
1	5'-AGAGTTTGATCCTGGCTCAG-3'	R	8-27 (16S)
14	5'-TGTACACACCGCCCGTC-3'	R	1334-1350 (16S)
16	5'-AAGGAGGTGATCCAGCCGCA-3'	C	1486-1467 (16S)
17	5'-ATTAGCTCAGGTGGTTAG-3'	R	6-23 ($tRNA^{ile}$)
18	5'-TTTGCGGCCGCTCTGTGTGC CTAGGTATCC-3'	C	76-45 (23S)

a, Primer sequences corresponding to the RNA sequence are indicated by R; primers complementary to the RNA sequence are indicated by C.
b, Nucleotide position in the corresponding rRNA sequence of *Synechococcus* sp. PCC6301.

2.2.21 Restriction digestion of PCR-amplified DNA

PCR-amplified DNA (10μl, approximately 0.5-0.8μg) was digested, without further purification, with various restriction enzymes (*Dde* I, *Taq* I, *Alu* I, *Sau* 3A, *Hae* III, *Pvu* I, *Xba* I, *Sal* I, *Sac* I, *Ava* I, *Bam* HI, *Hind* II, *Hind* III, *Pst* I, *Bgl* II, *Eco* RV (from Boehringer Mannheim, Germany) and *Hinf* I, *Hha* I, *Hpa* II, *Rsa* I (from Sigma, England) according to the manufacturer's instructions. The products were separated on gels containing 2% (w/v) agarose NA (nucleic acids) + 1% (w/v) agarose MP (multi purpose) in TAE (mM Tris-HCl, mM NaAC, mM EDTA, pH 8.0) buffer. The gels were

stained with ethidium bromide and photographed (see 2.2.15). Appropriate molecular weight markers (123bp ladder, Sigma, England) were run on each gel.

2.2.22 Data handling by Binary Matrix and Similarity coefficients

Each band visualised on a gel was measured by using the Phoretix 1D software package developed by Phoretix International Ltd (Newcastle, UK). DNA band patterns were converted into a binary matrix: band present was indicated as 1, band absent was indicated as 0. Following band scoring similarity indices were calculated for further analysis. The method of Nei and Li (1979) was used as follows:

$$S = 2\,Nxy/(Nx+Ny)$$

Where S is the similarity index, Nxy is the total number of bands shared between sample x and y, Nx is the total number of bands in sample x, Ny is the total number of bands in sample y. Identical patterns will therefore equal 1 and totally unrelated patterns equal 0.

2.2.23 Phylogenetic analysis of PCR-amplified products

Each band visualised on a gel was measured by using the Phoretix 1D software package developed by Phoretix International Ltd (Newcastle, UK). Phenetic analysis of both Random Amplified Polymorphic DNA (RAPD) (section 2.219) and Restriction Fragment Length Polymorphisms (RFLPs) (section 2.2.20) data was performed by calculating pairwise genetic distances from a binary matrix. Distances were calculated by using the 14 algorithms supplied by the RAPDistance package developed by

Armstrong *et al.* (1994). The resulting trees were constructed by using the neighbor-joining method and the NJTREE program.

Phenetic analysis of RFLP data was also performed by using the DOLLOP program in the PHYLIP package. The consensus tree was constructed by using the CONSENSE program in the PHYLIP package.

2.2.24 Purification of PCR-amplified product and TA-Cloning

PCR-amplified products using primer pair 17/18 were directly purified using the Wizard PCR preps DNA purification system (Promega) according to the manufacturer's instructions. The DNA concentration was adjusted to 30ng/ul (for direct automatic sequencing, see section 2.2.26 method 2). The purified PCR product was ligated into a pGEM-T vector (Promega) or a pCR II vector (Invitrogen) to which was added a 3'-terminal thymidine to both ends. Recombinant plasmids were used to transform *E. coli* JM109 high efficiency competent cells (provided in the Promega cloning kit) or One Shot competent cells (provided in the Invitrogen cloning kit). White colonies (recombinant clones) were selected from LB/ampicillin/IPTG/X-Gal agar (Appendix A) plates and each was transferred to 5ml LB/ampicillin medium (Appendix A) and incubated for 24 hours at 37°C with shaking at 100 rpm in a GALLENKAMP incubator.

2.2.25 Isolation and purification of recombinant plasmid DNA

Recombinant plasmid DNA was isolated and purified by using Wizard minipreps DNA purification system, according to the manufacturer's instructions. The plasmid DNA concentration was adjusted to 500ng/ul with sterile ×1 distilled water.

2.2.26 DNA sequencing

Method 1: Sequence determination of directly purifed PCR products (section 2.2.24) was carried out by using the ABI PRISM dye terminator cycle sequencing kit according to the manufacturer's instructions. Each 20ul reaction mixture for cycle sequencing contained 100ng PCR product, 3.2pmol primer and 8.0ul terminator ready reaction mixture. The thermal cycling was achieved in a Perkin-Elmer Cetus Thermal Cycler according to the following programme: 25 cycles of 96°C for 30 s, 50°C for 15 s and 60°C for 4 min. Primer 18 (see Table 2.5) was used. The extension products were purified using a spin-column (Perkin-Elmer). Then the products were run on 6% (w/v) polyacrylamide gels (29:1 acrylamide:bisacrylaminde, purified and degassed with 1% (w/v) purification resin and 0.2 μm vacuum filter). The rDNA sequences were determined by using the automated DNA sequencer (ABI PRISM 377, UK).

Method 2: Sequence determination of cloned DNA (section 2.2.25) was carried out with the Sequenase 2.0 sequencing kit (USB, Amersham, UK) according to the manufacturer's instructions. The two primers (M13 forward (-21) and reverse (-26)) and ^{35}S-dATP were used. 6% (w/v) (19:1 acrylamide:bisacrylaminde) Sequagels (National Diagnostics, UK) were run in a vertical gel tank (Model S2, Life Technologies, UK) at 50W for 7h, by using a LKB BROMMA 2197 power pack. The

gel was autoradiographed at -80 °C for 36 h using Fuji medical X-ray film. The sequence was manually determined from the developed film.

2.2.27 DNA sequence analysis

Primary DNA sequences from PCR products were aligned to the published rDNA sequences for *Arthrospira* sp. PCC7345 (Accession No. X75044, Nelissen *et al.*, 1994), *Spirulina* sp. PCC6313 (Accession No. X75045, Nelissen *et al.*, 1994), *Synechococcus* PCC6301 (Accession No. X00346; Tomioka and Sugiura, 1984), *Trichodesmium* NIBB1067 (Accession No. X72871; Wilmotte *et al.*, 1994), *E. coli* (Accession No. J01702, Young and Steitz, 1978), *Cyanophora paradoxa* cyanelle (Accession No. M19493, Janssen *et al.*, 1987) and *Chondrus crispus* (red alga) chloroplast (Accession No. Z29521, Leblanc *et al.*, 1995) from the GenBank database using the CLUSTAL W program version 1.6 (http://www.ibcp.fr/clustalw.html).

Estimates phylogenies by the parsimony method:

Bootstrapping (1, 100, 500 runs) of aligned sequences was undertaken by using SEQBOOT program in the PHYLIP package. The parsimony trees were constructed by using DNAPARS program in the PHYLIP package. A consensus tree was constructed by using CONSENSE program in the PHYLIP package.

Estimates phylogenies by the maximum likelihood method:

The maximum likelihood trees were constructed by using DNAML program in the PHYLIP package with directly input aligned sequences (from CLUSTAL W).

Estimates phylogenies by the neighbour joining method:

The distance values were calculated by using DNADIST program in the PHYLIP package with directly input aligned sequences (from CLUSTAL W). The distances then were used in the distance matrix program (neighbour joining). The neighbour joining trees were constructed by using NEIGHBOR program in the PHYLIP package.

2.2.28 Primer design

Sequences to be used in designing primers should be specific for the cyanobacterial target region. The specificity of proposed primer sequences were checked by using Genbank data (BLAST e-mail: blast@ncbi.nlm.nih.gov) and by alignment of cyanobacterial 16S-23S spacer sequences (from this study).

The dissociation temperature of the primer/template duplex (Tm) can be calculated from the primer sequence from the formula:

$$Tm = 4 (G+C) + 2 (A+T).$$

PCR primers that ensure Tm of 54 °C or higher provide the best chance for maintenance of specificity and efficiency (Dieffenbach et al., 1993).

General requirements for primer pairs are: 1) be similar in size between 18 to 25 bases in length, 2) 40%-60% GC content with a 3'-terminal "G/C clamp" (at least one or two 3' G's and /or C's), 3) be free of repetitive motifs, 4) similar Tm values, 5) complementary sequences should not be found between the primer pair (Roux, 1995).

Specific oligonucleotide sequences were synthesized (Perkin-Elmer) for use as primers SF/SR for PCR (see section 6.2.3).

Chapter 3

Isolation, Purification and Identification of Cyanobacteria

3.1 Introduction

The isolation and purification of cyanobacteria from natural environments is important for the study of their taxonomy and characteristics. Methods for the isolation and purification of cyanobacteria have been reviewed by Walsby for planktonic species (1981), Castenholz (1981) for thermophiles, by Waterbury and Stanier (1981) for organisms from marine and hypersaline environments, and by Rippka *et al.*(1981) for strains of diverse origin (cited in Rippka, 1988). After preliminary experimentation the isolation method of Rippka (1988) was used.

Following successful isolation, cyanobacteria should be identified by a name, which serves as an indicator of the respective phenotypic properties and is therefore crucial for scientific communication. Unless the organisms have not been previously described, their names have to be chosen from an existing system of classification. Since cyanobacteria were first recognised more than 200 years ago, a bewildering array of genera and species has been created by botanists and ecologists. Classification was based either on the properties observable on samples collected from the natural habitat or on those extractable from dried herbarium specimens. Furthermore, many genera and species underwent repeated taxonomic revisions, leading to a large number of synonyms that only botanical experts are capable of unravelling.

Although the relationship between cyanobacteria (as blue-green algae) and the bacteria has been recognised for more than a century, the classification of natural cyanobacterial populations was established from a range of morphological

characteristics and these have been reviewed by Whitton (1992) and further details are given in Chapter 1.

Morphological evaluations by light microscopy enable a continuity in observation from the unaided eye, through the use of a dissecting microscope (10-100×), to a magnification of a compound light microscope (100-1000×). This scope has been extended, to include applications of greater magnifications (up to 1,000,000×) of external and internal structures, using scanning and transmission electron microscopes. Within the last two decades, ultrastructural data has accumulated, providing a new source of useful taxonomic information (e.g. Lang and Whitton 1973; Rippka 1974; Jensen 1985; Hoffman 1988; Anagnostidis and Komarek 1988).

At the level of taxonomic distinction between genera, the traditional systems of cyanobacteria placed a high value on cell division patterns, colony formation and relationship to extracellular envelopes and sheaths. Differences in cell shapes and dimensions were used largely to distinguish between species within each genus. this practice caused difficulties in classification by introducing a tendency to assign organisms with different cell organisation but similar cell arrangements to the same generic identify.

A new and deeply reorganised taxonomic revision was published by Anagnostidis and Komarek (1985, 1988, 1990) and Komarek and Anagnostidis (1986, 1989). These treatises are valuable contributions towards a modern system of cyanobacterial classification, particularly since an attempt was made to take into account not only the knowledge gained by botanists over the last 30 years but also

that of bacteriologists over a similar period. This has led to the creation not only of numerous new generic names, but also to the nomenclatural change of some traditional genera (Rippka and Herdman, 1992). The generic limits adopted tend to be quite narrow, leading to smaller and more homogeneous genera than in most other botanical revision. The authors also made an extensive review of the literature and integrated all the biochemical, ultrastructural and molecular characters available with their considerable taxonomic experience.

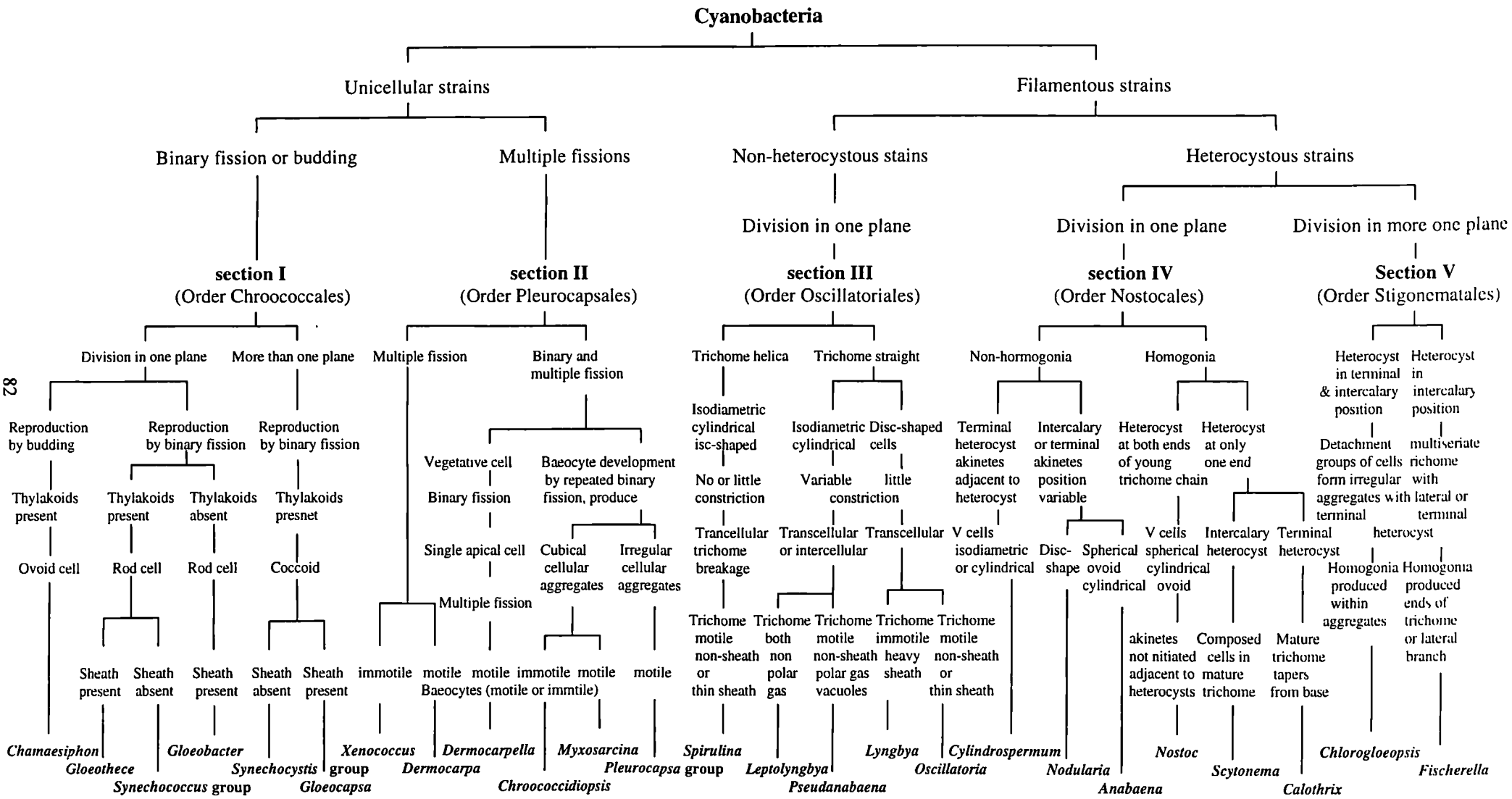
Stanier *et al.*(1978) proposed to place the nomenclature of cyanobacteria under the rules of the International Code of Nomenclature of Bacteria, under which a living type strain, allowing modern comparative analysis, designates both species and genus. The second, more immediate, solution for the identification of axenic cyanobacteria was the proposed "working classification" of Rippka *et al.*(1979), which certainly will have to be modified as more complete and stable data on fine structural, physiological, biochemical, and genetic characteristics become available. The system is based on axenic, clonal cultures and the use of characters from a range of disciplines (Stanier, 1971; Kenyon *et al.,* 1972; Rippka *et al.* 1974; Waterbury, 1977,1978; Herdman *et al.* 1979), rather than morphology alone. In this system, cyanobacteria are divided into five major sections (or Orders, Castenholz and Waterbury, 1989). The criteria used to define Orders are based on morphology as botanical revision (see Table 1.2). At the level of taxonomic distinction between genera where some families have poor morphological differentiation, other characters like ultrastructure and physiology were also considered, the characters chosen are simple and defined as " present or absent". For example, *Gloeobacter* is defined as a unicellular organism lacking thylakoids, *Gloeothece* is defined as a unicellular organism with rod-shape, present sheath and thylakoids. The genera of

each Order are described in Appendix C. At the level of species classification, a little more information is still required to describe all the species. Some part of this system was changed at genera level by Castenholz and Waterbury (1989), and Rippka and Herdman (1992). In section I, the genera *Chamaesiphon* (cells ovoid, reproduction by budding), *Gloeobacter* (cells rod-shaped, thylakoids absent, sheath present, reproduction by one plane binary fission) and *Gloeothece* (cells rod-shaped, thylakoids present, sheath present, aerobic nitrogen fixation) were retained as defined by Rippka *et al.* (1979). Waterbury and Rippka (1989) employ the term "group", rather than genus, for two large and heterogeneous assemblages. The *Synechococcus* group and the *Synechocystis* group, both of which clearly warrant further generic division; each group containing 5 clusters (Rippka and Herdman, 1992). A group *Synechococcus* (Rippka *et al.*, 1979) with cell width >3μm and nitrogenase produced anaerobically has been recognised as a *Cyanothece* group by Waterbury and Rippka (1989) and genus *Cyanothece* (Komarek, 1976) by Rippka and Herdman (1992). The genus *Gloeocapsa* (Rippka *et al.*, 1979) (*Gloeocapsa* group Waterbury and Rippka 1989) has been retained under a more restricted definition by Rippka and Herdman (1992). Genera *Chroococcus* and *Microcystis* were represented in the PCC by Rippka and Herdman (1992). In section two, all six genera of Rippka (1979) are same as described in the system of Waterbury (1989). The major change since the publications of Rippka *et al* (1979) and Waterbury (1989) is the recognition of the newly created genus *Stanieria* (Komarek and Anagnostidis, 1986; Rippka and Herdman, 1992), which replaces Dermocarpa (Rippka *et al.*, 1979 and Waterbury, 1989). In recent botanical revision (Komarek and Anagnostidis, 1986), section I and section II were placed into one order *Chroococcales*, which contains seven families and eighty-four genera. The genera *Chroococcidiopsis* and *Myxosarcina* in Rippka *et al* (1979), Waterbury (1989) and

Rippka and Herdman (1992) conform well to the botanical descriptions. In section III, significant change has been made since the publication of Rippka *et al.* (1979). The most significant change is the elimination of LPP groups A and B, made possible by the revisions of Anagnostidis and Komarek (1988). In the revisions of Castenholz (1989), all strains in LPP A and some strains in LPP B (Trichome immobile in persistent sheath) have been assigned to the genera *Lyngbya* (one trichome per sheath) and *Microcoleus* (two or more trichomes per sheath). Other LPP strains (trichome motile, no persistent sheath) have been transferred to *Pseudanabaena* (strong constriction), *Oscillatoria* and *Trichodesmium* (absent or feeble constriction). In the revisions of Rippka & Herdman (1992), the LPP A is renamed *Lyngbya*. Most of the strains previously assigned to the LPP group B have been assigned to the genera *Geitlerinema* and *Leptolyngbya*, and a few strains have been temporarily transferred to *Microcoleus*. The genera *Pseudanabaena* and *Oscillatoria* (Rippka et al., 1979) have been retained with minor changes. The genus *Spirulina* (Rippka et al., 1979) has been divided into two genera, *Spirulina* and *Arthrospira* in both revisions of Castenholz (1989) and Rippka & Herdman (1992). In section IV, The genus *Anabaena* has (Rippka *et al.*, 1979) been divided into *Anabaena* and *Aphanizomenon* (more elongate), other genera *Cylindrospermum*, *Nodularia*, *Nostoc*, *Scytonema* and *Calothrix* were retained without changes by Castenholz (1989). In the revision of Rippka & Herdman (1992), the genera *Cylindrospermum*, *Nodularia*, *Nostoc*, *Scytonema* and *Anabaena* have been retained as previously defined (Rippka *et al.*, 1979). The genus *Calothrix* Rippka *et al.*, (1979) has been changed to *Calothrix* group because of high genetic diversity, and some members of *Calothrix* Rippka *et al.*, (1979) have been transferred to *Tolypothrix* (a botanical genus that has been slightly redefined). *Anabaenopsis*, *Aphanizomenon* and *Microchaete* are genera newly represented in

the PCC by Rippka and Herdman (1992). In section V, the genera *Chlorogloeopsis* was retained unchanged, the genus *Fischerella* was retained under a more restricted definition, and other seven genera were added in the revision of Castenholz (1989). In the revision of Rippka and Herdman (1992), the nomenclature of the strains assigned to section V is unchanged from that employed by Rippka *et al.* (1979).

In this study, the method used to identify cyanobacteria from natural sources is based on the results of pure culture studies (Rippka *et al.*,1979). Fig 3.1 shows a flow diagram of this system. Cyanobacteria are divided into five major sections (Orders) according to the morphologically characterised form of reproduction (single cell or chain), differentiation of cells, and number and regularity of planes of cell divisions. Section I was defined as unicellular cyanobacteria that reproduce by binary fission or budding. Cells are coccoid to rod-shaped. Division occurs in one, two or three successive planes at right angles to one another or in irregular planes, resulting in cells occurring singly or in aggregates of varying size. Section II was defined as cyanobacteria that reproduce by the formation of small, spherical cells (baeocytes) produced through multiple fission of a vegetative cell and released through rupture of the fibrous outer wall of the parental cell. Enlargement of vegetative cells is always accompanied by progressive thickening of the fibrous outer wall layer. Section III was defined as filamentous cyanobacteria that undergo binary fission in a single plane and that produce "vegetative" cells only. Heterocysts and akinetes do not occur. Section IV was defined as filamentous cyanobacteria dividing exclusively by binary fission in one plane only and having the potential to produce heterocysts. False branching may occur. Section V was defined as filamentous cyanobacteria dividing by binary fission in more than one plane and having the potential to produce heterocysts. True branching occurs. Characters used


Cyanobacteria
Unicellular strains
Filamentous strains
Binary fission or budding
Multiple fissions
Non-heterocystous stains
Heterocystous strains
Division in one plane
Division in one plane
Division in more one plane
section I
(Order Chroococcales)
section II
(Order Pleurocapsales)
section III
(Order Oscillatoriales)
section IV
(Order Nostocales)
Section V
(Order Stigonematales)
Division in one plane
More than one plane
Multiple fission
Binary and multiple fission
Trichome helica
Trichome straight
Non-hormogonia
Homogonia
Heterocyst in terminal & intercalary position
Heterocyst in intercalary position
Reproduction by budding
Reproduction by binary fission
Reproduction by binary fission
Vegetative cell
Binary fission
Single apical cell
Baeocyte development by repeated binary fission, produce
Isodiametric cylindrical isc-shaped
Isodiametric cylindrical
Disc-shaped cells
Terminal heterocyst akinetes adjacent to heterocyst
Intercalary or terminal akinetes position variable
Heterocyst at both ends of young trichome chain
Heterocyst at only one end
Detachment groups of cells form irregular aggregates with terminal heterocyst
multiseriate richome with lateral or terminal
Thylakoids present
Thylakoids present
Thylakoids absent
Thylakoids presnet
No or little constriction
Variable little constriction
Cubical cellular aggregates
Irregular cellular aggregates
Trancellular trichome breakage
Transcellular or intercellular
Transcellular
V cells isodiametric or cylindrical
Disc-shape
Spherical ovoid cylindrical
V cells spherical cylindrical ovoid
Intercalary heterocyst
Terminal heterocyst
Homogonia produced within aggregates
Homogonia produced ends of trichome or lateral branch
Ovoid cell
Rod cell
Rod cell
Coccoid
Multiple fission
Trichome motile non-sheath or thin sheath
Trichome both non polar gas
Trichome motile non-sheath polar gas vacuoles
Trichome immotile heavy sheath
Trichome motile non-sheath or thin sheath
akinetes not nitiated adjacent to heterocysts
Composed cells in mature trichome
Mature trichome tapers from base
Sheath present
Sheath absent
Sheath present
Sheath absent
Sheath present
immotile
motile
motile
immotile
motile
motile
Baeocytes (motile or immtile)
Chamaesiphon
Gloeothece
Synechococcus group
Gloeobacter
Synechocystis group
Gloeocapsa
Xenococcus
Dermocarpa
Dermocarpella
Chroococcidiopsis
Myxosarcina
Pleurocapsa group
Spirulina
Leptolyngbya
Pseudanabaena
Lyngbya
Oscillatoria
Cylindrospermum
Nodularia
Anabaena
Nostoc
Scytonema
Calothrix
Chlorogloeopsis
Fischerella

to define genera are mainly based on morphology. In section I & II, for the genera with poor morphological differentiation, other characters like ultrastructure (present or absent thylakoids) and physiology (motility of spores) were also used. The purified isolates were assigned in Section I -- *Synechococcus* group (Unicellular coccoid to rod-shaped cyanobacteria that divide by binary fission in a single plane. The cells are <3 μm in diameter, contain photosynthetic thylakoids located peripherally, and lack structured sheaths), Section II -- *Chroococcidiopsis* (Cyanobacteria that undergo repeated binary fission in three planes to produce more or less regular cubical cells in an aggregate which is followed by the massive release of immotile baeocytes. The baeocyte initiates growth by enlarging symmetrically into a spherical vegetive cell that, just before the onset of binary fission, attains a size that is characteristic and constant for any given strain), and Section III -- *Pseudanabaena* (Filamentous cyanobacteria which have conspicuous constrictions at the cross-walls. Cells are longer than broad to isodiametric and are often barrel-shaped. The diameter of trichome ranges from about 1 to 3 μm. Trichome are usually straight and motile. Most strains have polar gas vacuoles), *Leptolyngbya* (Filamentous cyanobacteria, cylindrical trichome slightly to intensely waved, slightly constricted at the cross walls. Ability to form sheaths around the trichome dependent on the environmental conditions, false branching lacking, cells ± isodiametric or longer than wide, gas vesicles lacking, thylakoids peripherally arranged, filaments more or less involved into tangled flaky clusters) and *Phormidium* (Filamentous cyanobacteria, cylindrical trichome slightly to intensely waved, unconstricted or slightly constricted at the cross walls. Ability to form sheaths around the trichome dependent on the environmental conditions, false branching lacking, cells ± isodiametric or shorter or longer than wide, gas vesicles lacking, thylakoids perpendicular to the walls, thallus expandcd, extensive, more or

less fine). Some characters (cells length:width, special mucilaginous envelopes around cells, mucilage, colonial aggregates, shape of colonies, colour of colonies, thylakoids arrangement) of Anagnostidis and Kamarek (1986, 1988, 1989, and 1990) were also used.

3.2 Results

3.2.1 Isolation condition

Optimal media and conditions were chosen, with four different media and two incubation conditions used for isolation. Eight samples were isolated by using direct and indirect methods (see 2.2.4). Table 3.1a shows that organisms from Raby pool grew very quickly in BG11, as well as $BG11_{10}$. For growth in liquid media, BG11 appears slightly better than $BG11_{10}$, but for solid media, $BG11_{10}$ appears slightly better than BG11. For medium C, the organisms always grew slower, appearing a few days later than in other media, but more cyanobacteria grew in liquid media, and cyanobacterial growth was faster than green algae. As shown in table 3.1b, the cultures growing in room conditions were very slow compared to those growing in the light cabinet.

3.2.2 Simple recognition of cyanobacteria

Recognition of cyanobacteria from media is greatly aided by their characteristic colour, and is mainly determined by the relative amounts of the major light-harvesting, water-soluble pigments phycocyanin and phycoerythrin (Glazer, 1988), minor colour contributions being due to chlorophyll a and carotenoids (Sandmann, 1988). Fig. 3.2 shows absorption spectra of phycobiliproteins of cyanobacteria (from Lazaroff, 1973; Anagnostidis and Komarek, 1988).

Table 3.1 Comparison of organism growth under different condition

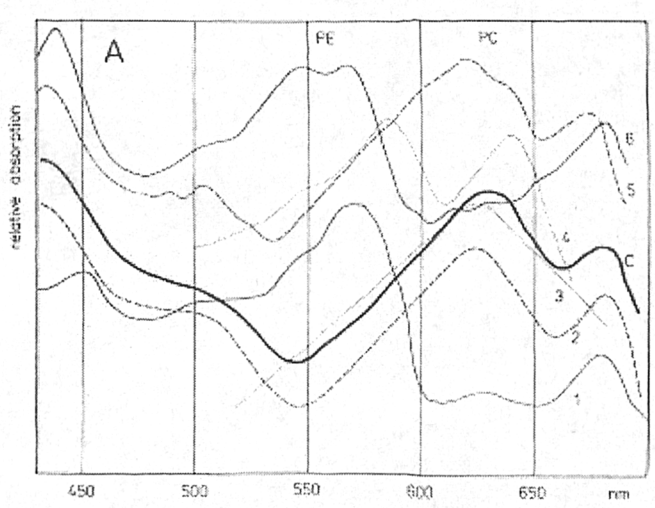
a). light cabinet (at 25-30 °C, with CO_2 and two warm white fluorescence lights, shaking at 100 rpm)

		Sample number *							
Media	Type	L1	L2	L3	L4	L5	P6	P7	P8
BG11	Solid	3	10	4	2	3	2	2	2
	Liquid	2	8	5	3	5	3	3	3
BG11/10	S	2	7	4	2	3	2	2	2
	L	3	10	6	4	4	3	3	3
ASM-1	S	3	---	---	3	7	2	2	2
	L	4	---	---	7	4	3	3	3
C	S	4	15	5	2	4	2	2	2
	L	5	14	12	4	6	4	3	3

b). room (at 15-20 °C, with indirect day light from window, no CO_2 supply, shaking at 100 rpm)

		Sample number *							
Media	Type	L1	L2	L3	L4	L5	P6	P7	P8
BG11	S	6	20	18	10	10	6	6	3
	L	7	21	18	4	4	3	3	3
BG11/10	S	6	20	17	10	10	6	6	3
	L	7	27	21	5	5	4	4	4
ASM-1	S	7	---	---	10	10	8	7	4
	L	8	---	---	7	7	4	4	4
C	S	7	27	21	10	10	7	5	4
	L	7	---	18	7	7	4	4	4

* Result is the time (days) till medium turn to visible green colour, the large number indicate slow growing.



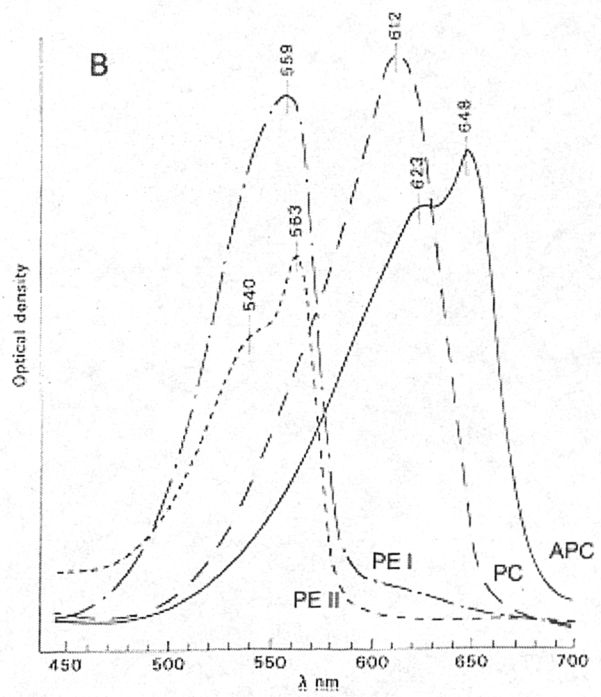


Figure 3.2 Absorption spectra of phycobiliproteins from cyanobacteria.

A: Absorption spectra from whole cell cyanobacteria. C-- common spectrum of cyanobacteria; 1-- red *Phormdium ectocarpi*; 2-- *Oscillatoria* sp. 3-- *Arthrospira maxima*; 4-- *Arthrospira africanum*; 5-- *Planktothrix agardhii*; 6-- red *Planktothrix rubescens* (from Anagnostidis and Komarek, 1988).

B: Biliproteins of *Nostoc muscorum* A prepared from freeze-thaw extracts of cells, precipitated by ammonium sulphate and speparated on calcium phosphate gel. APC-- allophycocyanin; PC-- phycocyanin; PE I-- phycoerythrin I; PE II-- phycoerythrin II (from Lazaroff, 1973).

Samples from natural environments may contain cyanobacteria (oxygenic photosynthetic prokaryotes, containing carotenoids, chlorophyll a and phycobiliproteins), prochloron (oxygenic photosynthetic prokaryotes, containing carotenoids and chlorophll a, b, but do not contain phycobiliproteins), photosynthetic bacteria (prokaryotes, containing carotenoids, but do not contain chlorophyll a and phycobiliproteins) and eukaryote algae (non-prokaryotes, most of them not containing phycobiliproteins). A direct scan of whole cell samples by UV-spectrophotometer (see 2.2.5 method), can differentiate the above organisms. For cyanobacteria, spectra were obtained with more than two peaks at 400-500nm, 615-650nm (and 560-580 nm) and 660-680nm which are carotenoids, phycobiliproteins and chlorophyll a. Respectively for prochloron, two peaks were obtained at 400-500nm for carotenoids and at 650-680nm for chlorophyll a, b. For green algae, two peaks were obtained at 400-500nm for carotenoids and at 650-680nm for chlorophyll a, b, similar to the prochloron result. Then critical examination of the samples under the microscope, ensure that they are prokaryotes. This method has been proved reliable by O_2-evolution measurements (for method see 2.2.6) and pigments (broken cell) test (for method see 2.2.7). The absorption spectra of cyanobacteria with phycocyanin, cyanobacteria with phycocyanin and phycoerythrin, and green-algae, measured on whole cells and a cell-free extract, are shown in Fig. 3.3 and 3.4. The 620nm absorbance maxima were obtained from eight isolates which were isolated from sample sites of Raby pool (see Fig. 2.2 Plate 1). Fig. 3.5 shows the relative composition of coloured isolates from different type of water sample.

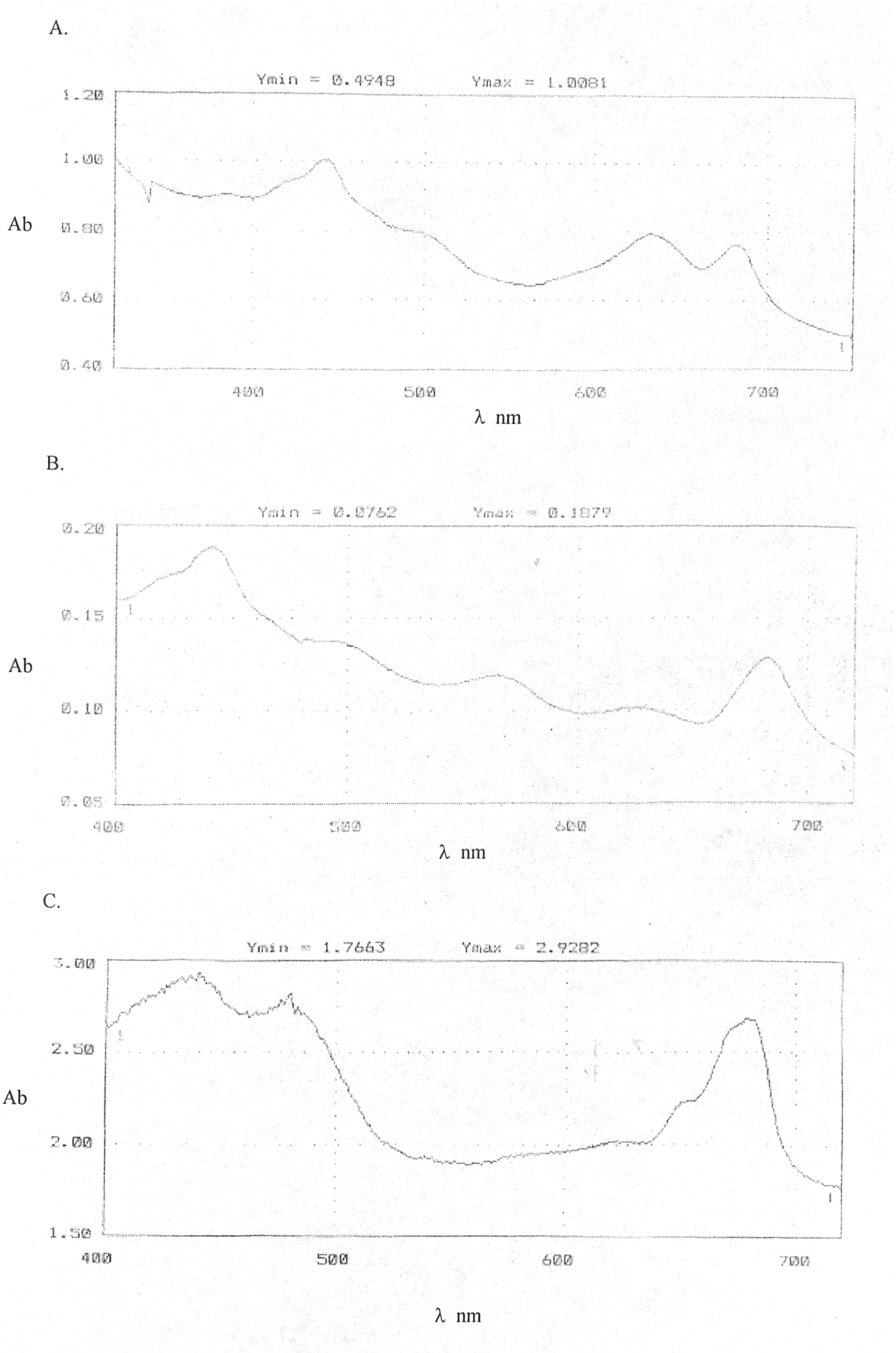


Figure 3.3 Absorption spectra (of whole cells) of different organisms.

A, *Synechococcus* LJM 003; B, *Pseudanabaena* sp. LJM 007; C, green algae.

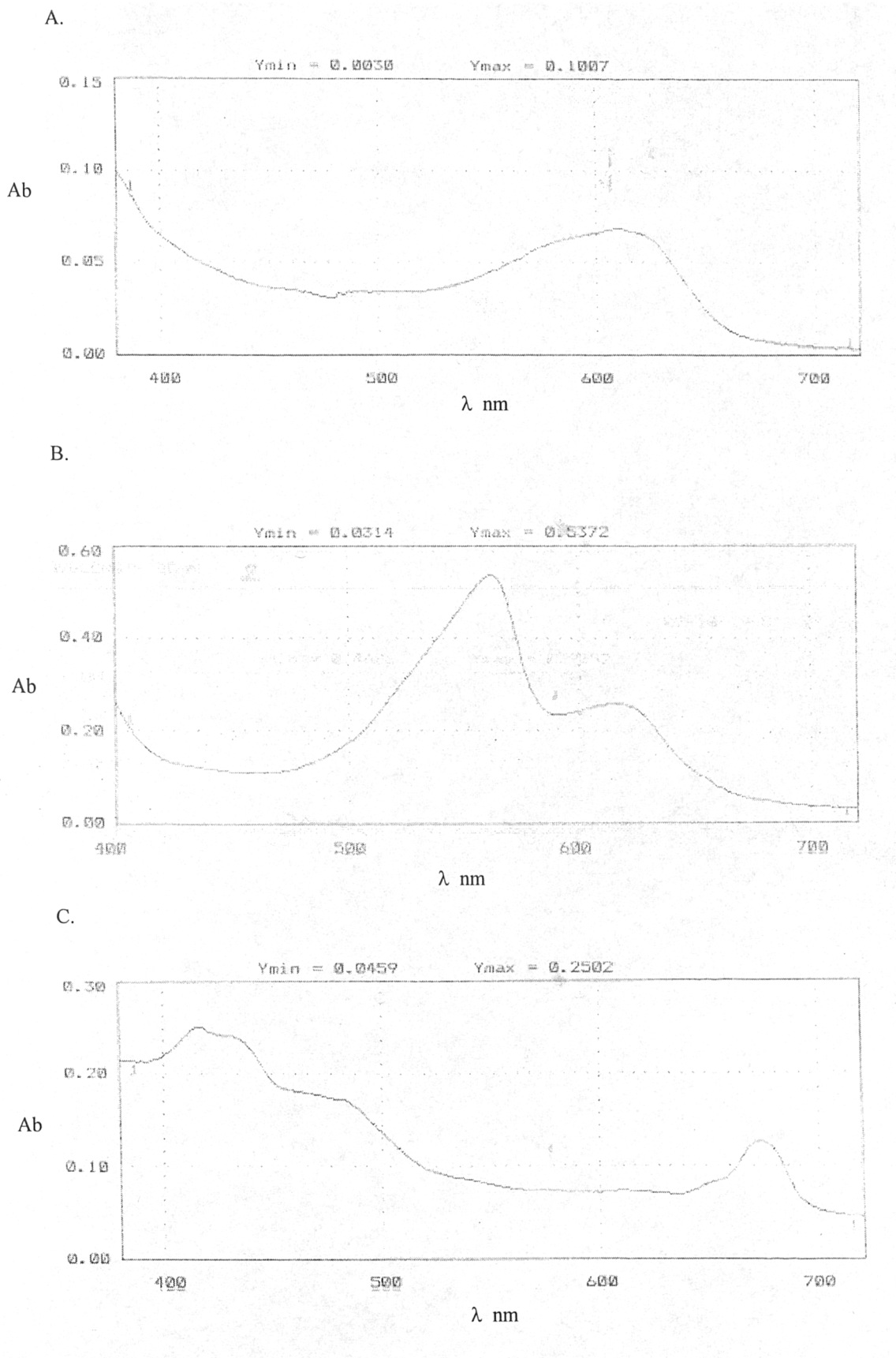


Figure 3.4 Absorption spectra (of cell-freeextracts) of different organisms extraction. A, *Synechococcus* LJM 003; B, *Pseudanabaena* sp. LJM 007; C, green algae.

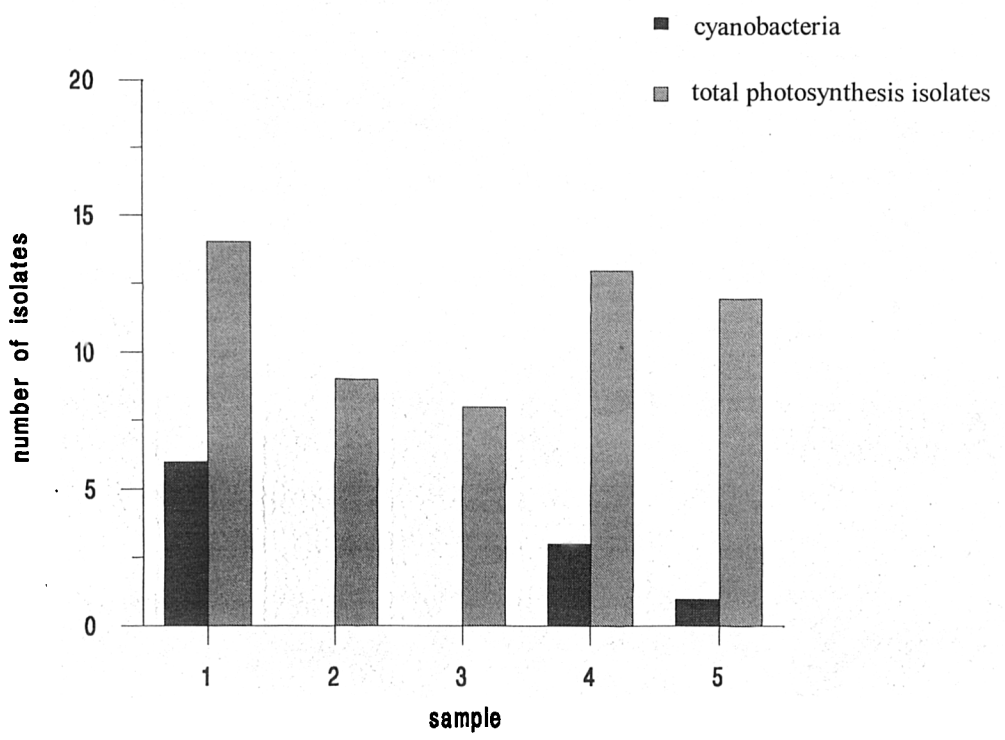


Figure 3.5 The number of cyanobacterial isolates and the total photosynthetic isolates isolated from different types of water sample from Raby pool by usir direct isolation. The indicating of water samples was listed in table 2.3.

3. 2.3 Purification of isolates

Single colonies of each cyanobacterial sample were subcultured repeatedly until pure cultures were obtained. Purification of the cyanobacteria which produce sheath or aggregates proved the most difficult by repeat subculture. Antibiotics, such as Ampicillin (1mg/ml) was added to BG11 medium. A cyanobacterial culture from the mid-exponential phase of growth was incubated in such medium in dark for 16h. Then such culture was incubated in BG11 without Ampicillin to the mid-exponential phase of growth, and sub-cultured repeatedly on to BG11 agar plates which content Nystatin (0.02g/l) until pure cultures were obtained. The temperature was kept at room temperature.

3. 2.4 Classification of isolates from different sampling sites.

Identification to generic level was done following the method of Rippka *et al* (1979), Castenholz and Waterbury (1989) and Komarek and Anagnostidis (1986 and 1988). Specific names, following classical phycological texts are also included, because they have been used widely and could therefore aid the comparison between these strains and those reported elsewhere. Each isolate is described in the following text. Ultrastructure and micrograph of each isolates are given. The characters used to differentiate between various isolates are summarised in Table 3.2.

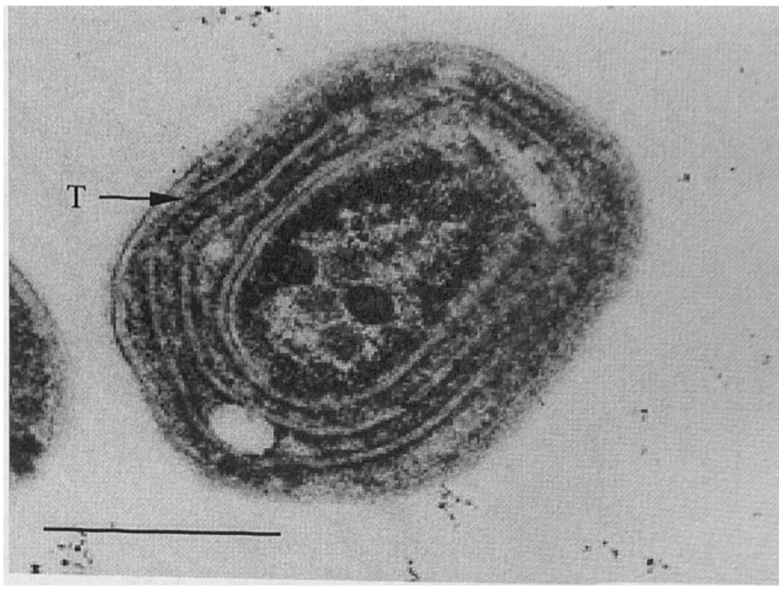
1. *Synechococcus* group

LJM 002

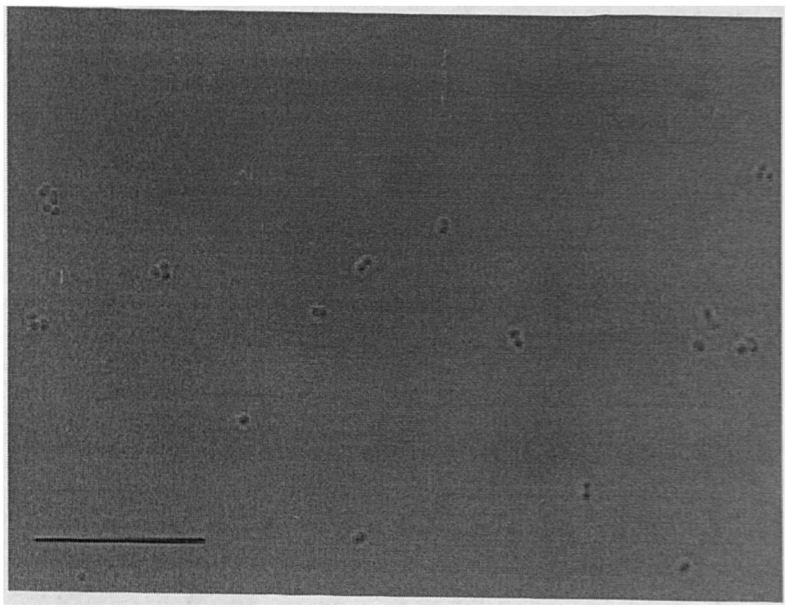
Unicellular short oval to short rod-shaped cyanobacteria that divide by binary fission in a single plane. The cells are 0.6--0.8 μm in diameter, cell length:width are 1.2--1.8, photosynthetic thylakoids present located peripherally, and lack structured sheaths and gas vesicle (see Fig. 3.6 a). Cells are motile with irregular type under light microscopy, do not contain PE or synthesise nitrogenase, and are obligately photoautotrophic. Colonies are pea green colour and spherical or irregular shape, and form soft colonial aggregates.

Figure 3.6. Micrograph of *Synechococcus* LJM 002. a) transmission electron micrograph of a thin section of strain LJM 002, bar, 0.5 μm; b) micrograph of cells or colonies of strain LJM 002, bar, 20 μm. T, thylakoid.

a.



b.

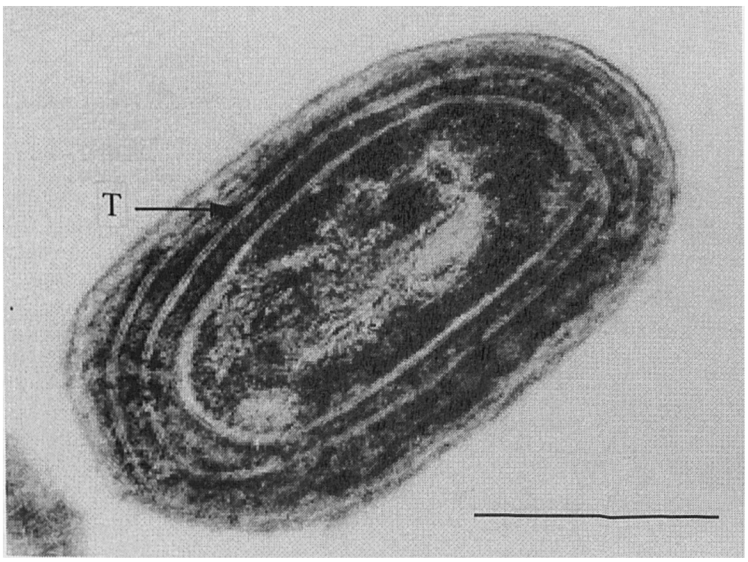


LJM 003

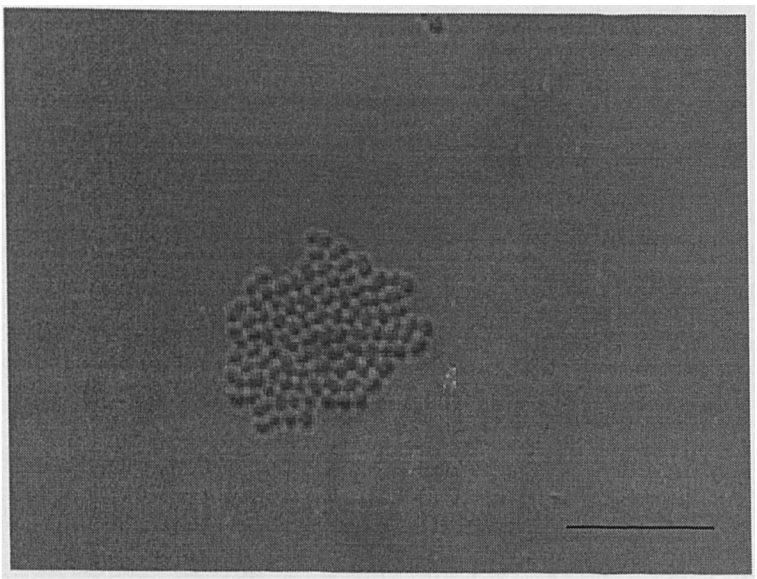
Unicellular short oval to short rod-shaped cyanobacteria that divide by binary fission in a single plane. The cells are 0.6--0.8 μm in diameter, cell length:width are 1.2--2.0, photosynthetic thylakoids present located peripherally, and lack structured sheaths and gas vesicle (see Fig. 3.7 a). Cells are motile with irregular type under light microscopy, do not contain PE or synthesise nitrogenase, and are obligately photoautotrophic. Colonies are pea green colour and spherical or irregular shape, and form very soft colonial aggregates.

Figure 3.7. Micrograph of *Synechococcus* LJM 003. a) transmission electron micrograph of a thin section of strain LJM 003, bar, 0.5 μm; b) micrograph of cells or colonies of strain LJM 003, bar, 10 μm. T, thylakoid.

a.



b.

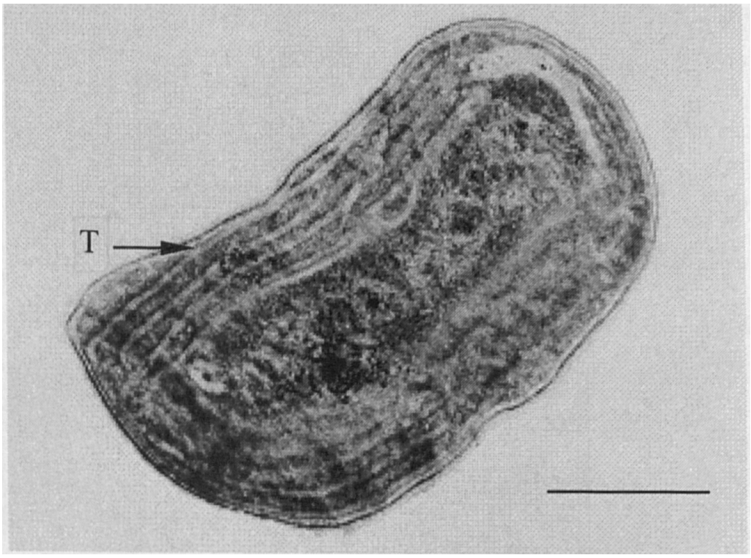


LJM 004

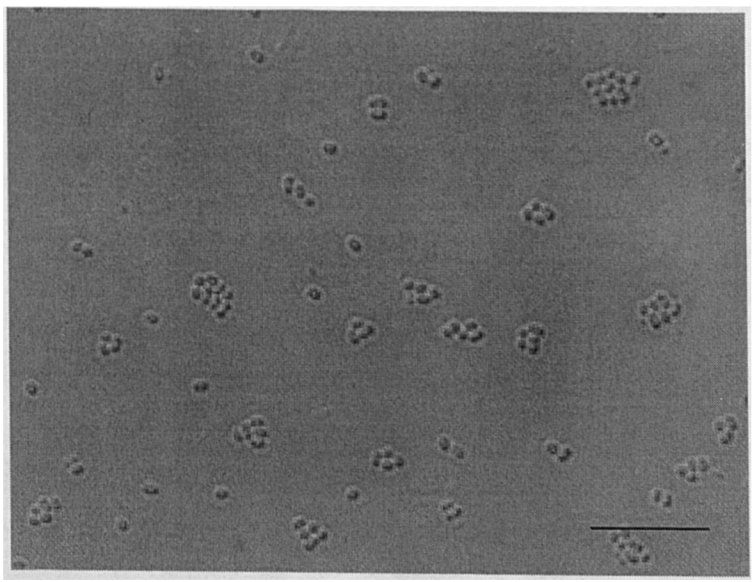
Unicellular short oval to short rod-shaped cyanobacteria that divide by binary fission in a single plane. The cells are 0.6--0.8 μm in diameter, cell length:width are 1.2--2.0, photosynthetic thylakoids present located peripherally, and lack structured sheaths and gas vesicle (see Fig. 3.8 a). Cells are motile with irregular type under light microscopy, do not contain PE or synthesise nitrogenase, and are obligately photoautotrophic. Colonies are light pea green colour and spherical or irregular shape, and form soft colonial aggregates.

Figure 3.8. Micrograph of *Synechococcus* LJM 004. a) transmission electron micrograph of a thin section of strain LJM 004, bar, 0.5 μm; b) micrograph of cells or colonies of strain LJM 004, bar, 10 μm. T, thylakoid.

a.



b.

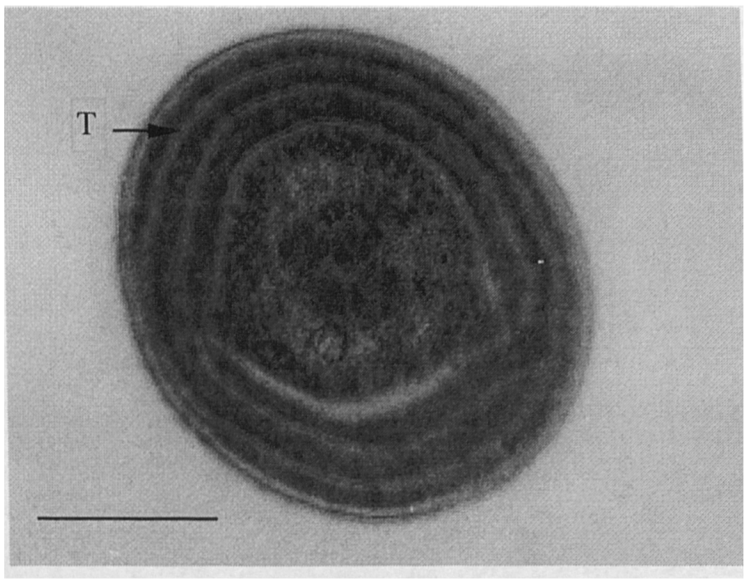


LJM 020

Unicellular short oval to short rod-shaped cyanobacteria that divide by binary fission in a single plane. The cells are 0.6--0.8 μm in diameter, cell length:width are 1.2--2.2, photosynthetic thylakoids present located peripherally, and lack structured sheaths and gas vesicle (see Fig. 3.9 a). Cells are motile with irregular type under light microscopy, do not contain PE or synthesise nitrogenase, and are obligately photoautotrophic. Colonies are pea green colour and spherical or irregular shape.

Figure 3.9. Micrograph of *Synechococcus* LJM 020. a) transmission electron micrograph of a thin section of strain LJM 020, bar, 0.25 μm; b) micrograph of cells or colonies of strain LJM 020, bar, 20 μm. T, thylakoid.

a.



b.

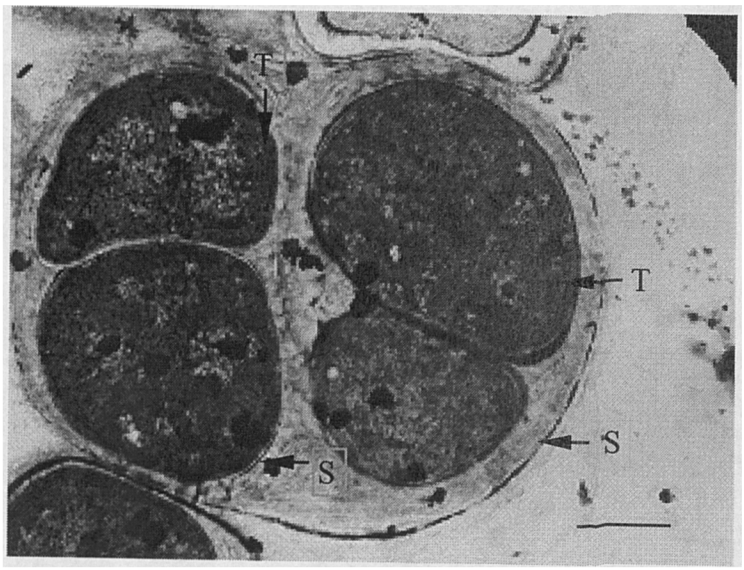
2. *Chroococcidiopsis*

LJM013

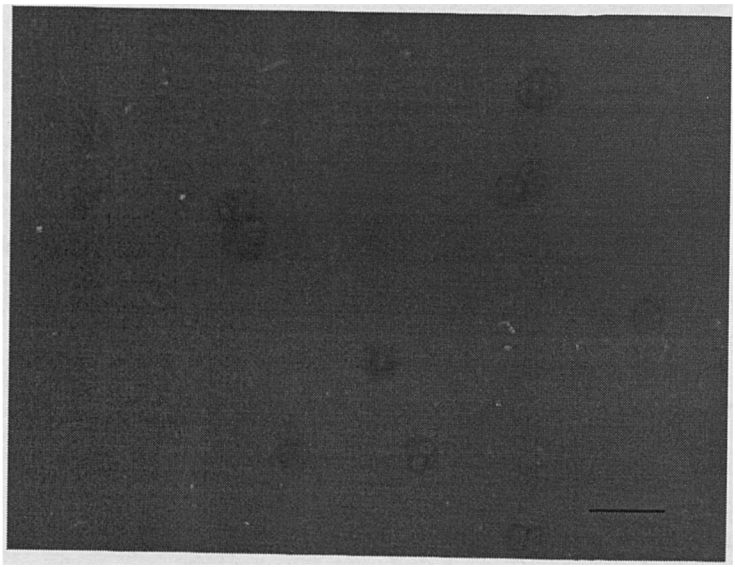
Cyanobacteria that divide by continual binary fission in three planes to produce more or less regular cubical cells of the aggregate followed by the massive release of immotile baeocytes. The baeocyte initiates growth by enlarging symmetrically into a spherical vegetive cell, just before the onset of binary fission. The baeocyte is 3--3.5 μm in diameter, the vegetative cell is 4.5--5 μm in diameter. Photosynthetic thylakoids present located irregularly (*most*) or peripherally, and strong structured sheaths and gas vesicles (see Fig. 3.10 a). Cells do not contain PE, but synthesise nitrogenase, and are photoheterotrophically positive for fructose. Colonies are dark pea green colour and of irregular shape.

Figure 3.10. Micrograph of *Chroococcidiopsis* LJM 013. a) transmission electron micrograph of a thin section of strain LJM 013, bar, 1.0 μm; b) micrograph of cells or colonies of strain LJM 013, bar, 20 μm. S, sheath; T, thylakoid.

a.



b.



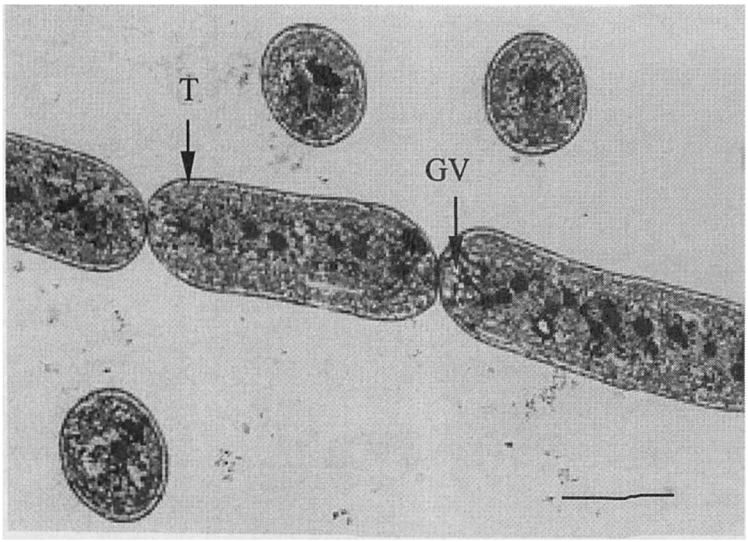
3. *Pseudanabaena*

LJM 001

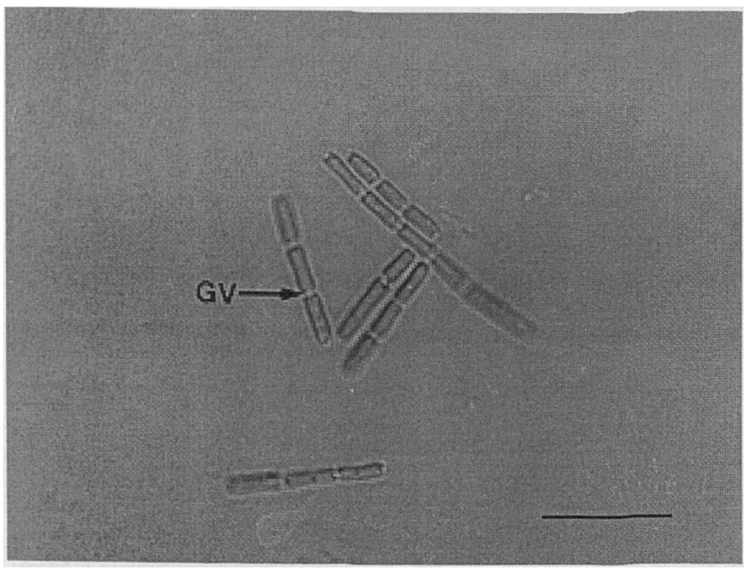
Non-heterocystous filamentous cyanobacteria which have conspicuous constrictions at the cross-walls. The diameter of trichome ranges from about 1.1 to 1.3 μm, cell length:width are 2.2--3.0, photosynthetic thylakoids present located peripherally, and lack structured sheaths, strains have polar gas vacuoles (see Fig. 3.11 a). Trichomes are straight and slightly motile under light microscopy, do not contain PE or synthesise nitrogenase, and are obligately photoautotrophic, 4--10 cells in one trichome. Colonies are pea green colour and "creeping" mats type.

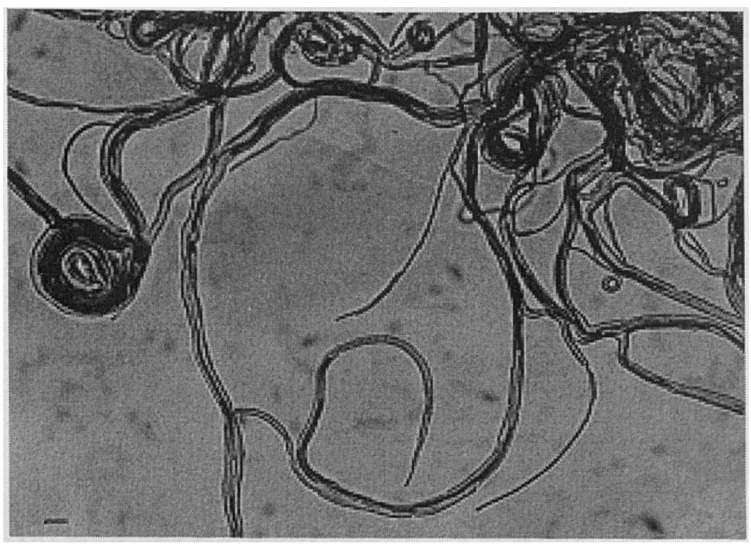
Figure 3.11. Micrograph of *Pseudanabaena* LJM 001. a) transmission electron micrograph of a thin section of strain LJM 001, bar, 1.0 μm; b) micrograph of cells or colonies of strain LJM 001, bar, 10 μm. G, gas vesicle; T, thylakoid.

a.



b.



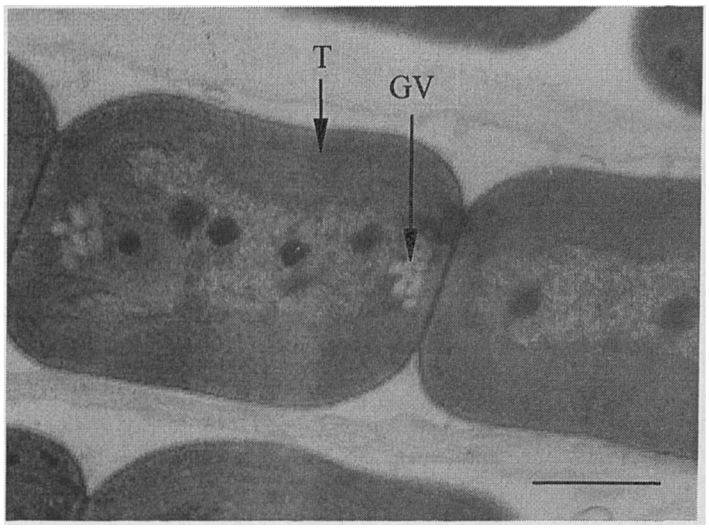


LJM 005

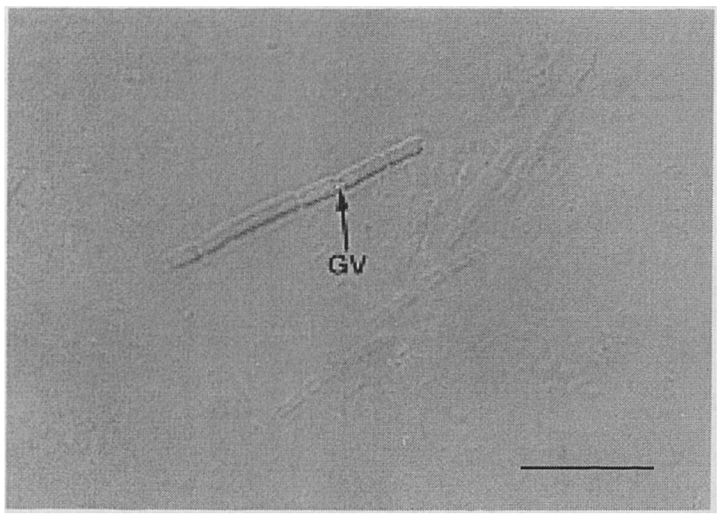
Non-heterocystous filamentous cyanobacteria which have conspicuous constrictions at the cross-walls. The diameter of trichome ranges from about 1.1 to 1.3 μm, cell length:width is 1.8--2.8, photosynthetic thylakoids present located peripherally, and lack structured sheaths, strains have polar gas vacuoles (see Fig. 3.12 a). Trichomes are straight and slightly motile under light microscopy, do not contain PE or synthesise nitrogenase, and are obligately photoautotrophic, 4--10 cells in one trichome. Colonies are pea green colour and "creeping" mats type.

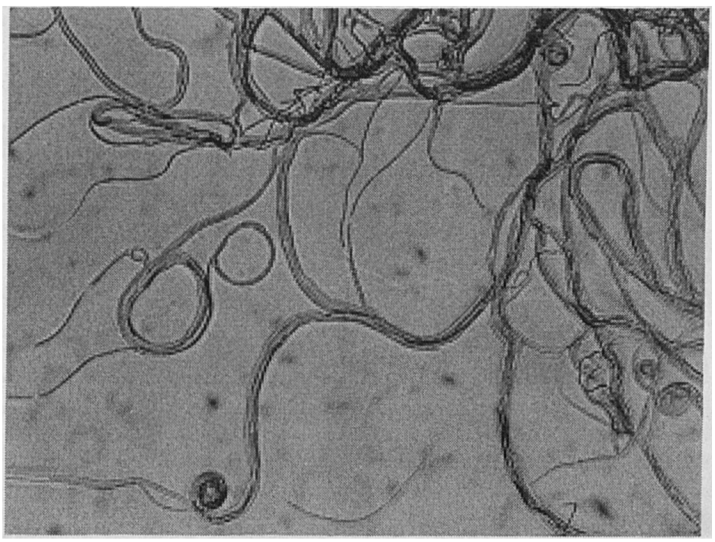
Figure 3.12. Micrograph of *Pseudanabaena* LJM 005. a) transmission electron micrograph of a thin section of strain LJM 005, bar, 0.5 μm; b) micrograph of cells or colonies of strain LJM 005, bar, 10 μm. G, gas vesicle; T, thylakoid.

a.



b.



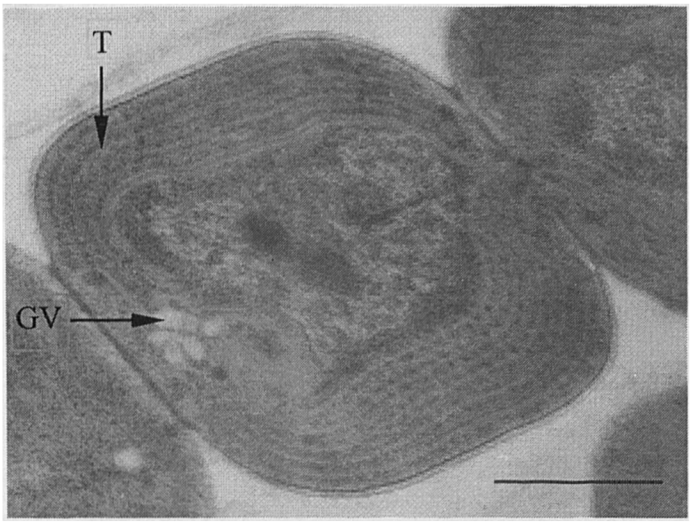


LJM 006

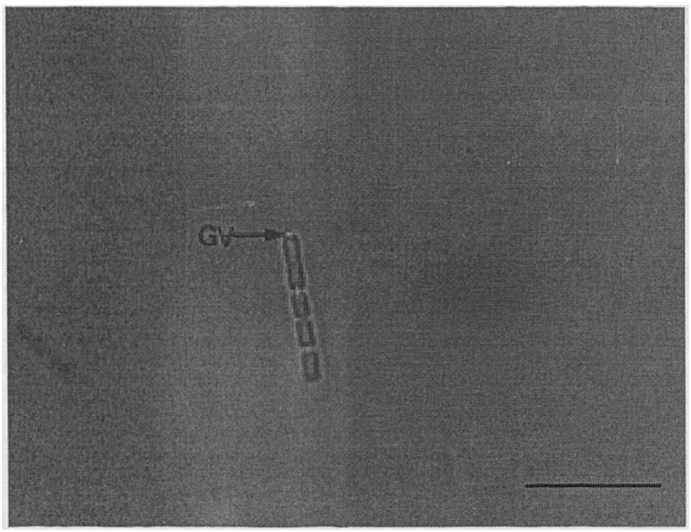
Non-heterocystous filamentous cyanobacteria which have conspicuous constrictions at the cross-walls. The diameter of trichome ranges from about 1.4 to 1.5 μm, cell length:width is 1.2--2.0, photosynthetic thylakoids present located peripherally, and lack structured sheaths, strains have polar gas vacuoles (see Fig. 3.13 a). Trichome are straight and slightly motile under light microscopy, contain PE or synthesise nitrogenase, and are obligately photoautotrophic, 4--10 cells in one trichome. Colonies are black green colour and "creeping" mats type.

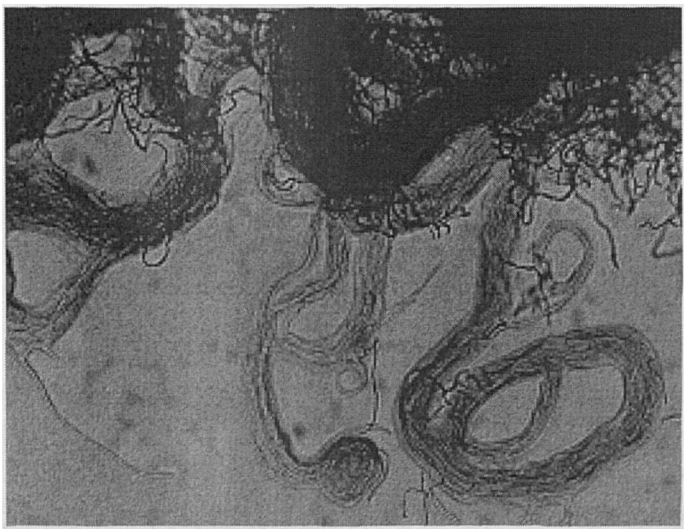
Figure 3.13. Micrograph of *Pseudanabaena* LJM 006. a) transmission electron micrograph of a thin section of strain LJM 006, bar, 0.5 μm; b) micrograph of cells or colonies of strain LJM 006, bar, 10 μm. G, gas vesicle; T, thylakoid.

a.



b.



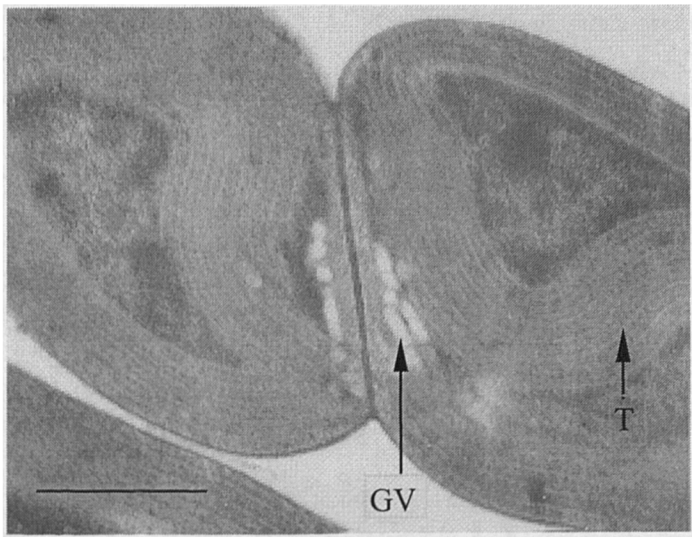


LJM 007

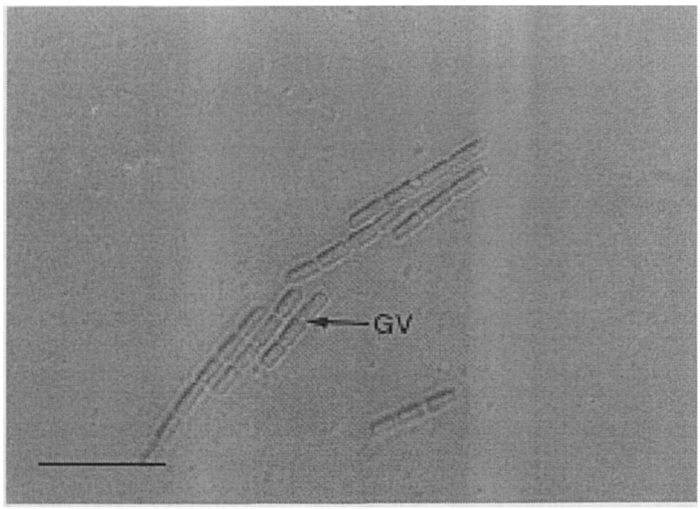
Non-heterocystous filamentous cyanobacteria which have conspicuous constrictions at the cross-walls. The diameter of trichome ranges from about 1.3--1.5 μm, cell length:width are 1.2--2.0, photosynthetic thylakoids present located peripherally, and lack structured sheaths, strains have polar gas vacuoles (see Fig. 3.14 a). Trichomes are straight and slightly motile under light microscopy, contain PE or synthesise of nitrogenase, and are obligately photoautotrophic, 4--10 cells in one trichome. Colonies are dark brown colour and "creeping" mats type.

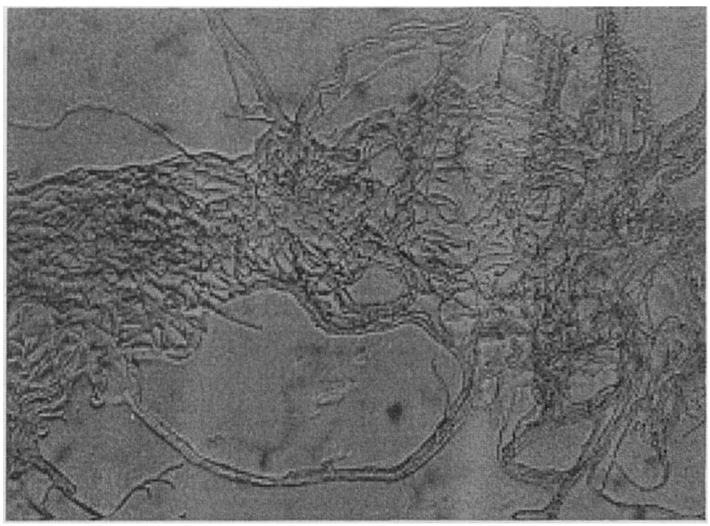
Figure 3.14. Micrograph of *Pseudanabaena* LJM 007. a) transmission electron micrograph of a thin section of strain LJM 007, bar, 0.5 μm; b) micrograph of cells or colonies of strain LJM 007, bar, 10 μm. G, gas vesicle; T, thylakoid.

a.



b.



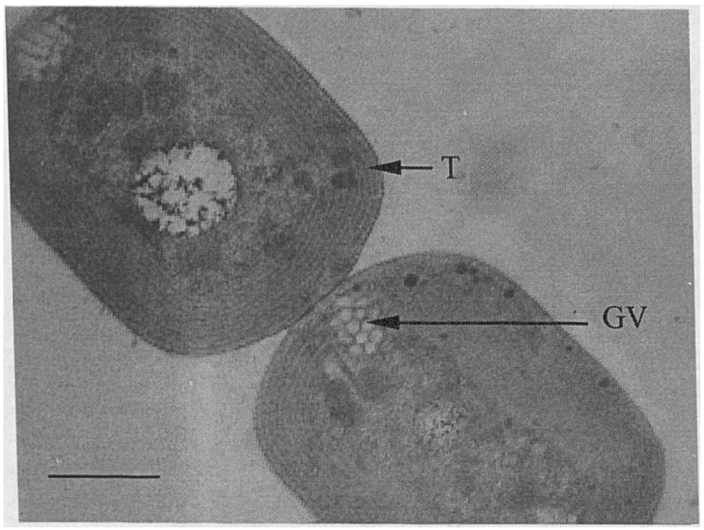


LJM 009

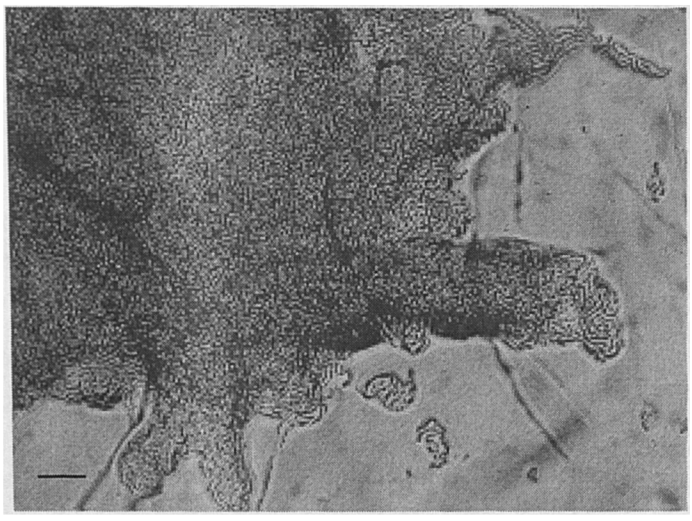
Non-heterocystous filamentous cyanobacteria which have conspicuous constrictions at the cross-walls. The diameter of trichome ranges from about 1.3 to 1.5 μm, cell length:width are 1.3--2.5, photosynthetic thylakoids present located peripherally, and lack structured sheaths, strains have polar gas vacuoles (see Fig. 3.15 a). Trichomes are straight and slightly motile under light microscopy, do not contain PE or synthesise nitrogenase, and are obligately photoautotrophic, 3--4 cells in one trichome. Colonies are pea green colour and "creeping" mats type.

Figure 3.15. Micrograph of *Pseudanabaena* LJM 009. a) transmission electron micrograph of a thin section of strain LJM 009, bar, 0.5 μm; b) micrograph of cells or colonies of strain LJM 009, bar, 40 μm. G, gas vesicle; T, thylakoid.

a.



b.

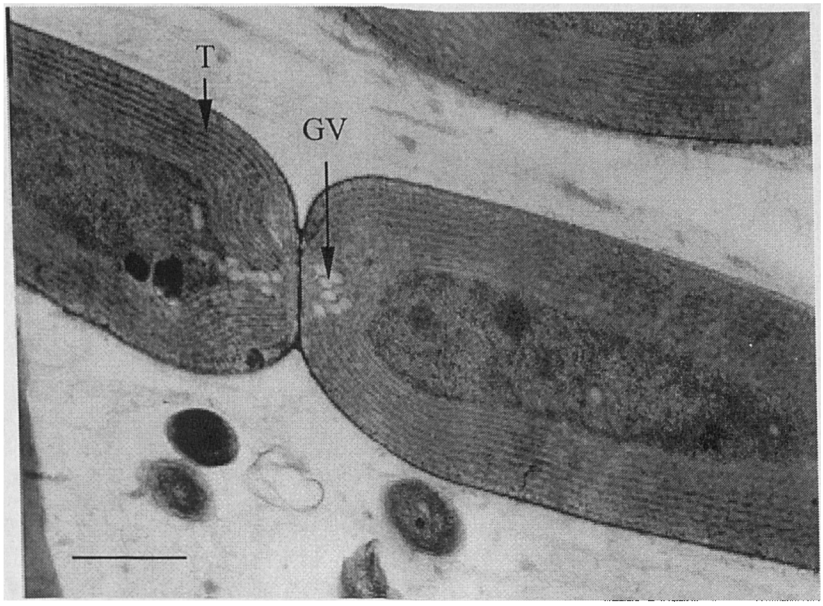


LJM 010

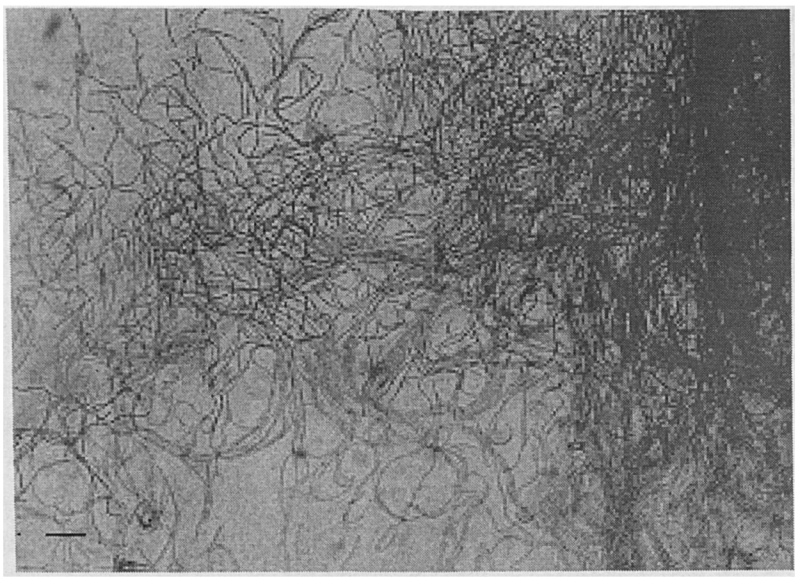
Non-heterocystous filamentous cyanobacteria which have conspicuous constrictions at the cross-walls. The diameter of trichome ranges from about 1.2 to 1.4 μm, cell length:width is 2.0--2.8, photosynthetic thylakoids present located peripherally, and lack structured sheaths, strains have polar gas vacuoles (see Fig. 3.16 a). Trichomes are straight and slightly motile under light microscopy, do not contain PE or synthesise nitrogenase, and are obligately photoautotrophic, 3--8 cells in one trichome. Colonies are pea green colour and "creeping" mats type.

Figure 3.16. Micrograph of *Pseudanabaena* LJM 010. a) transmission electron micrograph of a thin section of strain LJM 010, bar, 0.5 μm; b) micrograph of cells or colonies of strain LJM 010, bar, 40 μm. G, gas vesicle; T, thylakoid.

a.



b.

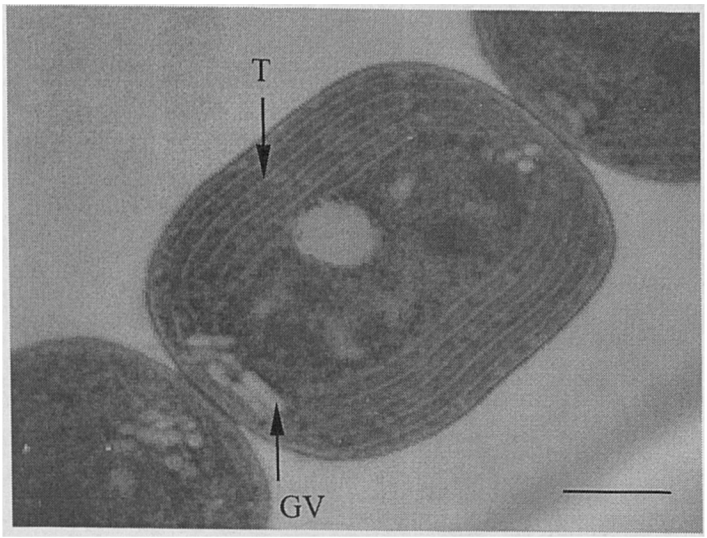


LJM 016

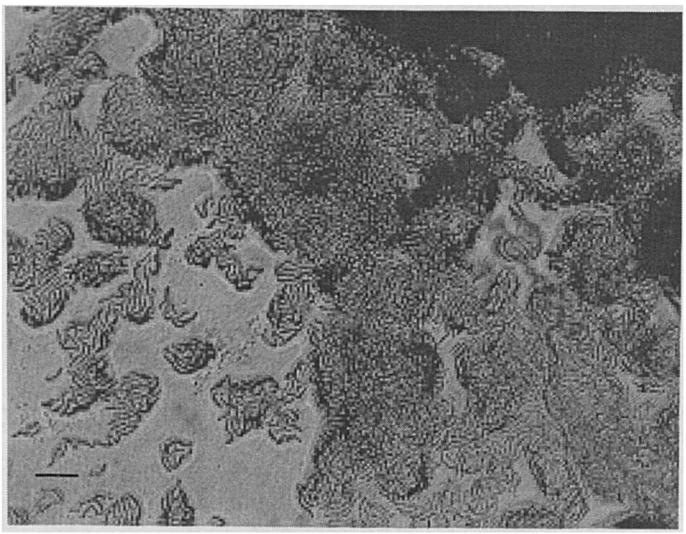
Non-heterocystous filamentous cyanobacteria which have conspicuous constrictions at the cross-walls. The diameter of trichome ranges from about 1.2 to 1.5 μm, cell length:width is 1.3--2.2, photosynthetic thylakoids present located peripherally, and lack structured sheaths, strains have polar gas vacuoles (see Fig. 3.17 a). Trichomes are straight and slightly motile under light microscopy, do not contain PE or synthesise nitrogenase, and are obligately photoautotrophic, 3--4 cells in one trichome. Colonies are pea green colour and "creeping" mats type.

Figure 3.17. Micrograph of *Pseudanabaena* LJM 016. a) transmission electron micrograph of a thin section of strain LJM 016, bar, 0.5 μm; b) micrograph of cells or colonies of strain LJM 016, bar, 40 μm. G, gas vesicle; T, thylakoid.

a.



b.

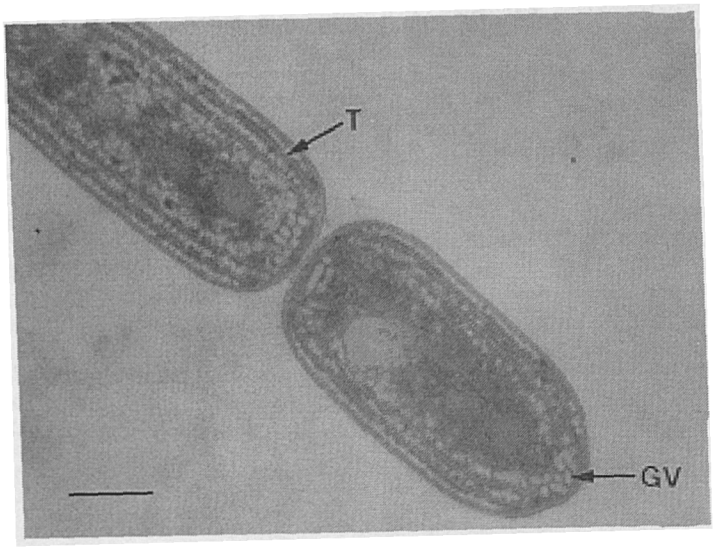


LJM 019

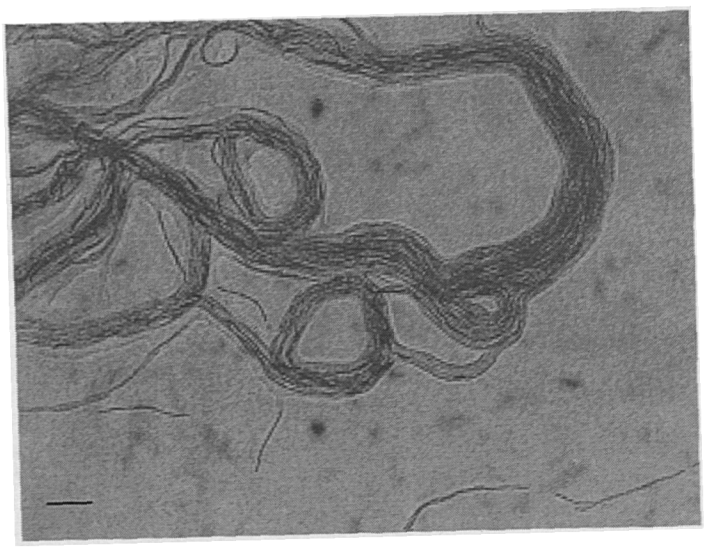
Non-heterocystous filamentous cyanobacteria which have conspicuous constrictions at the cross-walls. The diameter of trichome ranges from about 1.0 to 1.45 μm, cell length:width are 1.8--2.8, photosynthetic thylakoids present located peripherally, and lack structured sheaths, strains have polar gas vacuoles and (see Fig. 3.18 a). Trichomes are straight and slightly motile under light microscopy, do not contain PE or synthesise nitrogenase, and are obligately photoautotrophic, 4--6 cells in one trichome. Colonies are pea green colour and "creeping" mats type.

Figure 3.18. Micrograph of *Pseudanabaena* LJM 019. a) transmission electron micrograph of a thin section of strain LJM 019, bar, 0.5 μm; b) micrograph of cells or colonies of strain LJM 019, bar, 40 μm. G, gas vesicle; T, thylakoid.

a.



b.



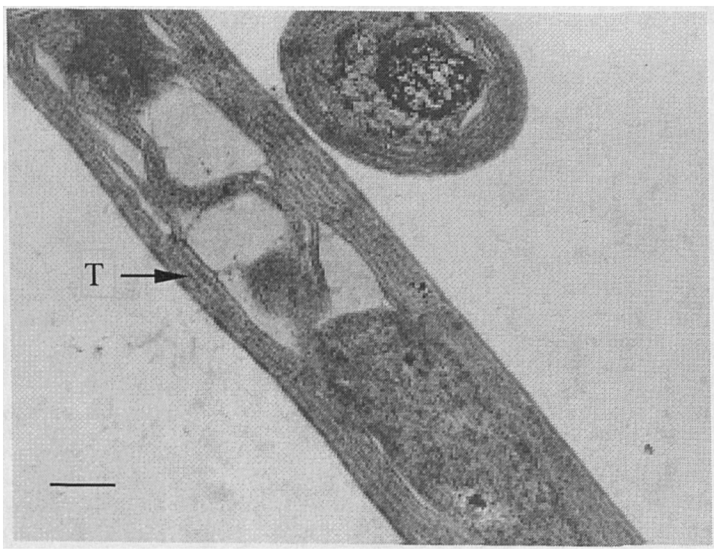
4. *Leptolyngbya*

LJM 012

Non-heterocystous filamentous cyanobactertia, cylindrical trichome immotile, but slightly waved at end, slightly constricted at the cross walls. Form thin sheaths around the trichome in dependence on the culture conditions, false branching lacking, cells much longer than wide, gas vesicles lacking, thylakoids peripherally arranged (see Fig. 3.19 a). The diameter of trichome ranges from about 1.2 to 1.5 μm, cell length:width are 2.0--2.8, Trichomes do not contain PE or synthesise nitrogenase, and are obligately photoautotrophic. Colonies are pea green colour and fascicles or mats type; filaments more or less involved into tangled flaky clusters

Figure 3.19. Micrograph of *Leptolyngbya* LJM 012. a) transmission electron micrograph of a thin section of strain LJM 012, bar, 0.5 μm; b) micrograph of cells or colonies of strain LJM 012, bar, 20 μm. G, gas vesicle; T, thylakoid.

a.



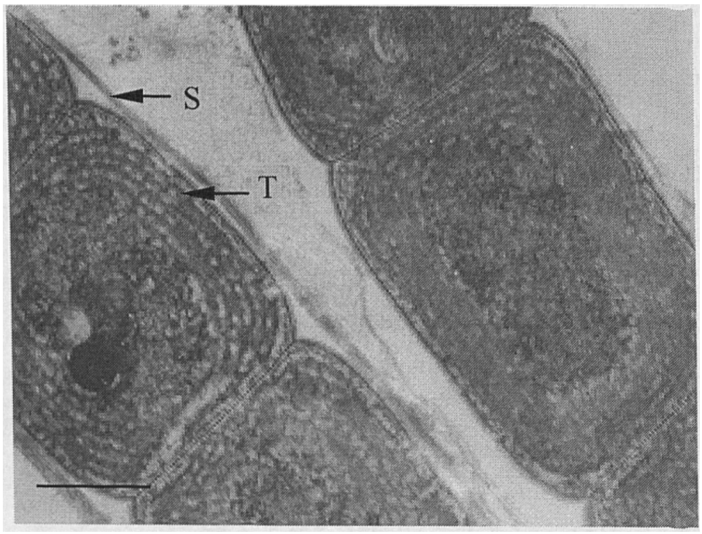
b.

LJM 014

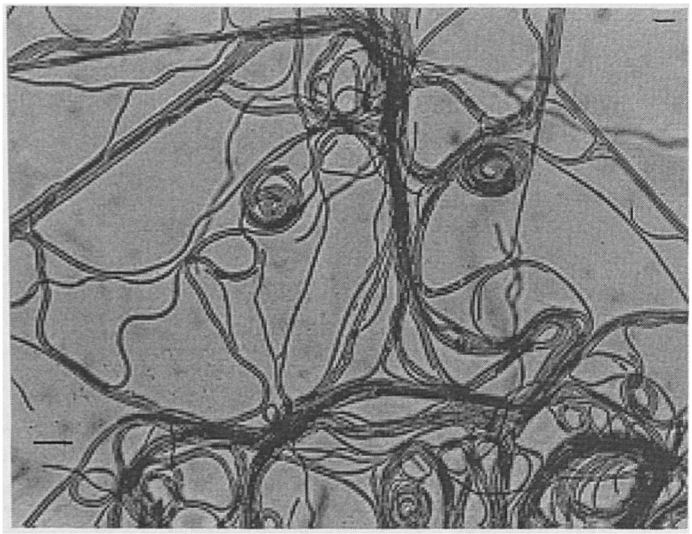
Non-heterocystous filamentous cyanobacteria, cylindrical trichome immotile, with small deep constrictions at the cross walls. Thick sheaths around the trichome, false branching lacking, cells ± isodiametric or shorter or longer than wide, lacking gas vesicles, thylakoids peripherally arranged (see Fig. 3.20 a). The diameter of trichome ranges from about 1.4 to 1.7 μm, cell length:width are 0.7--1.5, Trichomes contain PE or synthesise nitrogenase, and are obligately photoautotrophic. Colonies are black colour and fascicles or mats type. filaments more or less involved into tangled flaky clusters

Figure 3.20. Micrograph of *Leptolyngbya* LJM 014. a) transmission electron micrograph of a thin section of strain LJM 014, bar, 0.5 μm; b) micrograph of cells or colonies of strain LJM 014, bar, 40 μm. G, gas vesicle; S, sheath; T, thylakoid.

a.



b.



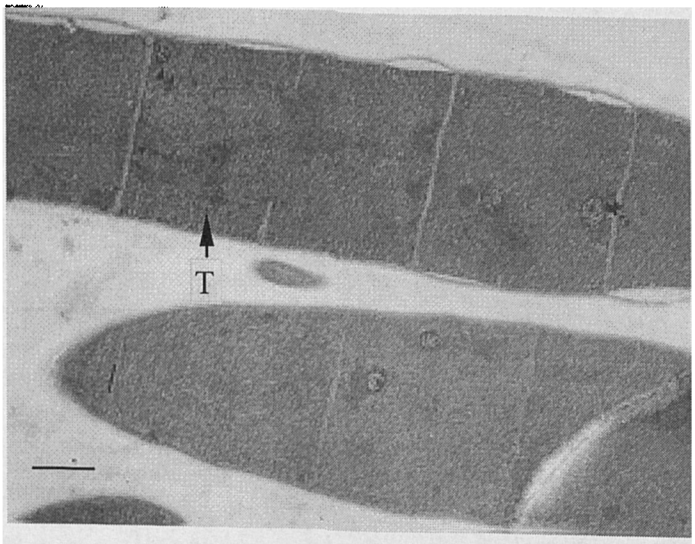
5. *Phormidium*

LJM 017

Non-heterocystous filamentous cyanobacteria, cylindrical trichomes waved at end, unconstricted at the cross walls. Ability to form thin sheaths around the trichome dependent on the culture conditions, lacking false branching, cells ± isodiametric or shorter or longer than wide; lacking gas vesicles, thylakoids perpendicular to the walls, thallus, extensive, fine, and compact up to leather-like. The diameter of the trichome ranges from about 3.0 to 4.0 μm, cell length:width is 0.8--1.1, Trichomes do not contain PE, but synthesise nitrogenase, and are photoheterotrophic positive for sucrose. Colonies are pea green colour and mats type.

Figure 3.21. Micrograph of *Phormidium* LJM 017. a) transmission electron micrograph of a thin section of strain LJM 017, bar, 1.0 μm; b) micrograph of cells or colonies of strain LJM 017, bar, 40 μm. G, gas vesicle; S, sheath; T, thylakoid.

a.



b.

Table 3.2 Properties of the cyanobacterial strains isolated from the lakes (Tai, Bala, and Windermere) and the Raby pool

	Unicellular				
	Synechococcus				*Chroococcidiopsis*
Properties	LJM002	LJM003	LJM004	LJM020	LJM013
Number of sample isolated from	1, 5	1, 4	4	10, 11	6, 7
Reproduction[a,b]	binary fission	binary fission	binary fission	binary fission	binary and multiple fission
Division in one plane[b]	+	+	+	+	--
Thylakoids[a]	+	+	+	+	+
Sheath layers[a]	--	--	--	--	+
Synthesis of PE	--	--	--	--	--
Synthesisof nitrogenase	--	--	--	--	+
Photoheterotroph					
Glucose	--	--	--	--	--
Fructose	--	--	--	--	+
Sucrose	--	--	--	--	--
Ribose	--	--	--	--	--
Glycerol	--	--	--	--	--
VB_{12} requirement	--	--	--	--	--
Cell width[b] (μm)	0.6-0.8	0.6-0.8	0.6-0.8	0.6-0.8	4.0-4.5

cont. Table 3.2

	Unicellular				
	Synechococcus				*Chroococcidiopsis*
Properties	LJM002	LJM003	LJM004	LJM020	LJM013
Cells (lg:lt)	1.2-1.7	1.2-2.0	1.2-2.0	1.2-1.8	1.0-1.3
Cell shape[b]	shortly oval	shortly oval	shortly oval	shortly oval	spherical
Cell motile[b]	++	++	++	++	--
Gas vacuoles[a,b]	--	--	--	--	--
Special mucilaginous envelopes around the cells	(+)	(+)	(+)	(+)	--
Mucilage (colonial)		--	--	--	-- +
Colonial aggregates	+++	++	++	+	+++++
Shape of colonies	spherical, irregular	spherical, irregular	spherical, irregular	spherical, irregular	irregular
Colour of colonies	pea green	pea green	light pea green	pea green	dark pea green

cont. Table 3.2

	Filamentous				
	Pseudanabaena				
Properties	LJM001	LJM005	LJM006	LJM007	LJM010
Number of sample isolated from	1	1	1	1	6, 7
Reproduction[a,b]	trichome breakage	trichome breakage	trichome breakage	trichome breakage	trichome breakage
Cells	vegetative	vegetative	vegetative	vegetative	vegetative
Thylakoids[a]	peripheral concentric	peripheral concentric	peripheral concentric	peripheral concentric	peripheral concentric
Sheath layers[a]	--	--	--	--	--
Synthesis of C-PE	--	--	+	+	--
Synthesisof nitrogenase	--	--	--	--	--
Photoheterotroph					
Glucose	--	--	--	--	--
Fructose	--	--	--	--	--
Sucrose	--	--	--	--	--
Ribose	--	--	--	--	--
Glycerol	--	--	--	--	--
VB_{12} requirement	--	--	--	--	--
Cell width[b] (μm)	1-1.1.3	1.1-1.3	1.4-1.5	1.3-1.5	1.2-1.4
No. of cells in a trichome	4-10	4-10	4-10	4-10	3-8

cont. Table 3.2

	Filamentous				
	Pseudanabaena				
Properties	LJM001	LJM005	LJM006	LJM007	LJM010
Cells (lg:lt)	2.2-3.0	1.8-2.8	1.2-2.0	1.2-2.0	2.0-2.8
Cell shape[b]	cylindrical	cylindrical	cylindrical	cylindrical	cylindrical
Cell motile[b]	(+)	(+)	(+)	(+)	(+)
Gas vacuoles[a,b]	+, polar	+, polar	+, polar	+, polar	+, polar
Special mucilaginous envelopes around the cells	(+)	(+)	(+)	(+)	(+)
Mucilage (colonial)		--	--	--	-- --
Colonial aggregates	+	+	+	+	+
Type of colonies	"creeping" mats	"creeping" mats	"creeping" mats	"creeping" mats	"creeping" mats
Colour of colonies	pea green	pea green	black	dark brown	pea green

cont. Table 3.2

	Filamentous				
	Pseudanabaena		*Leptolyngbya*		*Phormidium*
Properties	LJM009	LJM016	LJM012	LJM014	LJM017
Number of sample isolated from	6, 7	12	6, 7	6,7	12
Reproduction[a,b]	trichome breakage	trichome breakage	trichome breakage	trichome breakage	trichome breakage
Cells	vegetative	vegetative	vegetative	vegetative	vegetative
Thylakoids[a]	peripheral concentric	peripheral concentric	peripheral concentric	peripheral concentric	perpendic to the walls
Sheath layers[a]	--	--	+, thin	+++, thick	(+), thin
Synthesis of PE	--	--	--	+	--
Synthesisof nitrogenase	--	--	--	+	+
Photoheterotroph					
Glucose	--	--	--	--	--
Fructose	--	--	--	--	--
Sucrose	--	--	--	--	+
Ribose	--	--	--	--	--
Glycerol	--	--	--	--	--
VB_{12} requirement	--	--	--	--	--
Cell width[b] (μm)	1.3-1.5	1.2-1.5	1.2-1.5	1.4-1.7	3.0-4.0
No. of cells in a trichome	3-4	3-4	no limited	no limited	no limited

cont. Table 3.2

	Filamentous				
	Pseudanabaena				
Properties	LJM009	LJM016	LJM0012	LJM0014	LJM017
Cells (lg:lt)	1.3-2.5	1.3-2.2	2.0-2.8	0.7-1.5	0.8-1.1
Cell shape[b]	cylindrical	cylindrical	cylindrical	shortly cylindrical	shortly cylindrical
Cell motile[b]	+	+	++	++	++
Gas vacuoles[a,b]	+, polar	+, polar	--	--	--
Special mucilaginous envelopes around the cells	--	--	--	(+)	(+)
Mucilage (colonial)		--	--	--	+ --
Colonial aggregates	--	--	+	+	+
Type of colonies	"creeping" mats	"creeping" mats	mats	mats	mats
Colour of colonies	pea green	pea green	pea green	black	pea green

(+), weak and easily lost

PE, phycoerythrin

a, result of electron microscopy

b, result of light microscopy

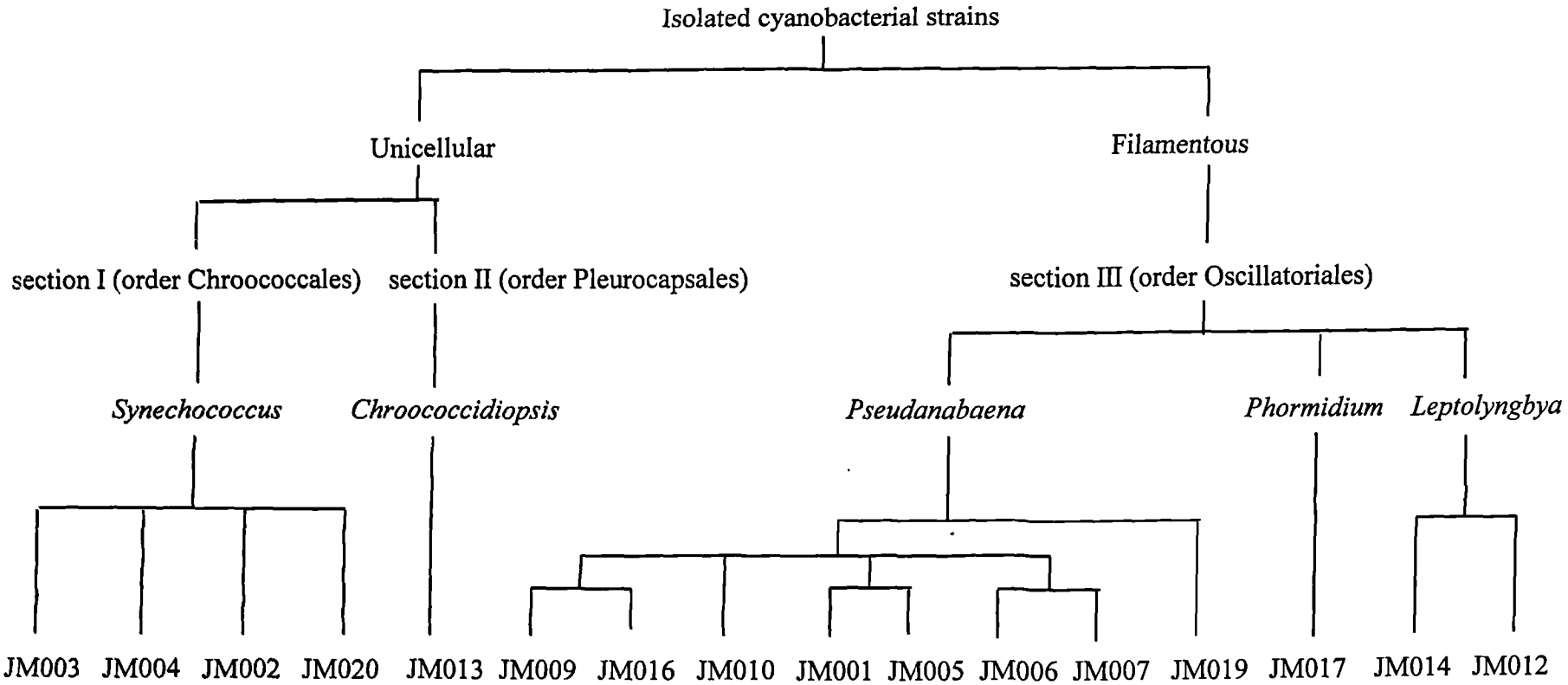


Figure 3.22. The taxonomic relationships of cyanobacterial isolates from different sampling sites. The relationship was listed by using classification methods of Rippka et al (1979), Castenholz and Waterbury (1989) and Anagnostidis and Komarek (1988).

The taxonomic relationships of isolates from different sampling sites is given in figure 3.22.

3.3 Discussion

For isolation of cyanobacteria, some of the more popular media and those utilised in major cyanobacterial culture collections are compared by Rippka (1988). Four different media, two methods and two incubation conditions were chosen here. The four different media were introduced successfully for the isolation of freshwater cyanobacteria. In our experiment, cyanobacteria and green-algae always grew together, and the number of green-algae was always more than cyanobacteria. Media C, BG11 and BG11/10 have been successfully employed for the isolation of cyanobacteria from samples, but no good results were obtained from Medium ASM-1, and Medium C was proved more sensitive for some cyanobacterial (*Pseudanabaena*) isolation. In Medium C, the organisms' initial growth was slower than in other media, possibly due to the formation of H_2O_2 on autoclaving (Van Baalen 1967).

The temperature for isolation suggested by Rippka should also be varied in ranges 5 to 10°C apart. Although most cyanobacteria from moderately temperate habitats will grow well at 20-30°C, many do not tolerate temperatures above 20°C. In our studies, same isolates were obtained from samples at 15-20°C and 20-30°C. The Pseudanabaena grew well at 20-30°C, but incubation above 30°C should be avoided, the temperature of isolation being similar to sampling niche temperature.

Cyanobacteria, particularly in the hands of bacteriologists, are normally kept under a continuous light regime rather than under a light-dark cycle, since early investigations by Allen and Arnon (1955) indicated that such a treatment is not harmful to their growth. In our research, this opinion has been confirmed. For example, continuous light can increase the growth speed of *Pseudanabaena*, and prevents the formation of typical colonial aggregates under 30°C.

For purification, two different bactericidal compounds (ampicillin and Nystatin) have been successfully employed for aggregated and sheathed cyanobacteria. But the treatment must be undertaken at room temperature.

Recognition of cyanobacteria from media will only come from: (1) critical examination of the samples under the microscope, to ensure that they are prokaryotes; (2) a demonstration by growth requirements or O_2-evolution measurements, to prove that they are not photosynthetic bacteria; (3) an analysis of their pigment content (cell free), to exclude the possibility that they may be prochloron-like organisms (Rippka, 1988). In our studies and review of previous work, we found one simple and direct method (the whole cell pigment test) to recognize cyanobacteria from axenic culture. Cyanobacteria, eucaryote algae and prochloron grew together in the media, when we used freshwater media to recover cyanobacteria from water courses. A number of workers have found that these organisms contain different pigments as previously stated (Rippka 1988, Lattimer 1957, Glazer 1988).

Previously some workers have used cell-free extracts and thylakoid membranes to test organisms for whether they contain phycobiliproteins and

chlorophyll (Rippka, 1974, 1988; Glazer 1988). The absorbance maxima of the pure chlorophyll a was obtained at 430 and 663nm (Goedheer 1966). The maxima of the absorbance of the pure chlorophyll b was obtained at 455 and 645nm (Hoffman &Werner 1966); phycobiliproteins are present in the form of hexameric complexes with three different linker polypeptides of 27,000, 33,000Da in intact phycobilisomes, the absorbance maxima being obtained at 620nm (Glazer 1988). In this study the same result was obtained by measurement on a cell-free extract and whole cells. Absorbance maxima of phycerythrin at 560-570 nm and phycocyanin at 600-620nm in cell-free extracts were obtained. One absorbance maximum at 565-575nm and two absorbance maxima at 600-700nm were obtained in whole cell samples; two are phycobiliprotein (565-575 and 620-630nm), one is chlorophyll a (660-670) (see Fig. 6.3).

For other green isolates, only one absorbance maximum was obtained, which appeared to be composed of two components as a shoulder appears on the peak (see Fig. 6.3). This method is quiet simple and can be used for recognition of cyanobacteria.

Fewer cyanobacteria species than algae species were obtained during this work. This could be due to the limited amount of sampling used and the selection of sampling sites.

Cyanobacteria species were not isolated from two water samples (ie, 2 and 3). This was due to their sparseness or absence in these samples. It is likely that cyanobacteria are not suited to the local condition, because of low light intensities and phytotoxic compounds produced by rotten woods and leaves. Another reason

for the lack of cyanobacteria in these five water samples could be the antagonistic effect of other microbial types on cyanobacterial growth or some species present were not readily isolated in the standard media. In our studies, we were not particularly interested in obtaining isolates of the different microbial types from the different environment, but more interested in isolating more different species of cyanobacteria which could be obtained from water samples. So in further isolations, we will collect samples from rich-sun light environments, and employ different media and conditions in isolation.

It was necessary to purify all these cyanobacterial isolates in order to carry out the physiological and genetic studies, and it proved very difficult to obtain axenic cultures, although success was achieved with certain species by using antibiotic treatments. This purification problem was not a novel one, Echlin and Morris, (1965) reported that the difficulties might be due to the close interrelationships between cyanobacteria and the contaminant micro-organisms, making their physical separation difficult.

Differences in structure and development permit the classification among cyanobacteria of five large sections (Rippka *et al*, 1979). These five sections correspond to major taxa recognised by phycologists (see Table 1.2). From section to genera, the most part of these genera do not correspond precisely to major botanical taxa. In *Synechococcus*, cells occur singly, in pairs or in short chains and are devoid of sheaths. Nageli (1849) proposed another genus, *Aphanothece*, for organisms like *Synechococcus* that develop as irregular aggregates united by a common slime layer. Although many strains of *Synechococcus* produce considerable quantities of extracellular slime in culture, none develops in the form of aggregates. A new genus Cyanothece has been

proposed by Komarek (1976) to accommodate some species hitherto placed in *Synechococcus.* Its principal distinguishing character is that cells occur either singly or in pairs, but never in chains. Since the extent of chain formation in organisms of the *Synechococcus* type is strongly dependent on the conditions of culture, so Komarek's proposal was not accepted by Rippka (1979) and Castenholz and Waterbury (1989). In this study, four isolates were placed into genus *Synechococcus,* cluster *Cyanobium* or cluster 5 according to Rippka (1979, 1992) and Castenholz and Waterbury (1989). The first problem of identification for these *Synechococcus* is too small cell size and small length:width (see Table 3.2) to observe cell division in one plane or in two or three plane by light microscope. A further problem is the difficulty of distinguishing between these strains. These strains were roughly separated by slightly different length:width, colonies colour and extent of softness of colonies aggregates. This result was very easily confirmed by RAPDs data (see section 5.6).

The specificity of genus *Pseudanabaena* was mentioned by several authors (Anagnostidis 1961; Rippka *et al.* 1979; Guglielmi and Cohen-Bazire 1982, 1984a,b; Anagnostidis and Komarek 1988; and Castenholz and Waterbury 1989). In this study, a combined approach was used. Eight isolates were placed into genus *Pseudanabaena* according to Rippka (1979) and Castenholz and Waterbury (1989), and seven of them into *Pseudanabaena* sp. according to Anagnostidis and Komarek (1988). The similar strains LJM001 and LJM005; LJM009 and LJM016 were separated by RAPDs data (see section 5.6). The strain LJM009 isolated from Taihu lake of China is extremely similar to strain LJM016 isolated from Bala lake of North Wales. What relationship exists between these two strains, could be very interesting.

The LPP group has been changed a lot in the revisions of Castenholz (1989). All strains in LPP A and some strains in LPP B (Trichome immobile in persistent sheath) have been assigned to the genera *Lyngbya* (one trichome per sheath) and *Microcoleus* (two or more trichome per sheath). Other LPP strains (trichome motile, no persistent sheath) have been transferred to *Pseudanabaena* (strong constriction), *Oscillatoria* and *Trichodesmium* (absent or feeble constriction). In the revisions of Rippka & Herdman (1992), the LPP A is renamed *Lyngbya*. Most of the strains previously assigned to the LPP group B have been assigned to the genera *Geitlerinema* and *Leptolyngbya*, and a few strains (more than one trichome per sheath) have been temporarily transferred to *Microcoleus*. Three isolates were placed into LPP group, LJM 012 and LJM 014 were placed into genus *Leptolyngbya*, LJM 017 was placed into genus *Phormidium* according to Anagnostidis and Komarek (1988). The differences of strain LJM 012 and LJM 014 are trichome waved or not, slightly constricted or little deep constricted at the cross walls, form thin sheaths or thick sheaths around the trichome, cells much longer than wide or ± isodiametric. The differences of *Leptolyngbya* LJM 012 and LJM 014 with *Phormidium* LJM 017 are thylakoids peripherally arranged or perpendic to the walls, slightly constricted to little deep constricted or unconstricted at the cross walls, ability to form sheaths around the trichome, and *Phormidium* cells are always shorter than wide. According to revisions of Castenholz (1989), all of these three strains would be placed into genus *Lyngbya*. It was not accepted in this study.

Chapter 4

Isolation of

DNA from Cyanobacteria

4.1 Introduction

Twenty years ago, little was known about the properties and organisation of the genome of the cyanobacteria, although a few studies had been published on the chromosomal (c) DNA of some unicellular cyanobacteria (Herdmann, 1976; Herdmann *et al.*, 1979). The first report documenting the presence of covalently closed circular (ccc) DNA in cyanobacteria used *Synechococcus* 6301 (Asato and Ginoza, 1973). Subsequently, plasmids were investigated in *Agmenellun quadruplicatum* PR-6 (Roberts and Koths 1979), a number of other unicellular cyanobacteria (Lau and Doolittle, 1979; van den Hondel *et al.*, 1979; Laudenbach *et al.*, 1983; Anderson and Eiserling, 1985) and in filamentous species (Simon, 1978; Friedberg and Seikffers, 1979; Reaston *et al.*, 1980). Lau and Doolittle (1979) indicated that different plasmids within the same strain and different plasmids within different strains might (but do not always) contain restricted regions of sequence homology. They suggested that these regions of homology may be analogous to the transposable genetic elements of bacterial plasmids. This together with indirect, but compelling, evidence for interspecific (or intergeneric) plasmid transfer, indicates that plasmids may play a role in the ecology and evolution of obligately autotrophic prokaryotes. Indeed plasmid profiles have proved useful in analysing genetic relatedness of various cyanobacteria (Lau and Doolittle, 1979; van den Hondel *et al.*, 1979; Lau *et al.*, 1980).

Early studies on cyanobacterial plasmids often involved the use of large-scale isolation involving caesium chloride and, in many cases, sucrose gradients (van den Hondel *et al.*, 1979; Lau *et al.*, 1980), although Simon (1978) has used

gentle lysis conditions, under which most chromosomal DNA remains membrane bound and can be removed by centrifugation.

To avoid such time-consuming steps, rapid mini-methods have been developed for resolving plasmid DNA (Friedberg and Seikffers, 1979; Lambert and Carr, 1982; Goyal, 1992). Chromosomal and plasmid DNA are often obtained from cells that are treated with lysozyme and lysed with a detergent. RNA and proteins are removed by RNase and protease treatments. The plasmid DNA is then released from the folded chromosomal complex by a shearing step or by RNase treatment (Kado and Liu, 1981). In this chapter some of the reported mini-methods have been modified and tested on laboratory strains and cyanobacterial isolates for the rapid isolation of plasmids and total DNA of suitable quality for restriction analysis and PCR. Initially methods were tested on laboratory cultures of *Synechococcus* PCC7942 (*Anacystis nidulans* R2), which is known to harbour two plasmids, pANl (large plasmid) and pANs (small plasmid) (van den Hondel *et al.*, 1979). In addition, plasmid profiles of selected cyanobacterial strains have been examined with a view to rapid identification of cyanobacteria.

4.2 Results

4.2.1 Comparison of methods for plasmid isolation

The presence of covalently closed circular DNA in *Synechococcus* PCC7942, was confirmed following purification of plasmid DNA from cleared lysates by CsCl-EtBr density-gradient centrifugation, as described in section 2.2.14.2. Two DNA bands were obtained, The lower band of plasmid DNA was analysed by

agarose gel electrophoresis and found to contain two ccc plasmid DNAs, of molecular sizes 48.5±1.5 kb (pANl) and 8±0.3 kb (pANs), and the plasmid sizes were confirmed by restriction, using restriction endonuclease *Bam* HI. The sizes estimated from this study are comparable to the sizes reported by Laudenbach *et al.* (1983) (48.5kb, and 8kb), Lau and Doolittle (1979) (46.1±1.2 kb and 8.08±0.075 kb) and van den Hondel *et al.* (1979) (49.85±1.5 kb and 7.93±0.225).

Five rapid mini-methods (see section 2.2.14.2) were compared for plasmid recovery from *Synechococcus* PCC7942. The yield of plasmid DNA was measured by using the Phoretix ID package (version 2.51) based on the intensity of UV induced-fluorescence of DNA bands on agarose gels. Electrophoresis of the plasmid preparations from *Synechococcus* PCC7942 (Fig. 4.1), indicated that method 1 and method 2 were both sensitive procedures. A very high yield for pANl (large plasmid) and very low yield for pANs (small plasmid) were obtained by method 1. Both pANl (27% yield of method 1) and pANs were obtained by method 2. Chromosomal DNA and RNA were eliminated in method 1 by heat-treatment (55°C for 2h) and incubation with RNase. For method 2, the chromosomal DNA and RNA were removed by incubation with RNase and overnight precipitation by NaCl. Method 3, method 4 and method 5 were very rapid procedures, but only a low yield pANs was obtained from *Synechococcus* PCC7942 by method 3; and a low yield of plasmid pANl by using method 4. No plasmids were obtained by method 5. When the cell sample was subjected to freezing followed by thawing before extraction of the DNA, the ratio of the ccc form of plasmid DNA to oc form was decreased. The use of frozen and thawed cells produced 0%-40% less of the large plasmid (pANl) and a similar amount of the small plasmid (pANs), together with 30%-50% more RNA compared with the unfrozen sample (Fig. 4.2).

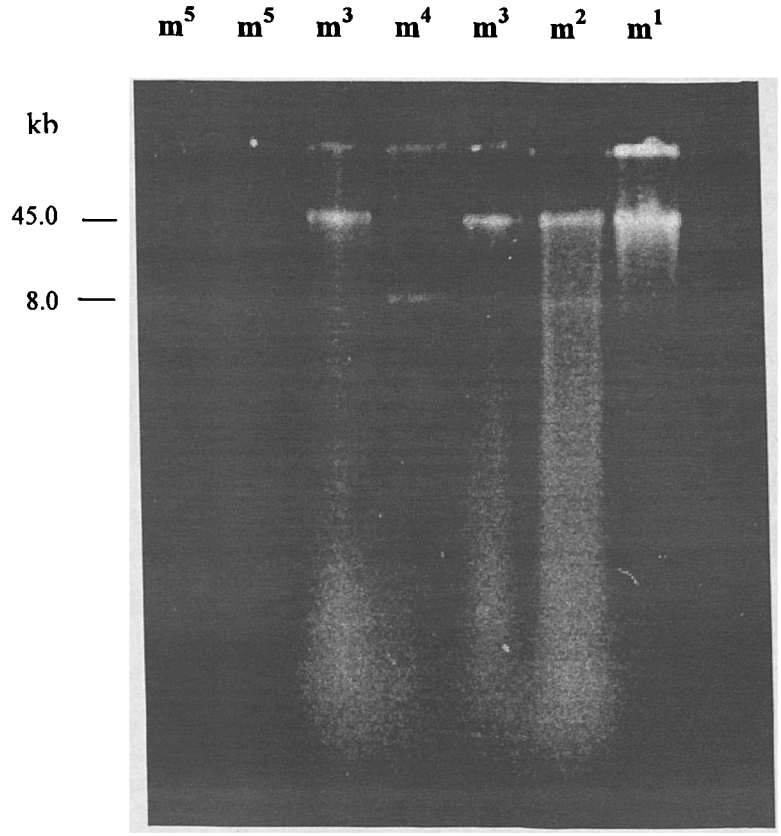


Figure 4.1. Agarose gel electrophoresis of plasmid DNA prepared by five rapid methods (lane m^1-m^5) from *Synechococcus* PCC7942. Separation was achieved on 0.8% (w/v) agarose horizontal gel as described in materials and methods (section 2.2.15).

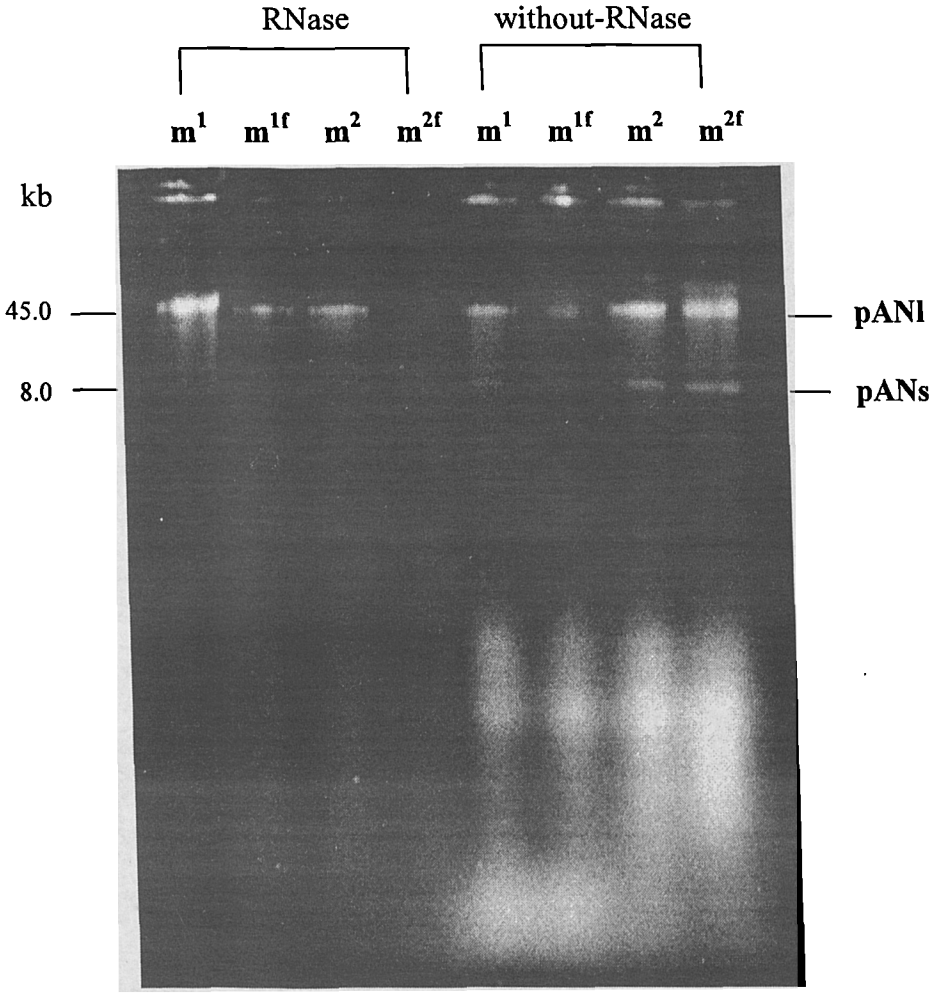


Figure 4.2. Agarose gel electrophoresis of plasmid DNA preparation. Plasmid prepared by two rapid methods: method 1 (lane m^1) and method 2 (lane m^2) from a fresh cell sample and from freeze-thawed cell sample (m^f) of *Synechococcus* PCC7942.

4.2.2 Analysis of extracted plasmid DNA

Electrophoresis of plasmids prepared from *Synechococcus* PCC7942 after heat-treatment showed the disappearance of the open circle (oc) and linear (l) DNA and simultaneous appearance of a faster-migrating band, presumably of single-stranded (ss) DNA, whereas no change occurred in the position of the ccc DNA bands (Fig. 4.3). The large plasmid, before heat treatment, comigrated with the chromosomal DNA. Upon heat treatment the plasmid remained at its original position, whereas the chromosomal DNA was completely denatured. Using plasmid DNA prepared by method 1, the pANl and pANs DNA bands did not change their position after heat treatment (pANs DNA band not clear in Fig. 4.3), suggesting that the plasmid DNA was in ccc form. For method 2, following this treatment the chromosomal DNA (c DNA) changed to ss DNA, and the plasmid DNAs were both in ccc form. For method 3, similarly some c DNA changed to ss DNA, whilst for method 4 heat treatment indicated that the large band was pANl in the oc form and pANs was ccc DNA.

4.2.3 Effect of culture age on plasmid isolation

Differences in the yield of plasmid DNA (on the basis of UV-induced fluorescence of DNA samples) were obtained with cultures of *Synechococcus* PCC7942 of different age (Fig.4.4). It can be seen, from five samples (1.0 mg) of different exponential phase cultures, that plasmid yield was a function of growth: In the early exponential phase (optical density at 720nm (OD_{720}) ~ 0.08-0.3), a high yield of pANl was obtained, but pANs was not found. For late exponential phase culture (OD_{720} >1.6-2.0), pANl and pANs both were obtained in low yield. In the

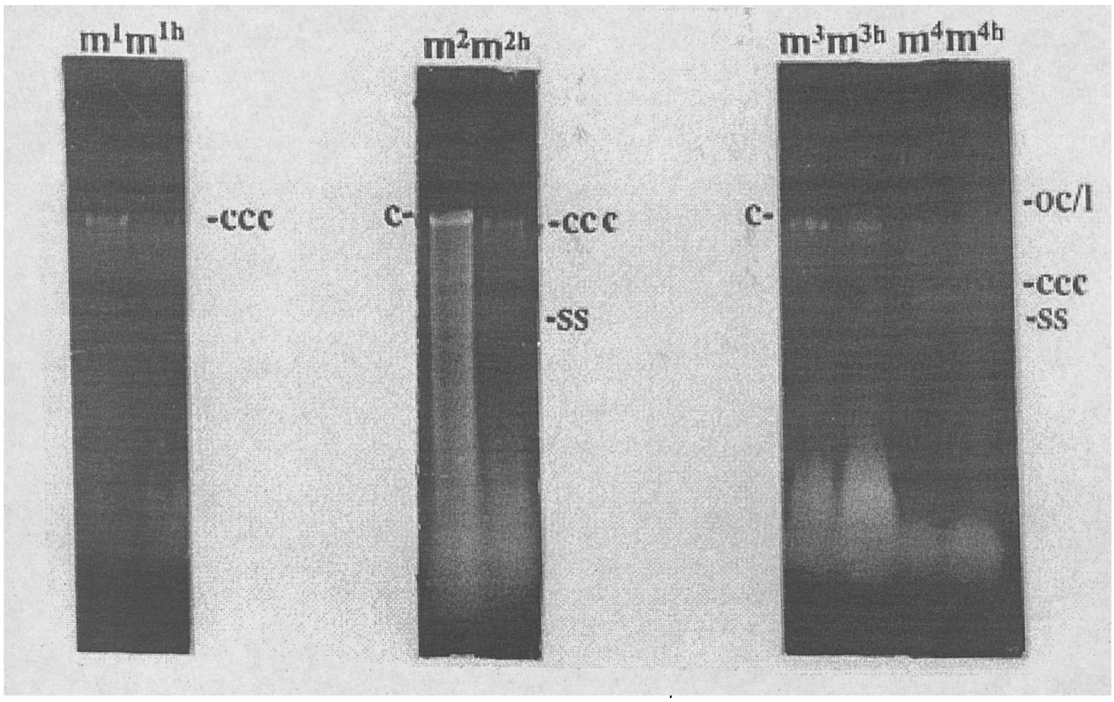
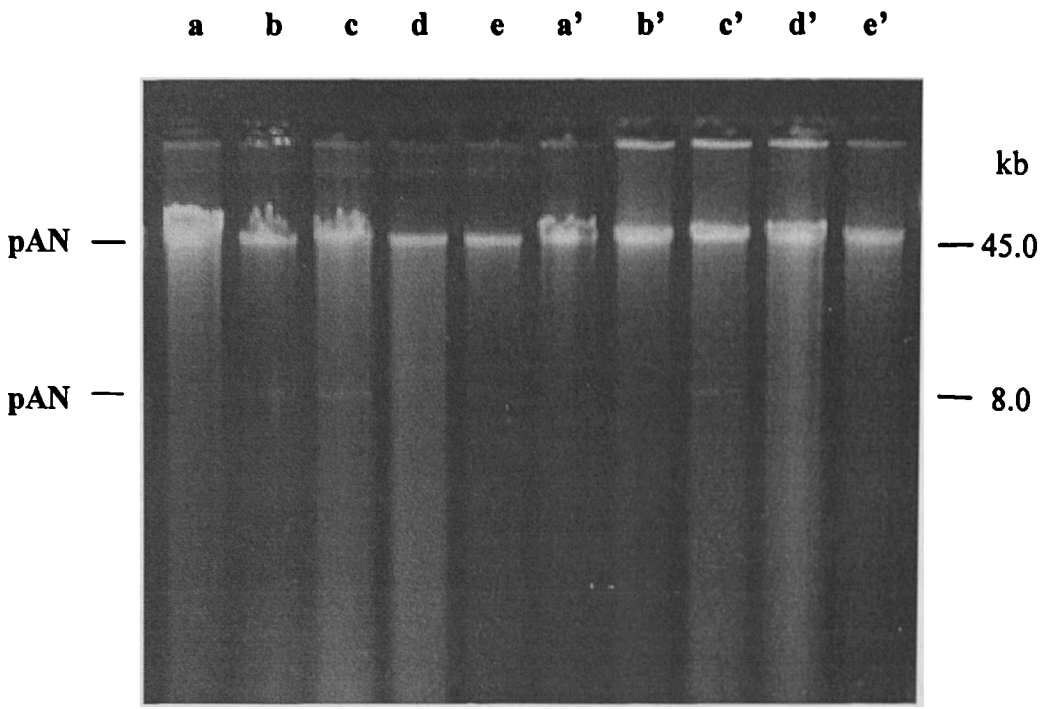
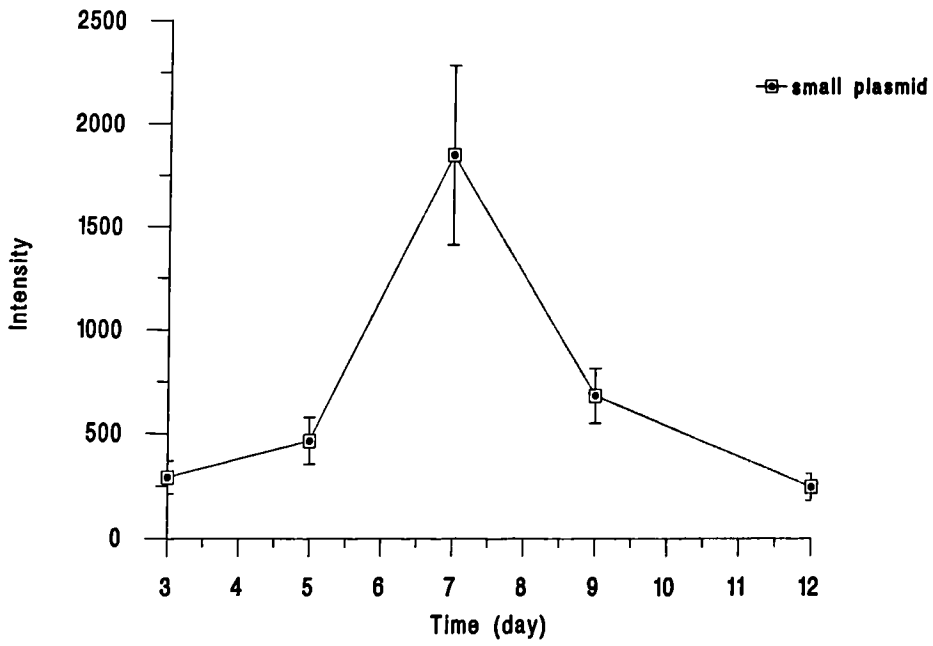


Figure 4.3 Agarose gel electrophoresis of characterisation plasmid by heat treatment. heat-treated (lane m^h) and unheated (lane m) ethanol precipitated plasmid DNA prepared by four rapid methods m^1-m^4 (Methods 1-4 as described in section 2.2.14.1) from *Synechococcus* PCC 7942. c, chromosomal DNA; ss, single-stranded DNA; oc/l, open circular/linear DNA; ccc, covalently closed circular DNA.

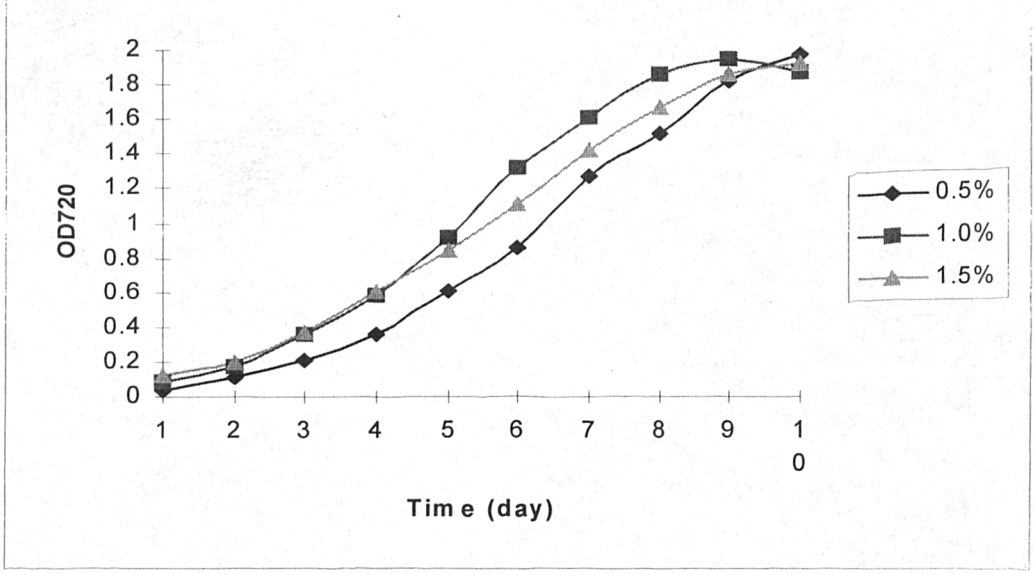
A.


a b c d e a' b' c' d' e'
kb
pAN
pAN
45.0
8.0

B.


2500
2000
1500
1000
500
0
Intensity
3 4 5 6 7 8 9 10 11 12
Time (day)
small plasmid

C (I).



C (II).

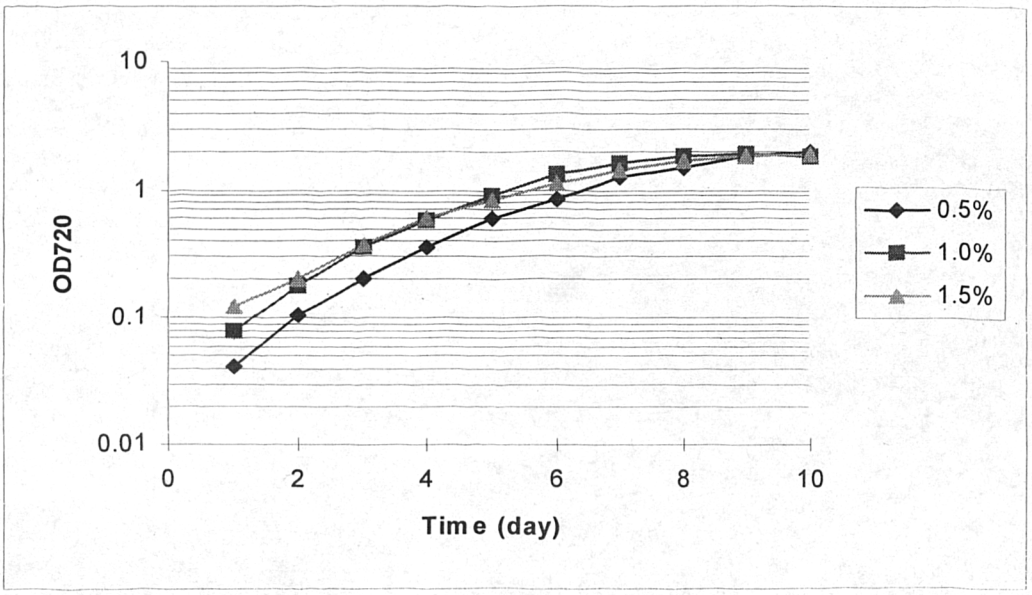


Figure 4.4 Effect of culture age on plasmid isolation. (A). Agarose gel electrophoresis of plasmid DNA prepared by method 1 from 1 mg cultures of *Synechococcus* PCC7942 at different ages. Lane a or a', at 3rd day (OD_{720}=0.206 or 0.358); lane b or b', at 5th day (OD_{720}=0.604 or 0.923); lane c or c', at 7th day (OD_{720}=1.262 or 1.597); lane d or d', at 9th day (OD_{720}= 1.795 or 1.924) and lane e or e', at 12th day (OD_{720}=2.071 or 2.121). Separation was achieved on 0.8% (w/v) agarose horizontal gel as described in section (2.2.15). (B). The different intensities of the small plasmid (pANs) isolated from a culture of *Synechococcus* PCC7942 at different ages. Each point is the mean ± SD from three separate experiments. The intensities of DNA bands were measured by using Phoretix 1D software. (C). Growth curve (I) OD_{720} versus time ((day) and (II) Log OD_{720} versus time (day)) of *Synechococcus* PCC7942 from three separate experiments inoculated with 0.5%, 1.0% and 1.5% stock culture (1mg dry weight cells per ml). Axis OD_{720} of 1.0 corresponds to approximately 10mg dry weight of cells per 10ml of culture (Lambert *et al.*, 1982).

mid-exponential phase (OD_{720} 0.3-1.6), the large and small plasmids were both routinely (5 replicates) obtained in high yield. Thus for further plasmid isolations, mid-exponential phase cultures were used.

4.2.4 Plasmid content of selected cyanobacteria

Different numbers and sizes of plasmid DNA were obtained from four laboratory strains and five isolates of cyanobacteria by using the rapid methods 1-5 (see Table 4.1). The sizes of the plasmids were confirmed by restriction with *Bam* HI, and using *Hind* III digested λ DNA as standards. For the isolates, *Synechococcus* sp.: LJM 002, 003 and 004, and *Pseudanabaena* sp.: LJM 001 and 005, no plasmids were obtained by any of the methods used. Plasmid DNA could be obtained from the unicellular laboratory strains by using methods 1, 2, 3, and 4. No plasmid DNA was obtained from the filamentous laboratory strains (*Nostoc* PCC7120 and *Nostoc palaeo*) tested by using methods 3, 4 and 5. For strain *Nostoc palaeo*, the smaller plasmid of 42 kb was not always obtained by any method used. It has previously been reported by Laudenbach (1983) that *Synechococcus* PCC 7942 contains two plasmids of 8.0, and 48.5 kb, and Simon (1978) reported that *Nostoc* PCC 7120 contained four plasmids of 3.3, 33, 42, 74 Megadaltons (MD). Two plasmids of similar size to those of 33MD (=49.5 kb) and 3.3MD (=4.95 kb) were obtained in this study.

Table 4.1 Plasmid content of selected cyanobacterial strains

Cyanobacterial strain [a]		Description [b]	Number of plasmids, (molecular size, kb) [c]				
			Method 1	Method 2	Method 3	Method4	Method5
Laboratory strain							
Synechococcus	PCC 7942	U	2(8, 48.5)	2(8,48.5)	2(8,48.5)	1(8)	0
Synechocystis	PCC 6308	U	1(48)	1(48)	1(48)	0	0
Nostoc	PCC 7120	HF	1(45)	2(4.5,45)	0	0	0
Nostoc	*Palaeo*	HF	1(55)	2(42,55)	0	0	0
Isolates							
Synechococcus	LJM 002	U	0	0	0	ND	ND
Synechococcus	LJM 003	U	0	0	0	ND	ND
Synechococcus	LJM 004	U	0	0	0	ND	ND
Pseudanabaena	LJM 001	F	0	0	0	ND	ND
Pseudanabaena	LJM 005	F	0	0	0	ND	ND

a, Strains were grown in medium BG11 as described in materials and methods section 2.1.3.

b, U: unicellular F: filamentous HF: heterocystous filamentous.

c, Methods 1, 2, 3, 4, 5 are described in section 2.2.24.1. ND, not determined.

4.2.5 Isolation of total genomic DNA

Total genomic DNA was isolated from cyanobacteria by the mini-methods of Porter (1988) and Neilan (1995) (as described in section 2.2.14.3). Both methods proved to be rapid and reliable (Fig. 4.5 A). Amounts of DNA recovered were within the range 4.5μg-8.5μg per ml for mid-exponential phase cultures of *Synechococcus* PCC 7942 using either method. The method of Neilan (1995) was found to be more effective than the method of Porter (1988) for removal of protein from the lysate. The total genomic DNA obtained from either method was suitable

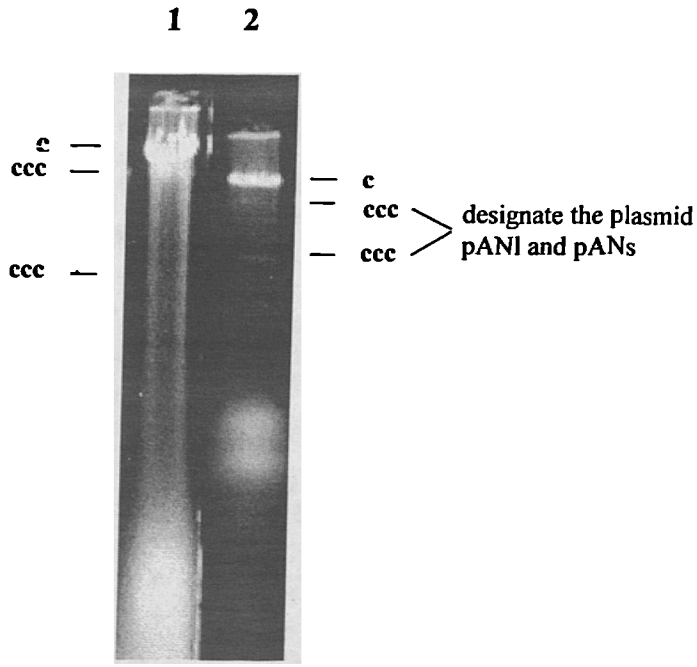
for amplification by PCR and the PCR-amplified products, using the random primer ABA-10 (see Table 2.4), were identical in each case (Figure 4.5 B). Identical amplified products were also obtained using either freshly isolated DNA or DNA stored for one year at -20 °C from Porter's method (Figure 4.5 C), indicating that the isolated DNA was stable.

It should be noted that genomic DNA obtained simply by cell lysis could also be amplified by PCR (see Fig. 4.5 B, lane 2).

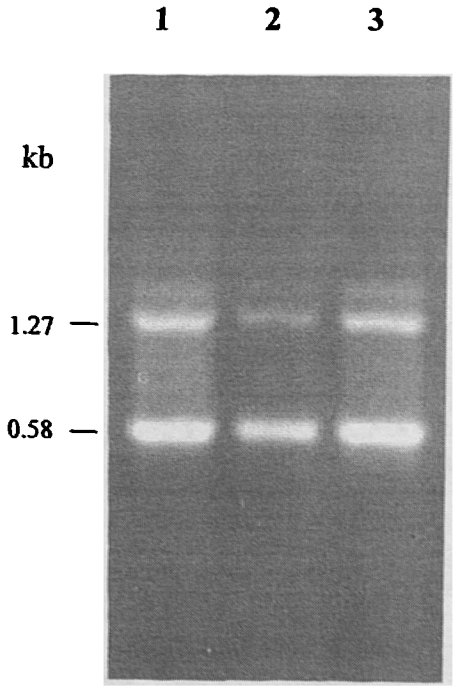
4. 2. 6. Restriction analysis of plasmid DNA and genomic DNA

Plasmid DNA obtained from *Synechococcus* PCC 7942 by method 2 and total genomic DNA obtained by method 1 (Porter, 1988) were restricted using *Bam* HI. Six distinct bands were obtained following restriction of the plasmid DNA, but only smears from the genomic DNA (Fig 4.6). This may due to the fact that too many bands were generated by restriction of the genomic DNA for effective resolution.

A.


1
2
c
ccc
ccc
c
ccc
ccc
designate the plasmid
pANl and pANs

B.


1
2
3
kb
1.27
0.58

C.

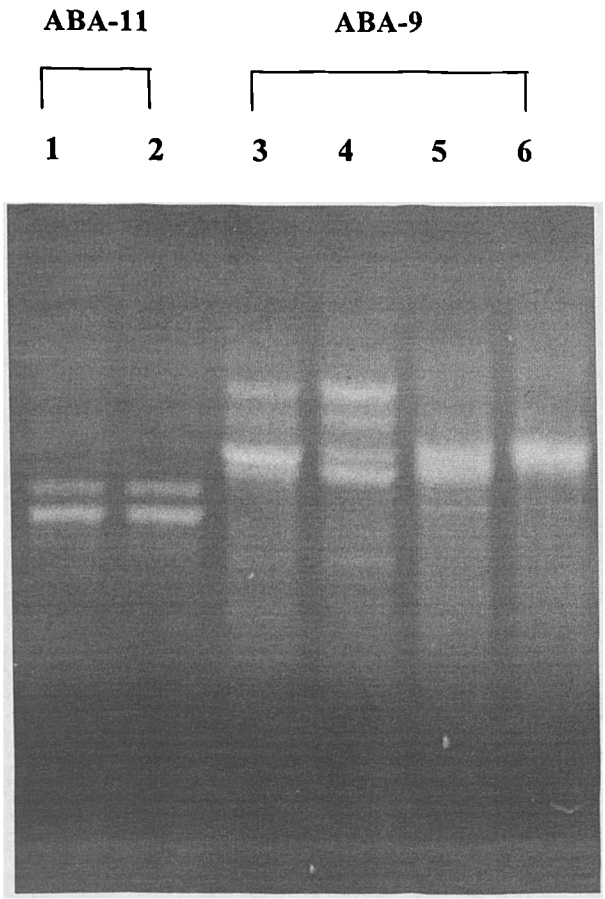


Figure 4.5 Agarose gel electrophoresis of total genomic DNA from *Synechococcus* PCC 7942. (A) Total genomic DNA isolated by different methods: Lane 1, Neilan's method (Neilan, 1995); 2, Porter's method (porter, 1988). (B). PCR-amplified products of total genomic DNA (obtaied from Porter's method or Neilan's method or direct cell lysis) by using random primer ABA-10 (as described in section 2.2.19). Lane 1, Porter's method; 2, direct cell lysis (section 2.2.14.3); 3, Neilan's method. (C). PCR-amplified products of total genomic DNA (isolated by Porter's method) by using random primer ABA-11, ABA-9 (section 2.2.19) to show the effect of storage. Lane 1 and 5, from freshly isolated DNA, 2 and 6 DNA, after one year storage (at -20 °C) from *Nostoc paleo*; Lane 3 from freshly isolated DNA from *Synechococcus* PCC 7942; 4, DNA after one year storage (at -20 °C) from *Synechococcus* PCC 7942.

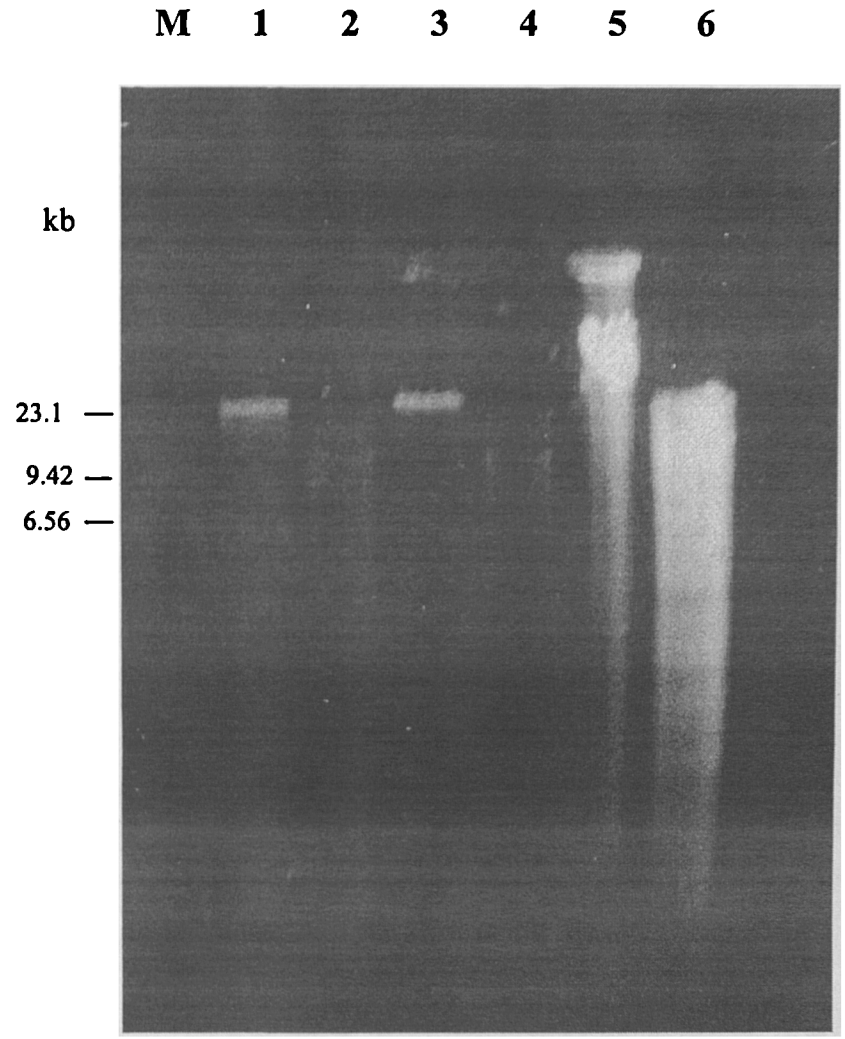


Figure 4.6 Agarose gel electrophoresis of *Bam* HI restricted plasmid and genomic DNA from *Synechococcus* PCC 7942. Lane 1, plasmid pANl; 2, *Bam* HI restricted plasmid; 3, plasmid pANl; 4, *Bam* HI restricted plasmid; 5, genomic DNA; 6, *Bam* HI restricted genomic DNA; M, λ *Hin*d III DNA.

4.3 Discussion

When isolating DNA from cyanobacteria, a major difficulty was to achieve gentle but efficient cell lysis of the different strains used. One reason for this difficulty may be that some cyanobacteria possess a thick outer sheath, usually of undetermined composition (Potts, 1984), to interfere with lysis. Efficient cell lysis is crucial to the study of plasmids particularly where they are be present in very low copy number.

Method 2 of the rapid mini methods for isolation of plasmid DNA, was shown to be the most successful using either unicellular or filamentous cyanobacteria. This method, which is based on the method of van den Hondel and co-workers (1979), has been developed here for application to a range of cyanobacterial strains. Lambert and Carr (1982) also found a modification of the method of van den Hondel and co-workers (1979) to provide reproducible results. In our studies, incubation with RNase and overnight precipitation by using NaCl proved most effective for removal of chromosomal DNA and gave better results than the method of Lambert and Carr (1982), which involved 2 h precipitation by using NaCl, without RNase treatment.

Method 1 proved to be a better procedure than the remaining methods. For unicellular strains large yields of the plasmids were obtained, but it did not prove very effective for all the filamentous strains tested. The effectiveness of methods 3 and 4 varied depending on the strain tested, whilst method 5 did not yield plasmid DNA from any of the cyanobacterial strains tested.

Differences in plasmid yield were obtained with cultures of the same strain, but at different stages of growth. Mid-exponential phase cultures (OD_{720} 0.3-1.6) produced optimal plasmid yields for all strains tested. Lambert and Carr (1982) also obtained maximal yields of plasmid DNA from exponential phase cultures. Two reasons for the lower yield from late-exponential phase (OD_{720} >1.6-2.0) cultures, could be due to the cell wall or sheath layer becoming thicker, thereby leading to inefficient lysis. Alternatively, variations in plasmid copy number during different growth phases could account for differences in plasmid yield.

The plasmid DNA isolated from mid-exponential phase cultures of cyanobacterial strains by method 2 was of suitable quality for restriction analysis. Further studies on cyanobacterial plasmids therefore employed such methodology for DNA isolation.

Two of the four laboratory strains of cyanobacteria studied here, namely *Synechococcus* PCC 7942 and *Nostoc* PCC 7120 were all known to possess plasmids (van den Hondel *et al.*, 1979; Lambert and Carr, 1982), and the methods employed here, except method 5, confirmed this. It has previously been reported by Simon (1978) that *Nostoc* PCC 7120 contains four plasmids of 3.3, 33, 42, 74 MD. Lambert and Carr (1982) have reported that one plasmid (3.0 MD) was obtained from *Nostoc* PCC 7120 by using a modification of the method of van den Hondel and co-workers, (1979). Two plasmids of similar size to those of 33MD (=49.5 kb) and 3.3MD (=4.95 kb) were obtained in this study. These findings indicate that rapid methods of plasmid isolation are limited, such that it is difficult to recover all plasmids in a particular cyanobacterial strain every time.

Different strains harboured different numbers and sizes of plasmids, irrespective of the degree of relatedness. Table 4.2, shows that less than 50% of cyanobacterial strains tested here contained plasmids. Although this represents only a small sample size, it is in line with previous work on plasmid profiles in different cyanobacteria (see for e.g. Lau, *et al.*, 1980; Lambert and Carr, 1982; Kohl *et al.*, 1988 and Goyal, 1992). Plasmid profiles may be of limited use in determining the relationships between strains that contain plasmids acquired through horizontal gene transfer; however they would not be useful for establishing classification schemes.

In this study all the strains examined were grown in BG11 medium under the same environmental conditions. Whether changes in environmental regimes may affect plasmid profiles remains to be determined.

Total genomic DNA suitable for PCR (see Fig. 4.4 B) was consistently isolated from cyanobacterial strains by the methods of Porter (1988) (see 2.2.14.3 method 1) and of Neilan (1995) (see 2.2.14.3 method 2). Both methods were reliable and reproducible. The genomic DNA isolated by the method of Porter (1988) was found to remain stable following long term storage (one year) at -20 °C. So further studies with genomic DNA employed this method.

Chapter 5

Identification of cyanobacteria by random amplified polymorphic DNA (RAPD) analysis

5.1 Introduction

Traditional techniques for identification and systematic of cyanobacteria have relied primarily on observed morphological characteristics. However, morphology may change depending on environmental conditions (Evans *et al.*, 1976) and the diversity of strains within a culture may be suppressed by selective culturing conditions (Doers and Parker, 1988). Photosynthetic pigment content, lipid composition, differentiated cell structures and other characters may also be subject to change because of the variable expression of cyanobacterial gene products in culture (Kenyon *et al.*, 1972; Klein *et al.*, 1973). The limitations of such methods have prompted the development of molecular biological techniques for identification of cyanobacteria.

The use of DNA markers of cyanobacteria in genetic mapping, genetic diagnostics, molecular taxonomy, and evolutionary studies has been well established (Wilmotte, *et al.*, 1984; Wood, *et al.*, 1990; Wilmotte, 1994; Neilan, 1995). The most commonly used DNA markers are restriction fragment length polymorphisms (RFLPs). Detection of RFLPs by DNA blot hybridisation is laborious and incompatible with applications requiring high throughput. Genetic tests based on the polymerase chain reaction (PCR) are rapid, sensitive and simple to perform. Previously required amounts of a specific gene could be determined by RFLPs or DNA-DNA hybridisation from cells, now even a single gene copy can be detected following nucleic acid amplification.

The polymerase chain reaction (PCR) is an *in vitro* method for amplifying selected nucleic acid (DNA or RNA) sequences (Mullis *et al.*, 1986; Mullis and Faloona, 1987; Mullis, 1990). The method consists of repetitive cycles of DNA

denaturation, primer annealing, and extension by DNA polymerase (Fig. 5.1). Two oligonucleotide primers flank the DNA segment to be amplified. They hybridise to opposite strands of the target sequence, such that synthesis proceeds across the region between the primers, replicating that DNA segment. The product of each PCR cycle is complementary to and capable of binding primers, and so the amount of DNA synthesised is doubled in each successive cycle (Saiki *et al.*, 1988).

Williams and co-workers (1990) and Welsh and colleagues (1990) have described a novel type of genetic marker that is based on DNA amplification by PCR, but requires no knowledge of target DNA sequence. Such markers, called RAPD (random amplified polymorphic DNA) markers, are generated by the amplification of random DNA segments with single primers of arbitrary nucleotide sequence. RAPD markers can be used for genetic mapping applications, as well as for genetic diagnostics. The assay is non-radioactive, requires only nanogram quantities of DNA, and is applicable to a broad range of species. In addition, the presence of a single RAPD band would be diagnostic for a sequence totalling 20bp in the target DNA (10 bases at each end of the DNA segment that is amplified). An average of five amplified bands per primer would mean that each primer is diagnostic for 5×20=100bp in the template. This is in contrast to an RFLP, which is diagnostic for only 12bp per probe-enzyme combination (Williams, *et al.*, 1993).

To perform a RAPD assay, a single oligonucleotide of an arbitrary DNA sequence is mixed with genomic DNA in the presence of a thermostable DNA polymerase and a suitable buffer, and then is subjected to temperature cycling conditions typical of the polymerase chain reaction. The products of the reaction depend on the sequence and length of the oligonucleotide, as well as the reaction

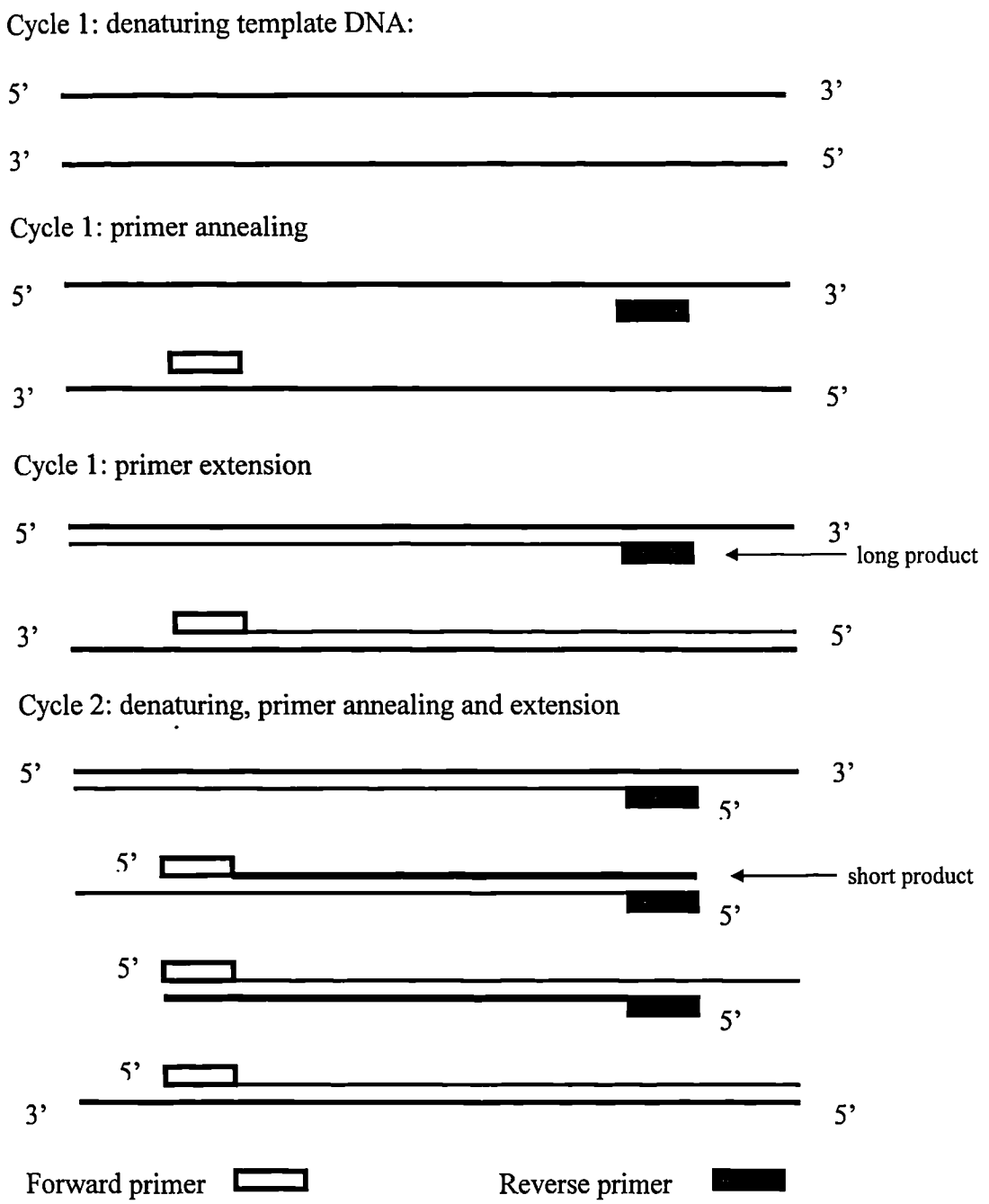


Figure 5.1 A diagrammatic representation of the PCR reaction. During the first cycle, template DNA is first denatured at 94 °C in the presence of primers. Lowering the temperature to 30-60 °C allows the primers to anneal to the template DNA. Extension occurs as the temperature is raised to 72 °C, to form the first two 'long products'. These long products have the PCR primer sequences at their 5' ends. During the second cycle, the two long DNA products made in the first cycle, now also become template DNA for primer binding and extension, to form two more long products, and two 'short products' that comprise only the DNA sequence between the two primers. After 30 cycles, well over 10^6 molecules of the specific short product should have been amplified.

conditions. At an appropriate annealing temperature during the thermal cycle, the single primer binds to sites on opposite strands of the genomic DNA that are within an amplifiable distance of each other (within a few thousand nucleotides), and a discrete DNA segment is produced. The presence or absence of this specific product, although amplified with an arbitrary primer, will be diagnostic for the oligonucleotide-binding sites on the genomic DNA (Fig. 5.2). In practice, the DNA amplification reaction is repeated on a set of DNA samples with several different primers, under conditions that result in several amplified bands from each primer. Polymorphic bands are noted, for example, between parents of a cross, and the polymorphisms can be mapped in a segregating population. Often a single primer can be used to identify several polymorphisms, each of which maps to a different locus (Welsh *et al.*, 1990; Williams *et al.*, 1993).

Williams and co-workers (1990) used shorter, arbitrary primers, 9 or 10 nucleotides in length, and low stringency cycles to amplify DNA polymorphisms from several eukaryotic and prokaryotic organisms. When separated by agarose gel electrophoresis and stained with ethidium bromide, up to about 10 products were detected. In most studies, polymorphic products were used as genetic markers. Hamelin and colleagues (1993) used seven random amplified polymorphic (RAPD) markers to identify the ascomycete *Gremmeniella abietina* between the North American and European fales. McCutcheon and co-workers (1993) applied the RAPD method to assess genotypic diversity in populations of *Rhabdocline parkeri* from Douglas fir. RAPD analysis has also been used to distinguish among clinical isolates of *Helicobacter pylori*, a bacterium whose long term carriage is associated with gastritis, peptic ulcers and gastric carcinomas (Akopyanz *et al.*, 1992). Furthermore, Kaukas and colleagues (1994) produced RAPD profiles, by using four

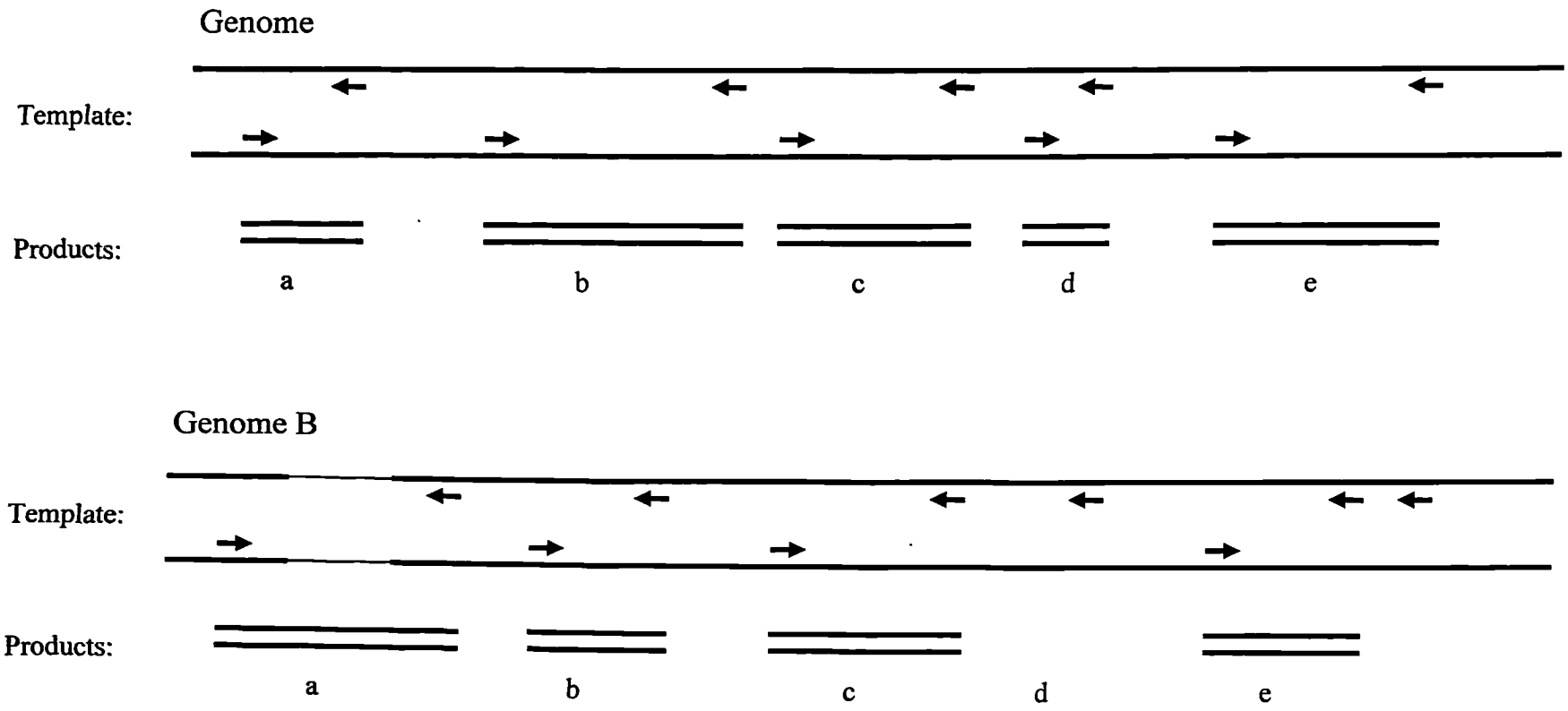


Figure 5.2 Origins of random amplification fragment length polymorphisms. Two related genomes are amplified with DNA polymerase and an arbitrary oligonucleotide primer (→) and resulting amplification products compared. In genome B an insertion (—) between priming sites produces a larger fragment a, while a deletion decreases the size of fragment b. Additionally, mutation in other priming sites eliminates fragment d and decreases the size of fragment e.

oligonucleotide primers, with genomic DNA from 15 isolates of *Schistosome*. Both inter- and intra-specific variation were noted. The results confirm that RAPD profiles may be used for both strain and species differentiation and for the generation of phylogenetic trees. In addition, the method has been used to study the cyanobacteria symbionts of *Azolla-Anabaena* (Eskew *et al.*, 1993). Fingerprints that were unique to the symbionts from three different *Azolla* sp. were generated and the maternal transmission of one symbiont was indicated. Neilan (1995) demonstrated the use of multiplex random amplified polymorphic DNA markers (MRAPDs) to distinguish between the genera *Anabaena* and *Microcystis*. This approach uses multiple random primers for DNA amplification. It was possible to discriminate among all the toxigenic cyanobacteria studied to three taxonomic levels of genus, species and strain. Clearly RAPD analysis is proving very promising for identification of a wide range of organisms to the strain level.

In this study, the RAPD technique has been developed and optimised for cyanobacterial DNA, with a view to its use to identify specific cyanobacterial strains. The RAPD markers generated have been applied to a phylogenetic analysis of the strains to assess genetic relatedness.

5.2 Results

5.2.1 Reaction conditions for RAPD-PCR

Titration of the reaction constituents was carried out to determine optimum conditions for DNA amplification by PCR using different template DNA concentration, primer concentration, magnesium ion concentration, dNTP

concentration and different annealing temperature. Template DNA concentrations in the range 0.55ng/μl (lane 9) to 8.4ng/μl (lane 5) gave clear, reproducible bands using Biolin-AmpliTaq polymerase (Fig. 5.3. A). Whilst for amplification using DyNAZyme II, template DNA concentrations in the range 17.2pg/μl (lane 13) to 1.1ng/μl of the reaction mixture (lane 10) gave clear, reproducible bands (Fig. 5.3. B). Template DNA concentration dependencies were not observed from 0.275ng/μl (lane 11) to 1.1ng/μl, but they were observed between 17.2pg/μl and 68.8pg/50μl (lane 12). High concentrations of genomic DNA (more than 8.4ng/μl) often resulted in smearing or in a lack of clearly defined bands (Fig. 5.3 A, lane 1, 2, 3, 4). At genomic DNA concentrations below 17.2pg/μl of the reaction mixture (see Fig.5.3 B, lane 13), some bands were lost, and new bands appeared in the RAPD profile.

For reproducibility, it is important to note that both the magnesium ion concentration and the annealing temperature affected the relative intensity of amplified bands. PCR-amplified products were obtained with a magnesium chloride concentration between 1.5 and 5mM, but not with a concentration of 0.5mM. As the magnesium concentration increased, some DNA segments were amplified more efficiently whilst others were amplified less efficiently (Fig 5.4). Therefore, the same magnesium chloride concentration (2.5 mM), which gave clear bands, was used for all amplifications. The annealing temperature can also affect the relative amount of some amplified bands (Fig. 5.5). Similar amplified bands were obtained with the annealing temperature at 30^{o}C or 34^{o}C. However, different amounts of some bands were obtained with the annealing temperature over 38^{o}C, as judged by band intensity. An annealing temperature of 42 ^{o}C was found to be too high to obtain reliable and reproducible amplification with many of the 10-mers tested.

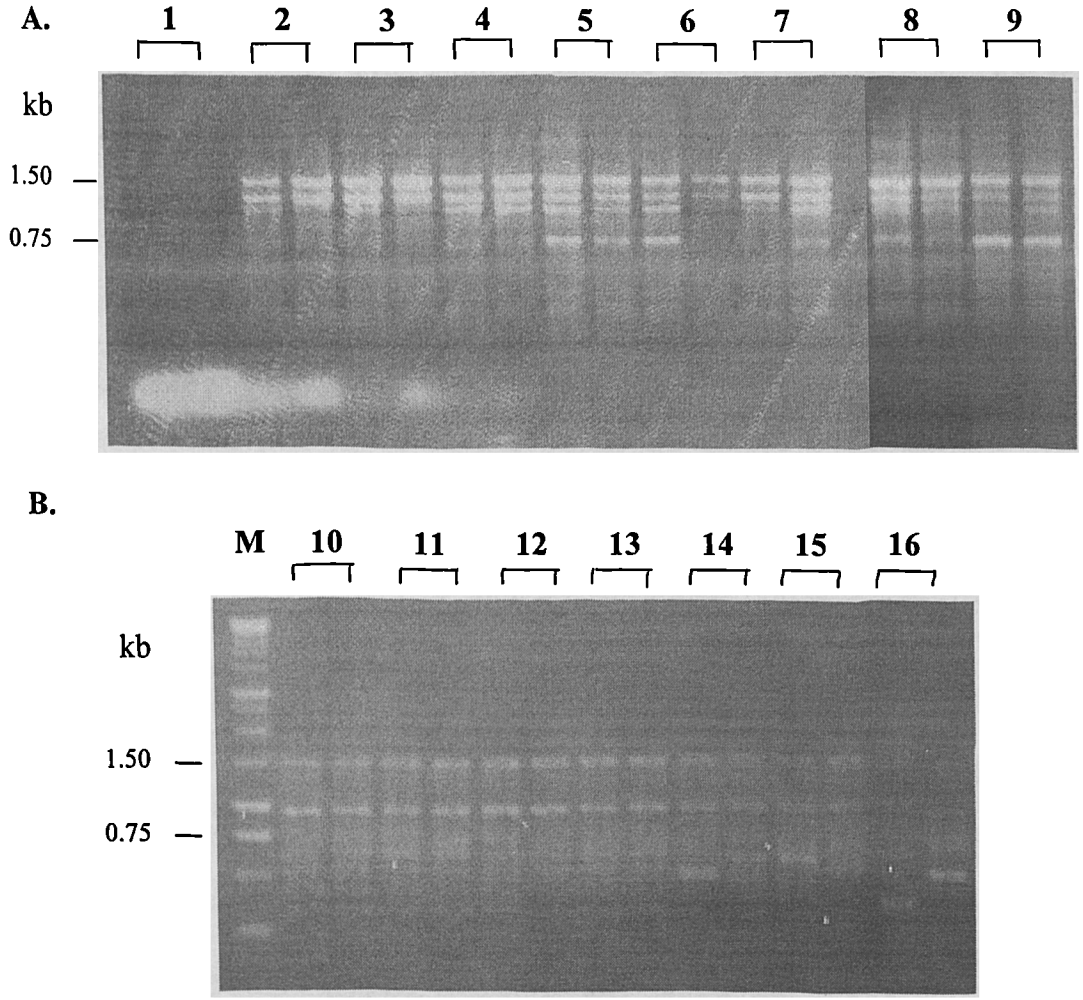


Figure 5.3. Agarose gel electrophoresis showing the effect of DNA concentration on the RAPD assay. Different concentrations of *Nostoc mus* DNA were amplified by program 1 (see section 2.2.19) with primer A-9. Each sample was amplified in duplicate to assess the reproducibility of the reaction. A, DNA samples amplified by Bioline-AmpliTaq. Lane 1, 133.6 ng/µl of the reaction mixture; 2, 66.8 ng/µl; 3, 33.4 ng/µl; 4, 16.7 ng/µl; 5, 8.35 ng/µl; 6, 4.2 ng/µl; 7, 2.1 ng/µl; 8, 1.05 ng/µl; 9, 0.525 ng/µl reaction; B, DNA samples amplified by Finnzymes-DyNAZyme II. Lane 10, 1.1 ng/µl of the reaction mixture; 11, 275 pg/µl; 12, 68.75 pg/µl; 13, 17.2 pg/µl; 14, 4.3 pg/µl; 15, 1.07 pg/µl; 16, 0.268 pg/µl.

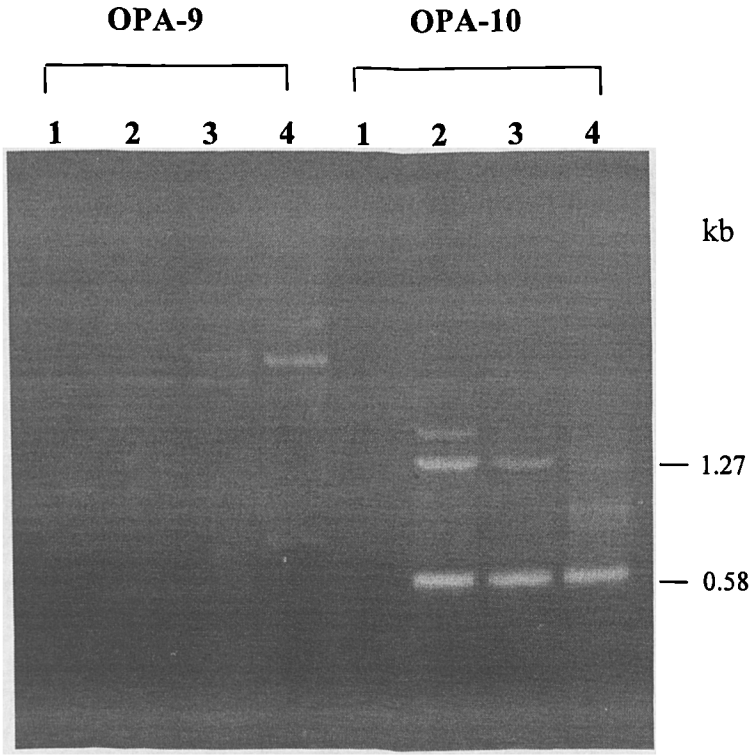


Figure 5.4. Agarose gel electrophoresis of RAPD products of *Synechococcus* PCC7942 DNA obtained with different magnesium concentrations. Lane 1-4: 0.5 mM, 1.5mM, 2.5mM and 5.0mM magnesium chloride in amplification reaction buffer. The products were amplified with the primer OPA-9 and OPA-10 under standard conditions with program 1 (see section 2.2.19).

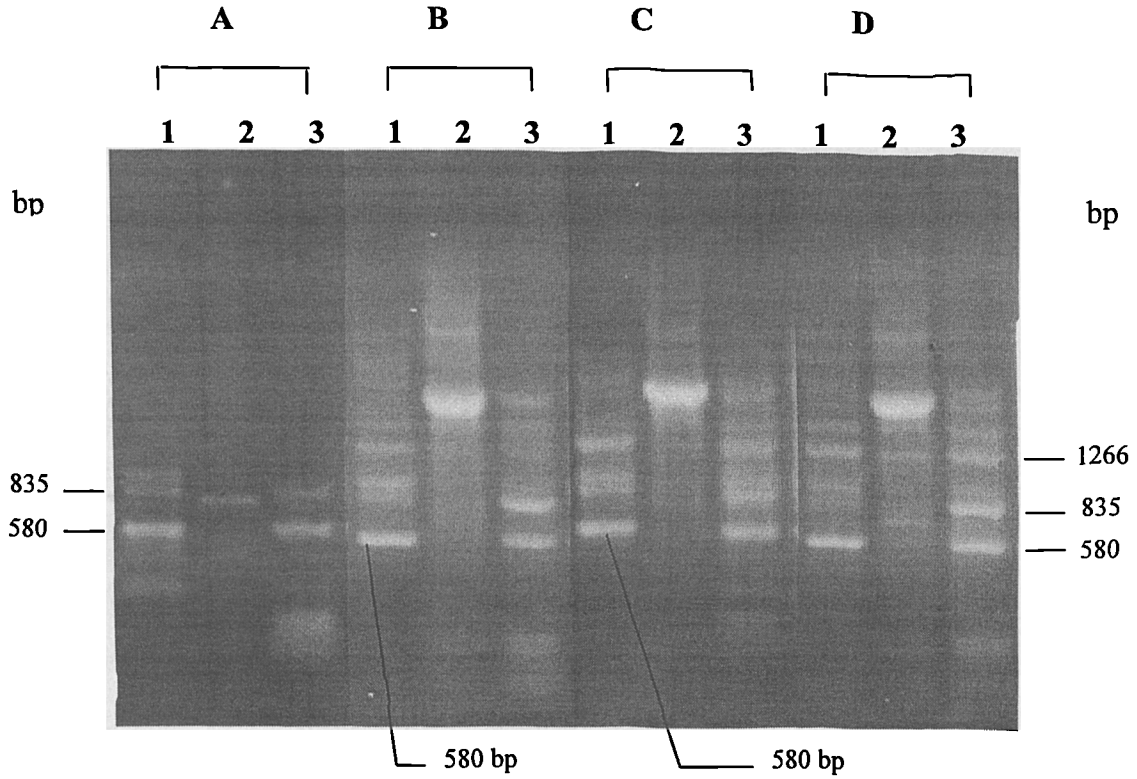


Figure 5.5. Agarose gel electrophoresis of RAPD products using different annealing temperatures for the primers. *Synechococcus* PCC7942 DNA was amplified at four different annealing temperatures: A, 42°C; B, 38°C; C, 34°C; D, 30°C. Standard conditions were used (program 4, section 2.2.19). Three different primers were used: lane 1, ABA-10; lane2, ABA-11 and lane 3, primer pair ABA-10+11.

Increasing the annealing temperature above 38 ^{o}C increased the stringency and in some cases altered the RAPD pattern, with fewer amplification products generated.

No effect of deoxynucleotide triphosphate concentration on the relative intensities of the amplified bands was noted. The same RAPD profiles were generated with deoxynucleotide triphosphate concentrations of 50, 100, 150, 200, 250 μM for each of the four bases (see Fig. 5.6). An average deoxynucleotide triphosphate concentration of 150μM for each of the four bases was chosen for generating RAPDs.

Primer concentrations within the range 0.11 to 0.95μM were most effective (see Fig. 5.7). At primer concentrations of 0.95μM or over, the intensity of the larger bands decreased and that of the smaller bands increased. Whilst at concentrations below 0.11μM, smaller sized bands were not produced in a concentration dependent manner, and the profiles were not reproducible. A concentration of AmpliTaq of 1 units per 50μl reaction as recommended by the manufacturers gave consistent amplification products for every species studied.

The DNA polymerase used affected the out-come of the amplification reaction. Different patterns of amplified bands were obtained when using different DNA polymerases (AmpliTic and AmpliTaq), and when using the same DNA polymerase from different manufacturers with a given DNA *template* and primer. DNA polymerases from four companies were compared. In each case the most intense RAPD bands were of the same size, however faint, variable bands were also generated (Fig. 5.8).

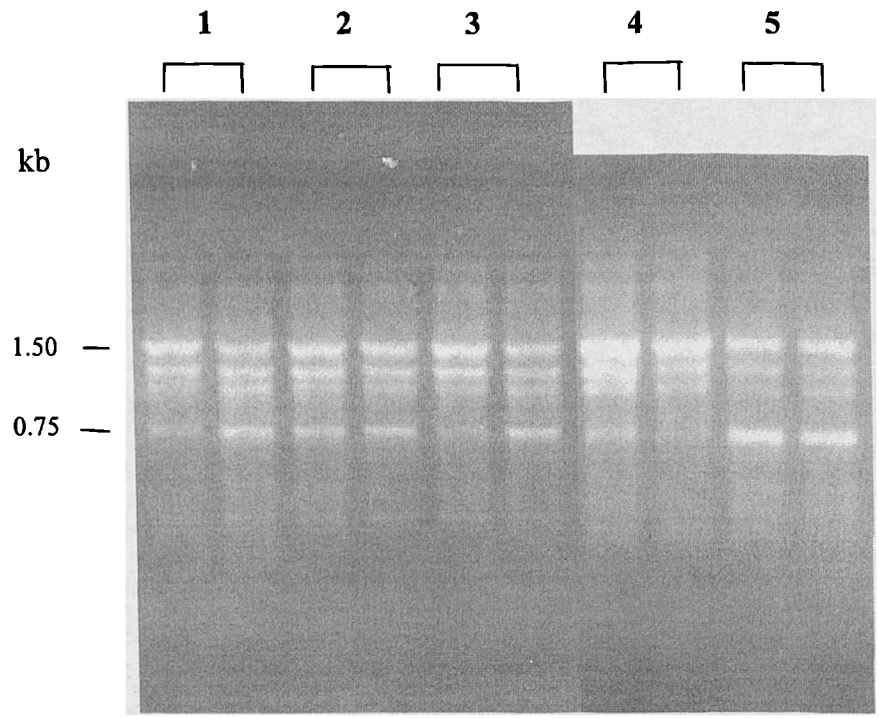


Figure 5.6. Agarose gel electrophoresis showing the effect of deoxynucleotide triphosphate concentration on the RAPD assay. *Nostoc mus* DNA was amplified by program 1 (see section 2.2.19) using primer A-9. Each sample was amplified in duplicate to assess the reproducibility of the reaction. Different deoxynucleotide triphosphate concentrations were used. Lane 1, 50μM; 2, 100 μM; 3, 150μM; 4, 200 μM; 5, 250 μM.

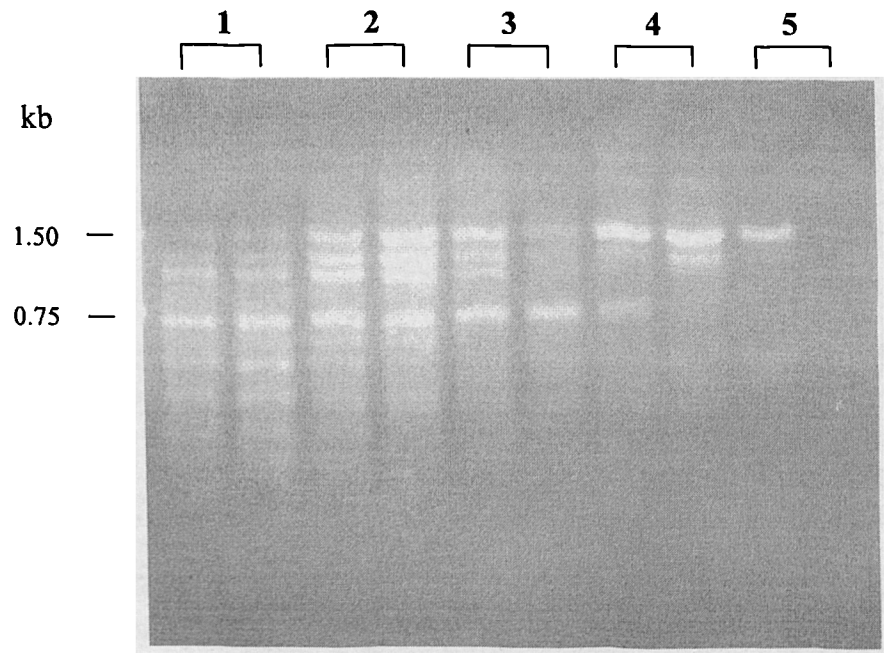


Figure 5.7. Agarose gel electrophoresis showing the effect of primer concentration on the RAPD assay. *Nostoc mus* DNA was amplified by program 1 with primer A-9 (see section 2.2.19). Each sample was amplified in duplicate to assess the reproducibility of the reaction. Different primer concentrations were used. Lane 1, 0.95 μM; 2, 0.32 μM; 3, 0.11 μM; 4, 0.03 μM; 5, 0.01 μM.

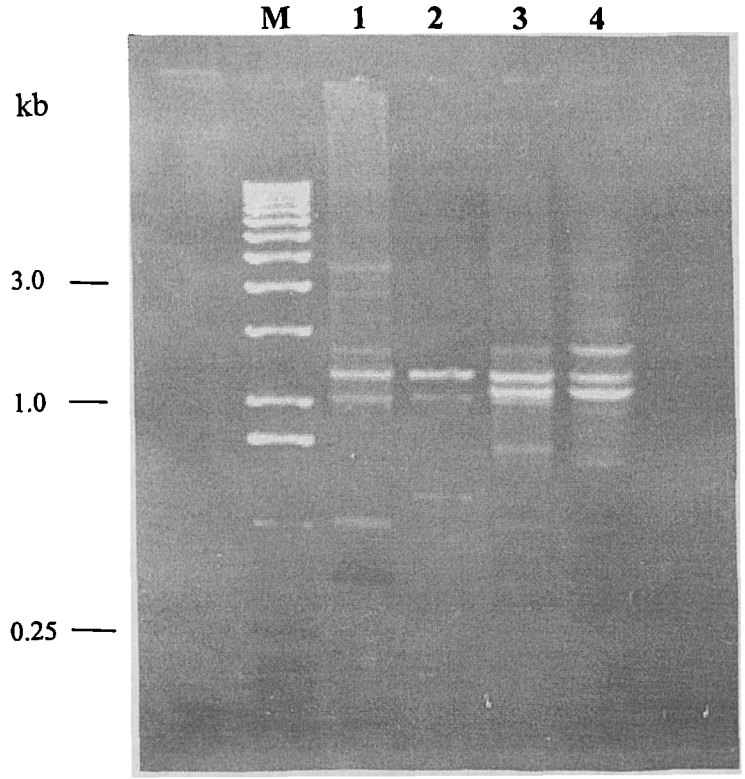


Figure 5.8. Agarose gel electrophoresis of RAPDs generated using different DNA polymerases. DNA from *Nostoc mus* was amplified by program 1 (see section 2.2.19) with primer ABA-11, using different DNA polymerases. Lane 1, AB-AmpliTic; 2, MBI-AmpliTaq; 3, Bioline-AmpliTaq; 4, Promega- AmpliTaq. M, Molecular size 1 Kb markers (Kb) are as indicated.

Five PCR programs were tested. Variation of amplified bands was noticed by using the different programs (Fig. 5.9). Program 4, gave clear bands for all the strains tested with either primer ABA-7 or ABA-10. The results from programs 1 and 2 were similar, except for amplification of *Anabaena cylindrica* DNA with primer ABA-10. No PCR products were detected for *Synechococcus* LJM003 with either primer ABA-7 or ABA10 by using program 3. Program 5 always gave slightly different larger sized fragments. The amplification reaction was performed separately on a Perkin-Elmer *Cetus DNA thermal* cycler and a HyBAID OmniGene temperature cycler using PCR program 1 (see section 2.2.19). More bands were obtained from the reaction using the HyBAID OmniGene temperature cycler (Fig. 5.10 A). It was found that the transition interval time on automatic setting was different for the two PCR machines: 72 s for the Perkin-Elmer and 82 s for the HyBAID cycler between the denaturation (94 °C) and annealing (34 °C) temperature. After setting the transition interval between melting and annealing temperature to 135 s (program 4 and 5) for both machines, the differences between the amplified products, using either machine, were reduced (Fig. 5.10 B).

The RAPD technique was optimised for the various cyanobacterial strains. Each PCR mix contained 1×PCR amplification buffer, 2.5mM $MgCl_2$, 150μM each of dATP, dCTP, dGTP and dTTP, 0.2μM primer, 0.1μg of template DNA, and 1 unit of AmpliTaq polymerase, in a total reaction volume of 50μl. Amplifications were performed in a Perkin-Elmer Cetus DNA thermal cycler (UK) using program 4 for 35 cycles (section 2.2.19).

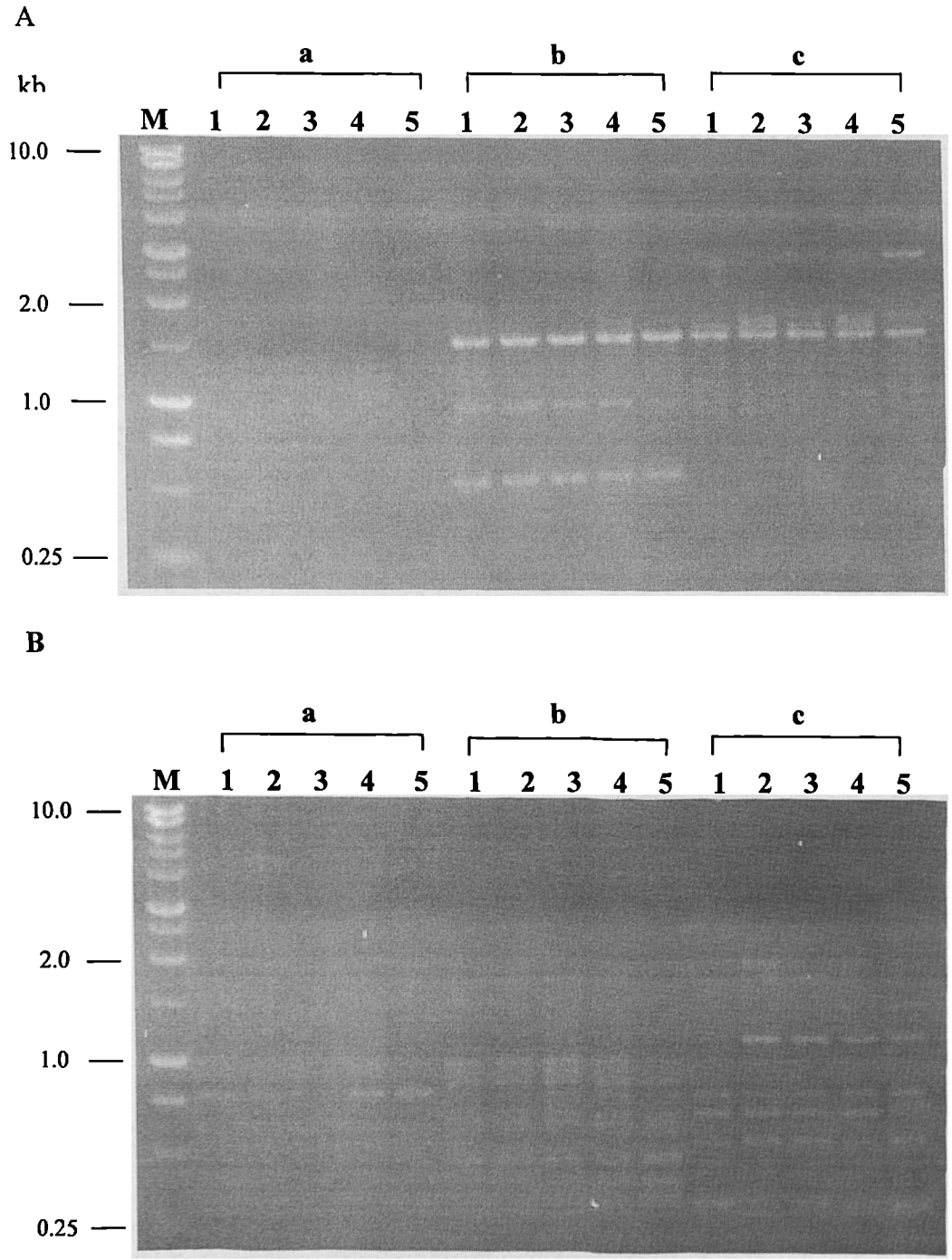


Figure 5.9. Agarose gel electrophoresis of RAPDs generated using different PCR programs. DNA samples from a, *Synechococcus* LJM003; b, *Pseudanabaena* LJM001; c, *Anabaena cylindrica* were amplified with primer (A) ABA-7 or (B) ABA-10, using DNA polymerase, Bioline-AmpliTaq (see section 2.2.19). Lane 1, program 1; 2, program 2; 3, program 3; 4, program 4; 5, program 5. M, 1 Kb marker.

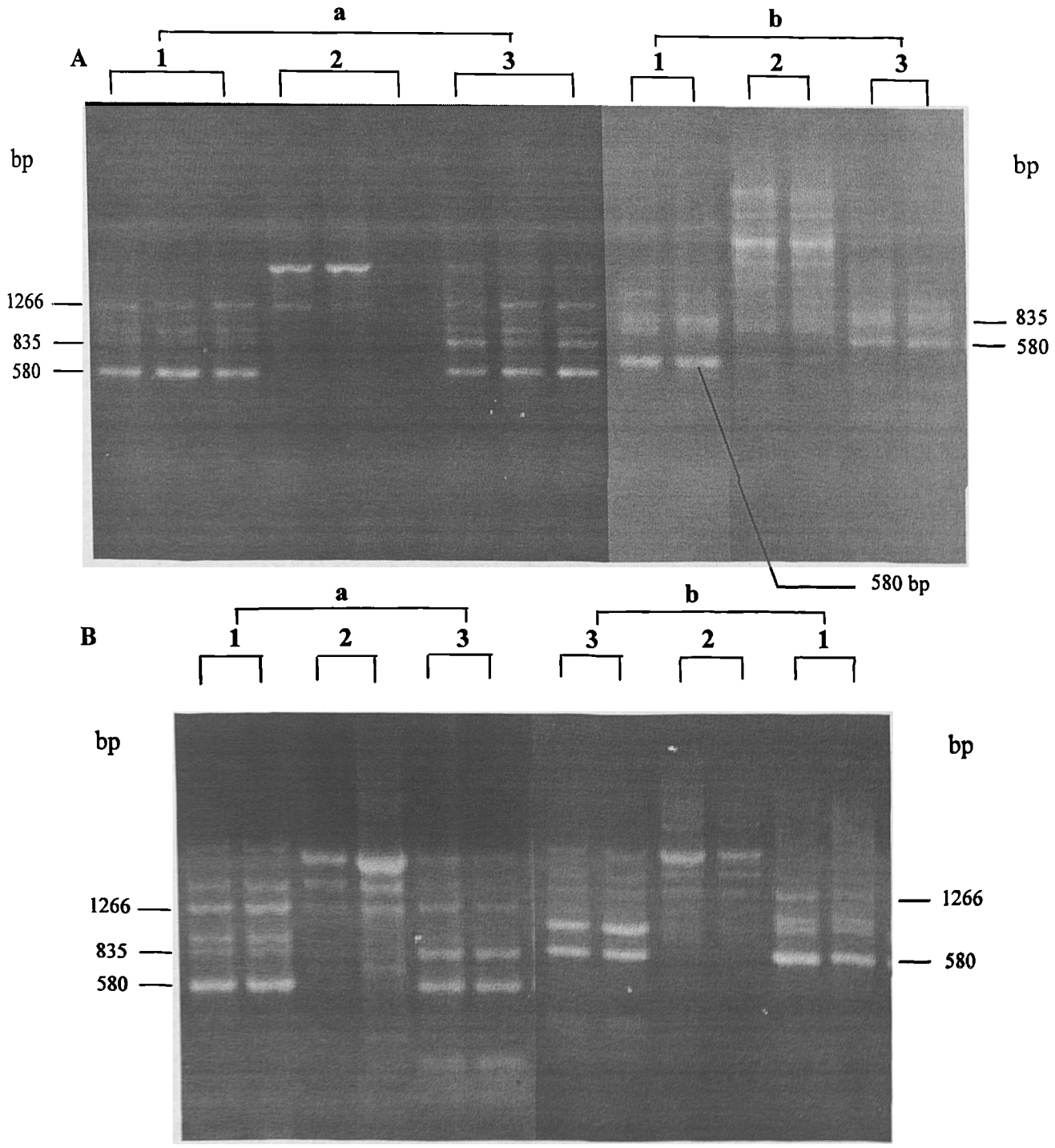


Figure 5.10. Agarose gel electrophoresis of RAPDs generated using different PCR machines and different programs. DNA samples from *Synechococcus* PCC7942 were amplified with different primers. Lane 1, ABA-10; 2, ABA-11; 3, ABA-10+11, and DNA polymerase, Bioline-AmpliTaq, using (a) Perkin-Elmer Cetus DNA thermal cycler and (b) HyBAID OmniGene temperature cycler. Fig. A, amplification using program 1; Fig. B, amplification using program 4 (see section 2.2.19).

5.2.2 Primer specificity

The specificity of primer sequences for amplifying DNA is very important for reproducibility of the RAPD profile. Primer AB1-11 generated two strong bands with DNA from *Nostoc mus*. These bands were not changed when the magnesium ion concentration and annealing temperature were changed (Fig.5.11). In order to increase reproducibility of the RAPD profile, combinations of two 10-mer oligonucleotides were tested on *Synechococcus* PCC7942 DNA at different magnesium ion concentrations (Fig. 5.12 and 5.13). In Figure 5.12, a similar DNA pattern was obtained with primer ABA-10 at magnesium chloride concentrations of 1.5, 2.5 and 5mM. A magnesium chloride concentration dependency was observed for DNA patterns amplified from primer ABA-9 and the combination ABA-9+10. In Figure 5.13, the combination of two 10-mer oligonucleotides did not increase the specificity of primers to generate a clear pattern. In the case of primers ABA-4 and 9 and AB1-1 and 17 none of the bands generated with the single primers remained when using primer pair ABA-4+9 or AB1-1+17. Furthermore, the pattern from the primer combinations was not as clear as the pattern from the single primers. With primer pair ABA-10/11, all the bands amplified from primer ABA-10 remained, and some new bands were generated.

The recommended primer for RAPDs under standard conditions, is the 10-base oligonucleotide primer containing 60%-80% G+C (Williams *et al.*, 1993). The ability of forty 10-base oligonucleotide primers (Kit AB1 1-20; Kit ABA 1-20; 60%-70% G+C; see Table 2.4) to amplify genomic DNA from different laboratory strains was tested. Different primer sequences were required for different cyanobacteria. A few primers of Kit AB1 (AB1-1, 7, 10, 11, 15, 17, 18) could

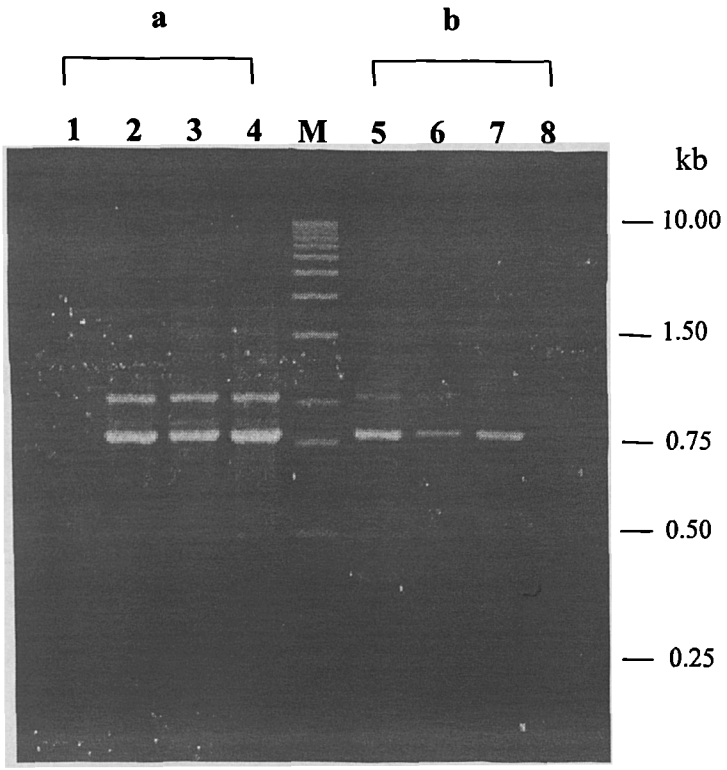


Figure 5.11. Agarose gel elctrophoresis of RAPD products of *Nostoc mus* DNA using (a) different magnesium concentrations and (b) different annealing temperatures. Lane 1, 0.5 mM; 2, 1.5mM; 3, 2.5mM; 4, 5mM $MgCl_2$. Lane 5, 30°C; 6, 34°C; 7, 38°C; 8, 42°C. The products were amplified with the primer AB1-11. M, 1kb marker.

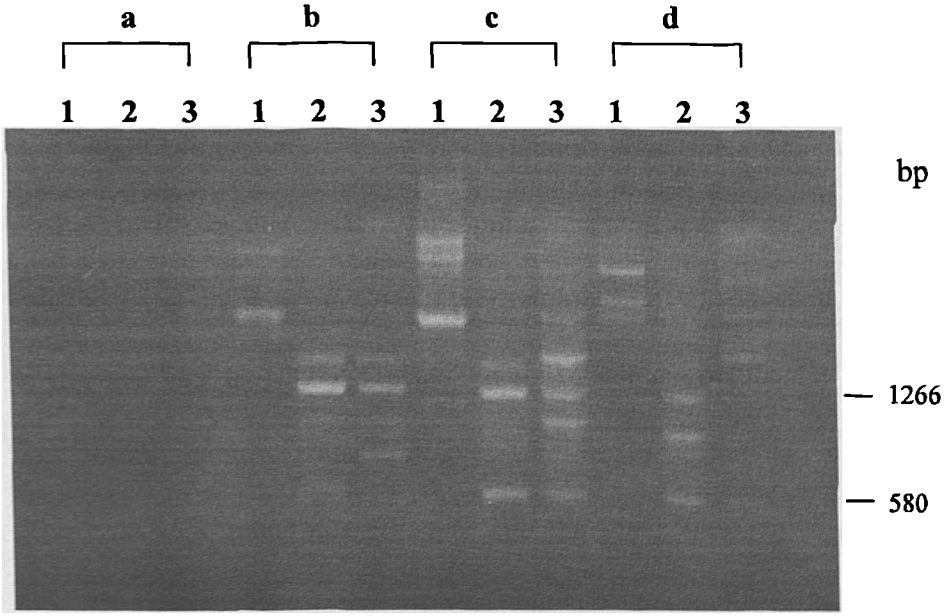


Figure 5.12. Agarose gel elctrophoresis of RAPD products of *Synechococcus* PCC7942 DNA with different magnesium concentrations and primer combinations. Lane 1, primer ABA-9; 2, primer ABA-10; 3, primer ABA-9+10. a, 0.5 mM; b, 1.5mM; c, 2.5mM and d, 5mM $MgCl_2$.

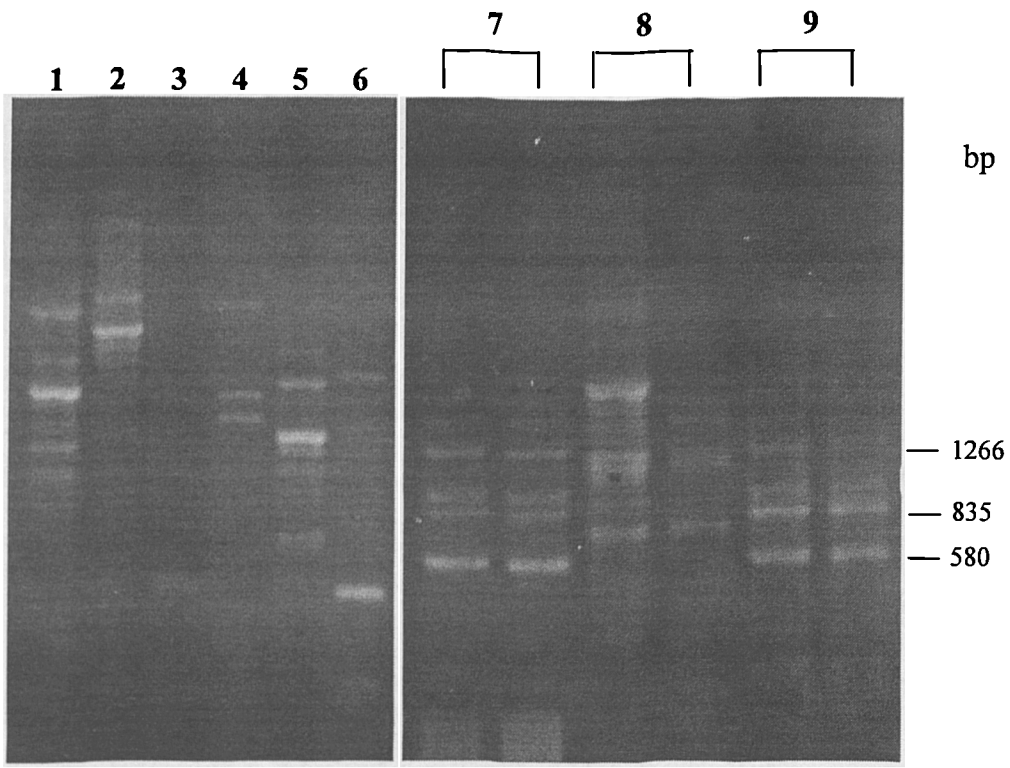


Figure 5.13. Agarose gel electrophoresis of RAPDs of *Synechococcus* PCC7942 DNA generated using different primers. Lane 1, primer ABA-4; 2, ABA-9; 3, ABA-4+9; 4, AB1-1; 5, AB1-17; 6, AB1-1+17; 7, ABA-10; 8, ABA-11 and 9, ABA-10+11, with DNA polymerase, Bioline-AmpliTaq, using the Perkin-Elmer Cetus DNA thermal cycler amplification, program 4 (see section 2.2.19).

amplify cyanobacterial DNA. Whilst most of the primers of Kit ABA (ABA-2, 3, 4, 5, 7, 8, 9, 10, 11, 12, 13, 14, 18, 19, 20) could amplify cyanobacterial DNA. Primers ABA-4, 7, 9, 10 and 14 always provided distinct bands for the different strains. Primer ABA-7 generated a more variable pattern, than the other primers, between closely related strains (Fig.5.14).

It was very difficult to choose a 10-base oligonucleotide primer specific to the wide diversity of cyanobacterial strains. All forty primers were compared, and primer ABA-7 and 10 were chosen for further study of RAPD profiles. Each amplification reaction was performed by using a single 10-base oligonucleotide primer, either ABA-7 or 10.

5. 2. 3. RAPDs of different cyanobacteria

Total genomic DNA of fifty different laboratory strains and isolates of cyanobacteria was amplified successfully by using primer ABA-7 and ABA-10. Reproducible genetic markers were produced that provided diagnostic fingerprints for each strain. In most cases a different RAPD pattern of amplified genomic DNA was generated (at least 2 bands) for each species tested (Fig 5.15). However, for strains of the same species, the same length of amplified product (or RAPD pattern) was obtained by using primer ABA-10. For example, the two *Nostoc MAC* strains exhibited an identical RAPD pattern, as did the two *Nostoc commune* strains. Different species and genera had different patterns using primer ABA-10, with the exception of *Nostoc ellipsospora, Anabaena* PCC 7120 and *Anabaena* ATCC 27892. An identical RAPD pattern was obtained for these cyanobacteria. Figure 5.15 also shows that all fresh water *Synechococcus* strains (PCC7942, LJM 002, 003, 004, 020) and the brackish water

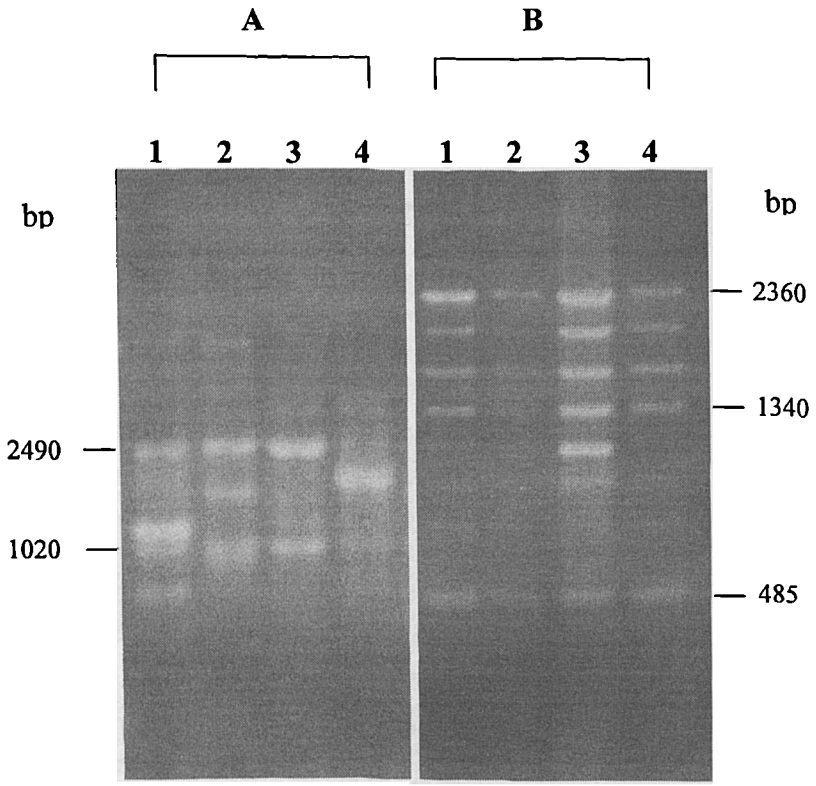
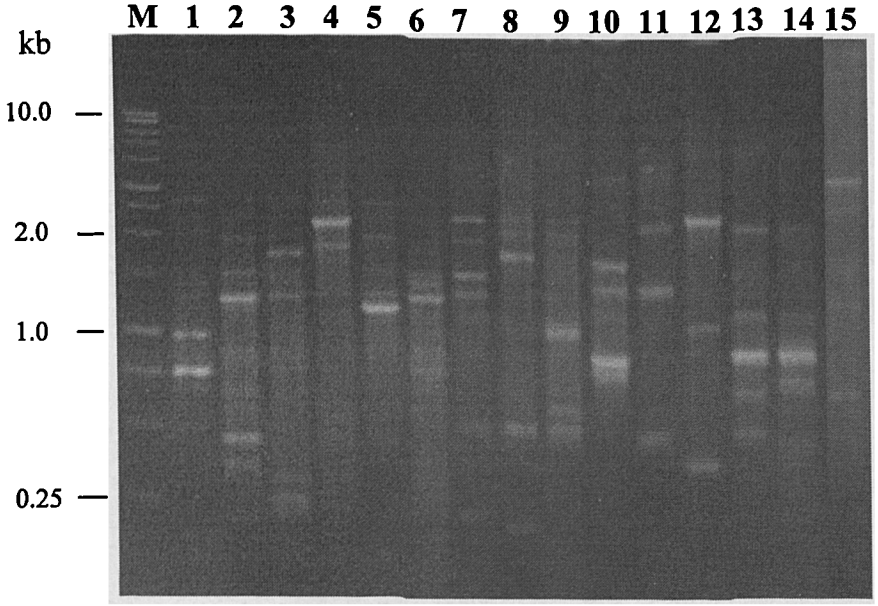


Figure 5.14. Agarose gel electrophoresis of RAPDs generated using total genomic DNA from different cyanobacterial strains and different primers. Lane 1, *Nostoc ellipsospora*; 2, *Anabaena* PCC7120; 3, *Anabaena* PCC7118; 4, *Anabaena* PCC7118 R32. A, primer ABA-7; B, primer ABA-10 used in separate amplifications. The DNA was amplified with AmpliTaq (Bioline) using standard conditions (see section 2.2.19).

Figure 5.15. Agarose gel electrophoresis of RAPDs of different cyanobacteria. Total genomic DNA from fifty cyanobacteria (1, *Synechococcus* LJM020; 2, *Synechococcus* LJM003; 3, *Synechococcus* LJM004; 4, *Synechococcus* LJM002; 5, *Synechococcus* PCC 7942; 6, *Synechococcus* CCAP 1479/7; 7, *Synechococcus* WH 7803; 8, *Pseudanabaena* sp. LJM009 9, *Pseudanabaena* sp. LJM016; 10, *Pseudanabaena* sp. LJM011; 11, *Pseudanabaena* sp. LJM001; 12, *Pseudanabaena* sp. LJM005; 13, *Pseudanabaena* sp. LJM006; 14, *Pseudanabaena* sp. LJM007; 15, *Pseudanabaena* sp. LJM019; 16, *Synechocystis* PCC 6803; 17, *Microsystis aeruginosa;* 18, *chroococcus prescottii;* 19, *Gloeocapsa* sp.; 20, *Chroococcidiopsis* LJM013; 21, *Leptolyngbya* LJM014; 22, *Oscillatoria animalis*; 23, *Phormidium autumnale*; 24, *Phormidium* LJM017; 25, *Leptolyngbya* LJM012; 26, *Nostoc palaeo*; 27, *Chlorogloeopsis fritschii*; 28, *Fisherella musicola.* 29, *Tolypothrix distorta;* 30, *Gloeotrichia* sp.; 31, *Aphanizomenon* gracile; 32, *Anabaena cylindrica*; 33, *Anabaena* sp. D0697; 34, *Anabaena flos-aquae* D0848; 35, *Anabaena* sp. D0752; 36, *Anabaena* sp. D0746; 37, *Anabaena* PCC 7118; 38, *Anabaena* PCC 7118 R3.2; 39, *Nostoc commune*; 40, *Nostoc commune* D0800; 41, *Nostoc MAC* R1; 42, *Nostoc MAC* PCC8009; 43, *Nostoc* sp. D0740; 44, *Nostoc linckia* D0734; 45, *Nostoc planctonicum*; 46, *Nostoc* sp. D0766; 47, *Nostoc piscinale* D0201; 48, *Nostoc muscorum*; 49, *Nostoc ellipsospora*; 50, *Anabaena* PCC 7120; 51, *Anabaena* PCC 7118) and two primers (A, ABA-7; B, ABA-10) were used in separate amplifications. The DNA was amplified with AmpliTaq (Bioline) using standard conditions (section 2.2.19).

A


M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
kb
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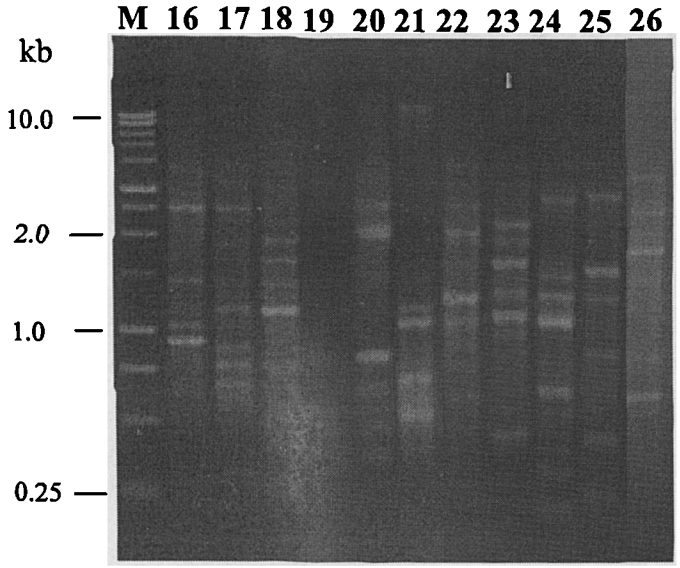
2.0
1.0
0.25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kb
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2.0
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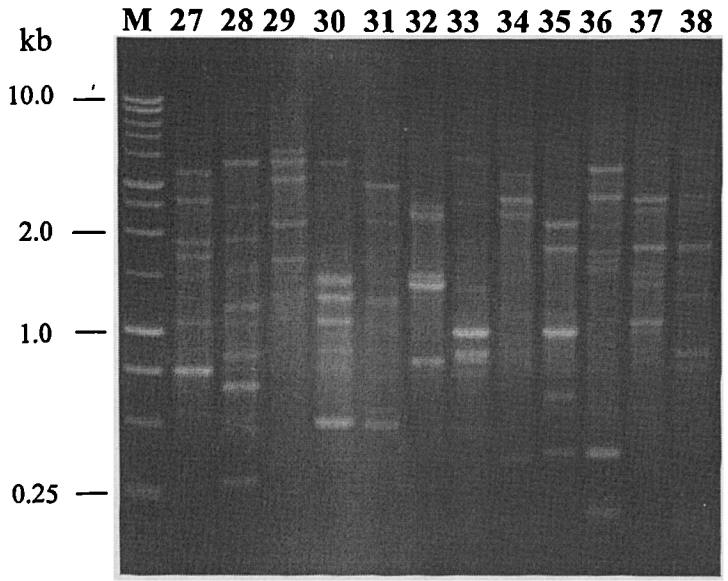
0.25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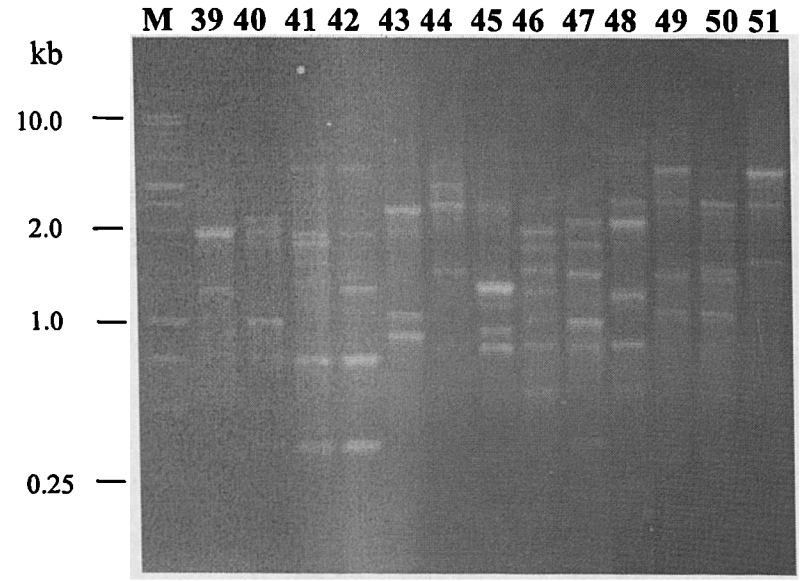
kb
10.0
2.0
1.0
0.25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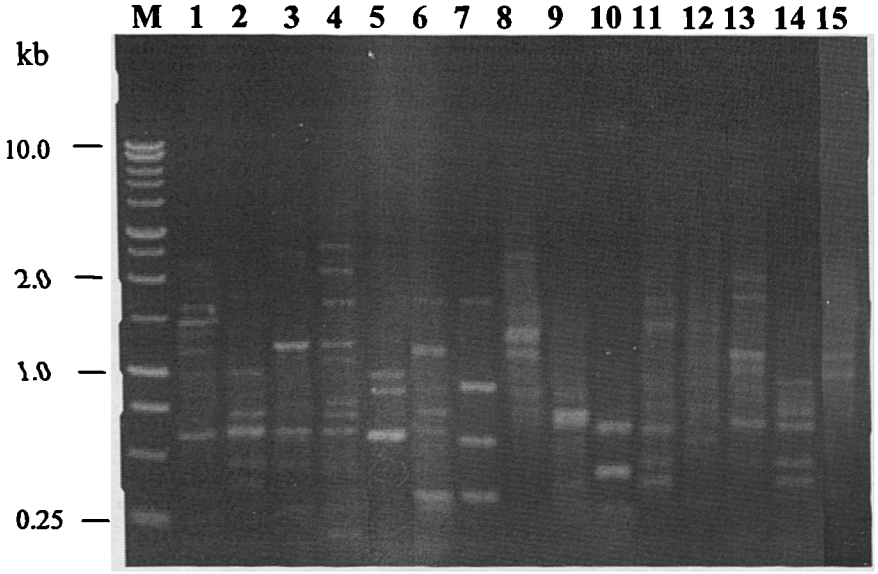
kb
10.0
2.0
1.0
0.25

B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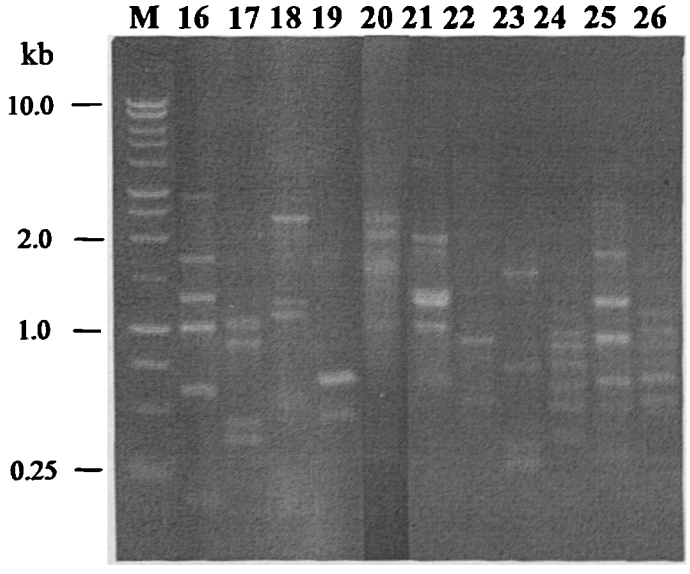
kb
10.0
2.0
1.0
0.25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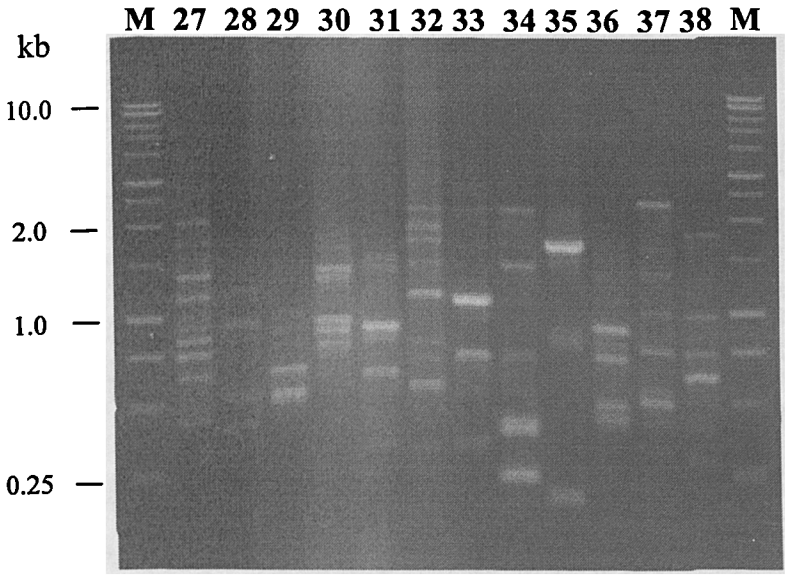
kb
10.0
2.0
1.0
0.25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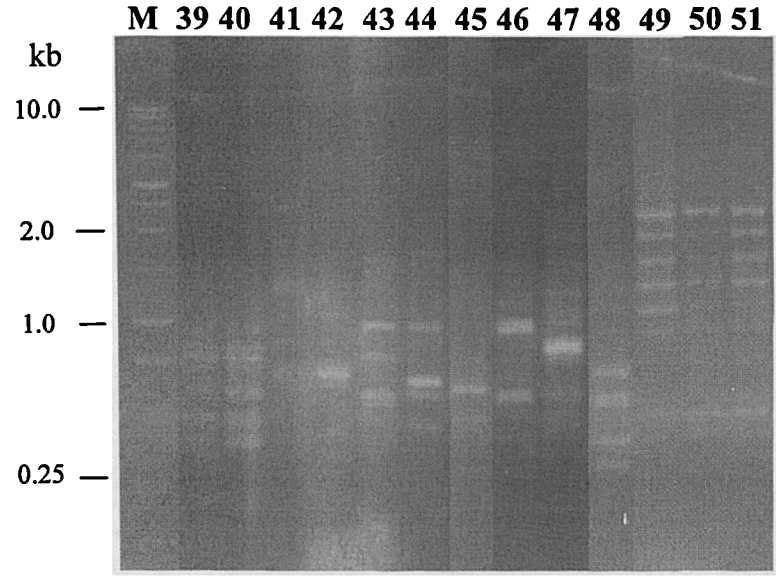
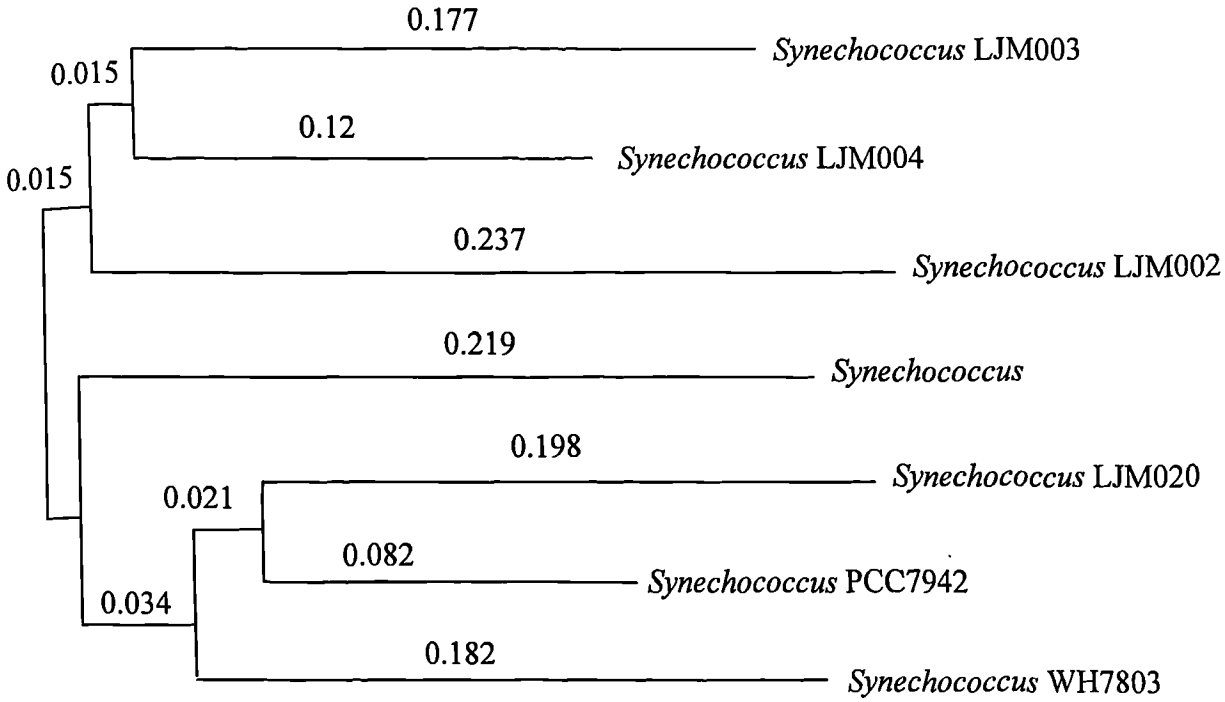
kb
10.0
2.0
1.0
0.25


M 39 40 41 42 43 44 45 46 47 48 49 50 51
kb
10.0
2.0
1.0
0.25

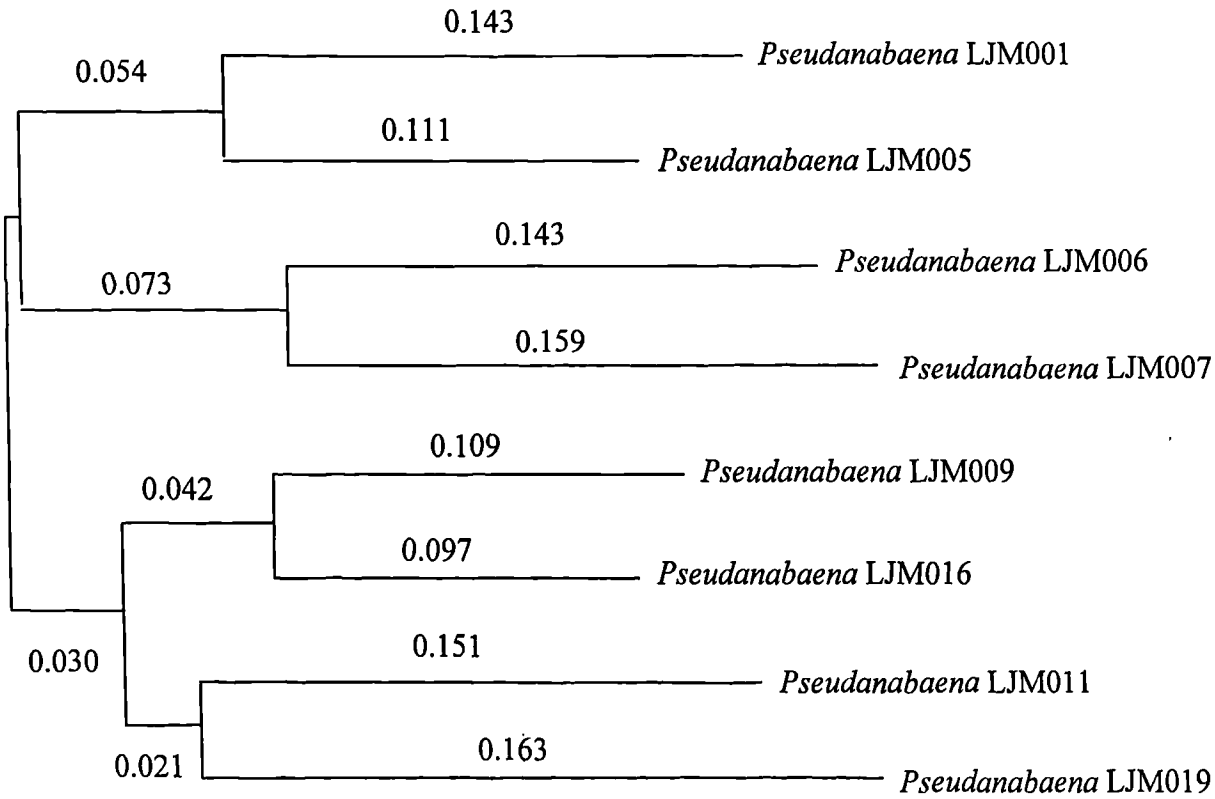
strain *Synechococcus* CCAP 1479/7 share a band of identical length (580 bp) using primer ABA-10, which was not found in the marine *Synechococcus* tested. Using primer ABA-7, different RAPD patterns, but with high similarity, were obtained for strains of the same species.

Dendrograms (Fig. 5.16) were constructed by using the RAPDistance package (Armstrong *et al.*, 1994) on the basis of pair-wise genetic distances with the combined RAPD data from the two different primers. The cyanobacteria were analysed in the following morphological groups: A, *Synechococcus* group; B, *Pseudanabaena* group; C, *Nostoc* group; D, remaining heterocystous strains; E, remaining non-heterocystous strains. Dendrogram C indicates a high similarity between *Nostoc ellipsospora, Anabaena* PCC 7120 and *Anabaena* ATCC 27892. Dendrogram A reveals a low similarity between the freshwater *Synechococcus* PCC 7942 and the marine *Synechococcus* WH 7803, and shows that the marine *Synechococcus* WH 7803 is not closely related to brackish water *Synechococcus* CCAP 1479/7. The similarity indices between the cyanobacterial strains of group A-D using primers ABA-7 and ABA-10 are shown in Table 5.1. The similarity indices for the cyanobacterial strains of different species were not over 0.5.

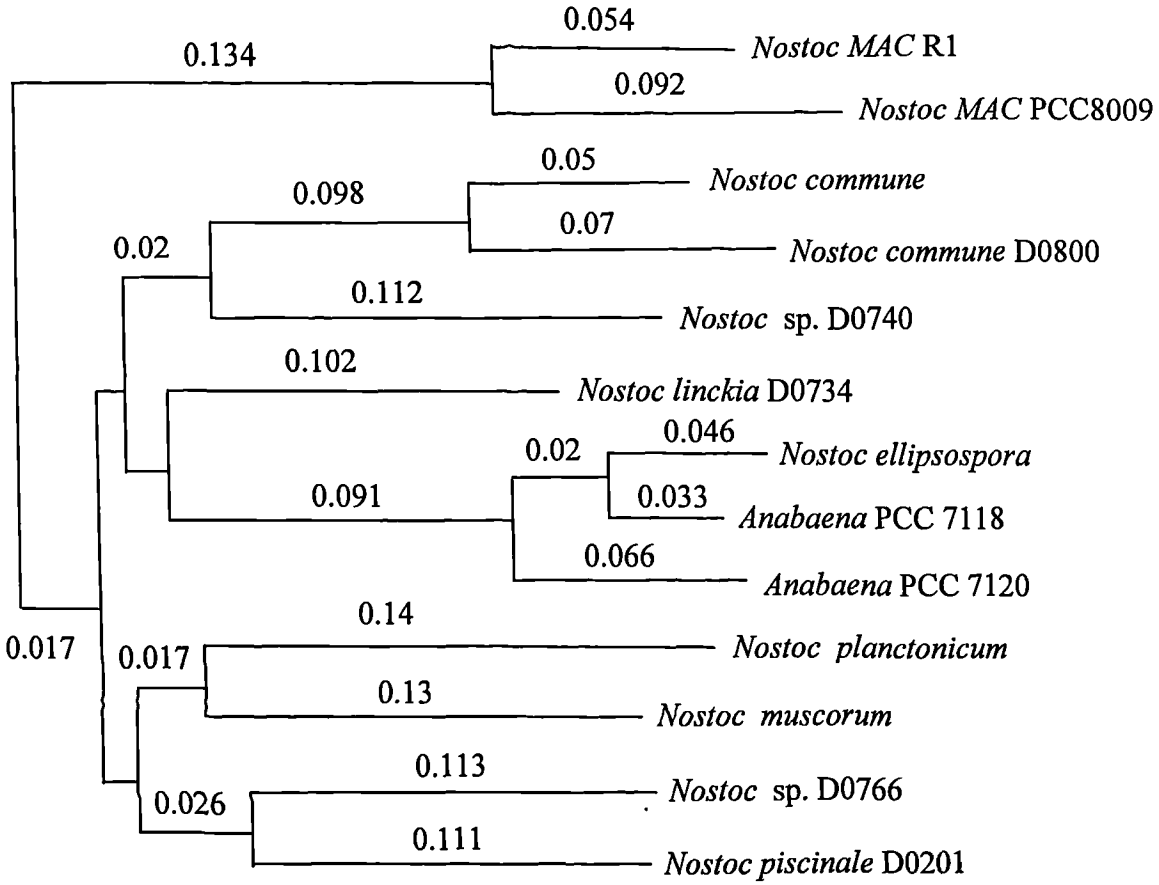
A.


0.177
Synechococcus LJM003
0.015
0.12
Synechococcus LJM004
0.015
0.237
Synechococcus LJM002
0.219
Synechococcus
0.198
Synechococcus LJM020
0.021
0.082
Synechococcus PCC7942
0.034
0.182
Synechococcus WH7803

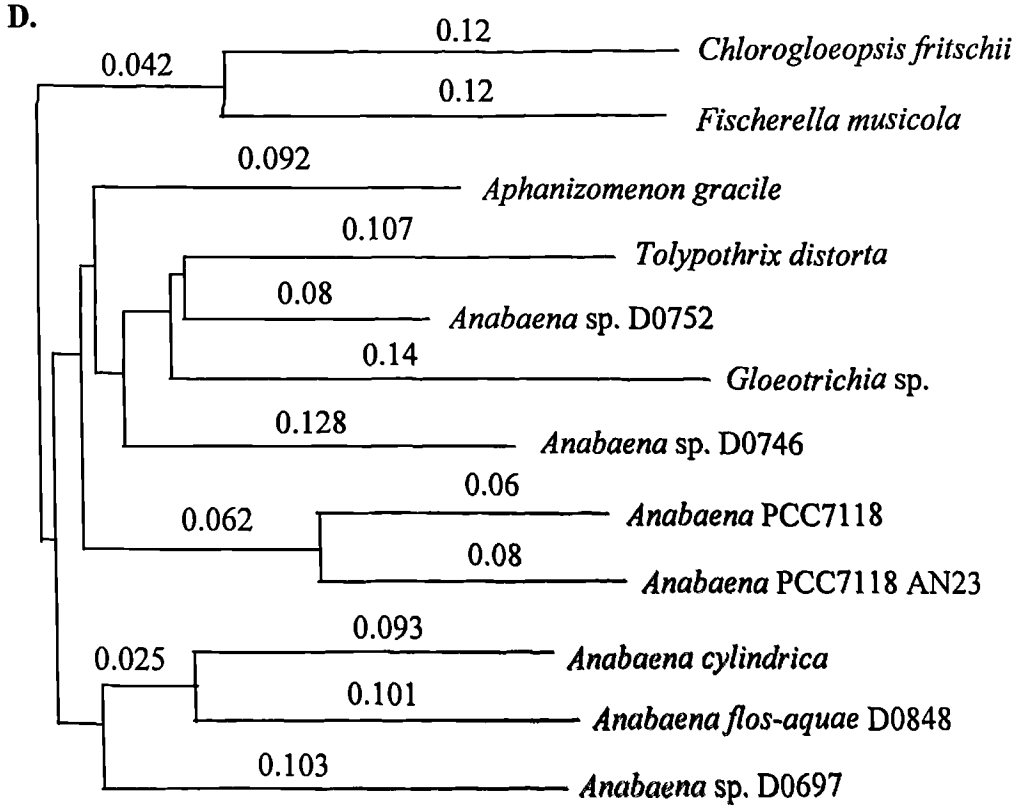
B.


0.143
Pseudanabaena LJM001
0.054
0.111
Pseudanabaena LJM005
0.143
Pseudanabaena LJM006
0.073
0.159
Pseudanabaena LJM007
0.109
Pseudanabaena LJM009
0.042
0.097
Pseudanabaena LJM016
0.030
0.151
Pseudanabaena LJM011
0.021
0.163
Pseudanabaena LJM019

C.


0.054
0.134
Nostoc MAC R1
0.092
Nostoc MAC PCC8009
0.05
0.098
Nostoc commune
0.07
0.02
Nostoc commune D0800
0.112
Nostoc sp. D0740
0.102
Nostoc linckia D0734
0.046
0.02
Nostoc ellipsospora
0.091
0.033
Anabaena PCC 7118
0.066
Anabaena PCC 7120
0.14
0.017
Nostoc planctonicum
0.017
0.13
Nostoc muscorum
0.113
Nostoc sp. D0766
0.026
0.111
Nostoc piscinale D0201

D.


0.12
0.042
Chlorogloeopsis fritschii
0.12
Fischerella musicola
0.092
Aphanizomenon gracile
0.107
Tolypothrix distorta
0.08
Anabaena sp. D0752
0.14
Gloeotrichia sp.
0.128
Anabaena sp. D0746
0.06
0.062
Anabaena PCC7118
0.08
Anabaena PCC7118 AN23
0.093
0.025
Anabaena cylindrica
0.101
Anabaena flos-aquae D0848
0.103
Anabaena sp. D0697

E.

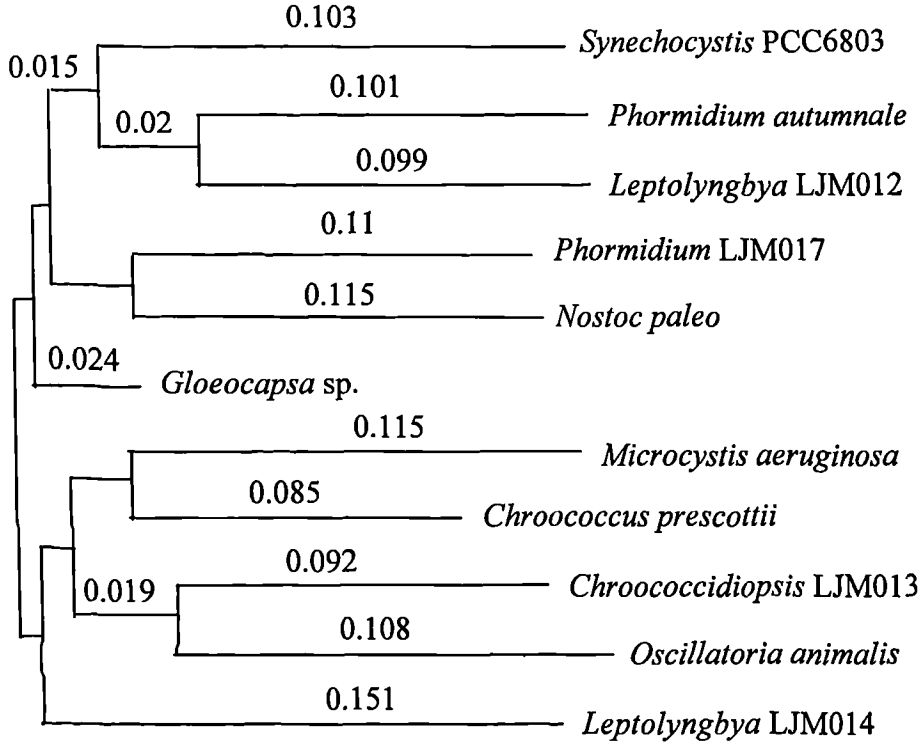


Figure 5.16. Dendrograms indicating genetic distances among cyanobacterial strains. The genetic distance from the node, based on the RAPD profiles of cyanobacterial strains, is indicated above each branch. The less closely related the strains are the greater the genetic distance. A, *Synechococcus* group; B, *Pseudanabaena* group; C, *Nostoc* group; D, remaining heterocystous strains; E, remaining non-heterocystous strains.

Table 5.1 The similarity indices of RAPDs for cyanobacterial strains with primer ABA-7 and primer ABA-10. A, *Synechococcus* group; B, *Pseudanabaena* group; C, *Nostoc* group; D, *Anabaena* group.

A.	Similarity Index[a]						
Strain	*S.* PCC7942	*S.* LJM020	*S.* LJM003	*S.* LJM004	*S.* LJM002	*S.* CCAP1479/7	*S.* WH7803
Synechococcus PCC7942	1	0.40	0.30	0.41	0.26	0.24	0.15
Synechococcus LJM020		1	0.26	0.32	0.18	0.32	0.15
Synechococcus LJM003			1	0.53	0.38	0.39	0.22
Synechococcus LJM004				1	0.45	0.41	0.18
Synechococcus LJM002					1	0.28	0.21
Synechococcus CCAP1479/7						1	0.30
Synechococcus WH7803							1

B.	Similarity Index[a]							
Strain	*P.* LJM009	*P.* LJM016	*P.* LJM011	*P.* LJM001	*P.* LJM005	*P.* LJM006	*P.* LJM007	*P.* LJM019
Pseudanabaena LJM009	1	0.52	0.26	0.32	0.42	0.24	0.18	0.27
Pseudanabaena LJM016		1	0.24	0.32	0.50	0.07	0.13	0.15
Pseudanabaena LJM011			1	0.32	0.15	0.12	0.19	0.28
Pseudanabaena LJM001				1	0.5	0.3	0.17	0
Pseudanabaena LJM005					1	0.18	0.12	0.07
Pseudanabaena LJM006						1	0.5	0.12
Pseudanabaena LJM007							1	0.18
Pseudanabaena LJM019								1

C.	Similarity Index[a]												
Strain	*N. commune*	*N. com.* D0800	*N. MAC* R1	*N.* PCC8009	*N.* D0740	*N.* D0734	*N. ellipsora*	*A.* PCC 7118	*A.* PCC 7120	*N. planctoicum*	*N.* D0766	*N.* D0201	*N. muscorum*
Nostoc commune	1	0.81	0.12	0.12	0.30	0.15	0.27	0.26	0.27	0.22	0.14	0.37	0.27
Nostoc commune D0800		1	0.12	0.06	0.23	0.15	0.18	0.26	0.18	0.30	0.21	0.27	0.27
Nostoc MAC R1			1	0.80	0.21	0.15	0.25	0.20	0.20	0.06	0.13	0.12	0.06
Nostoc MAC PCC8009				1	0.15	0.22	0.27	0.20	0.27	0.06	0.07	0.13	0.06
Nostoc sp. D0740					1	0.27	0.23	0.26	0.27	0	0.16	0.22	0.16
Nostoc linckia D0734						1	0.33	0.32	0.25	0.27	0.24	0.16	0.17
Nostoc ellipsospora							1	0.8	0.92	0.08	0.22	0.2	0.14
Anabaena PCC 7118								1	0.76	0.15	0.22	0.14	0.14
Anabaena PCC 7120									1	0.15	0.14	0.14	0.2
Nostoc planctonicum										1	0.24	0.14	0.23
Nostoc sp. D0766											1	0.28	0.26
Nostoc piscinale D0201												1	0.27
Nostoc muscorum													1

D.	Similarity Index[a]						
Strain	*A. cylindrica*	*A.* D0697	*A.* D0848	*A.* D0752	*A.* D0746	*A.* PCC7118	*A.* PCC7118 AN23
Anabaena cylindrica	1	0.22	0.34	0.14	0.12	0.37	0.30
Anabaena sp D0697		1	0.31	0.17	0.21	0.20	0.20
Anabaena flos-aquae D0848			1	0.15	0.20	0.19	0.28
Anabaena sp. D0752				1	0.14	0.27	0.27
Anabaena sp. D0746					1	0.12	0.19
Anabaena PCC7118						1	0.43
Anabaena PCC7118 AN23							1

a, Similarity indices were determined from the combined RAPD data using primer ABA-7 and ABA-10. 1 = identical; 0 = unrelated.

5.3 Discussion

The RAPD-PCR using random oligonucleotide primers of 10 nucleotides in length, has been applied to rapid identification of animal, plant, fungal, algae and bacterial strains (Welsh and McClelland, 1990; Williams *et al.*, 1990; Akopyanz *et al.*, 1992; McCutcheon *et al.*, 1993). RAPD profiling of these genomes has been widely accepted as a valid taxonomic and phylogenetic tool. In this study, results indicate that both the heterogeneity and the homogeneity of the RAPD pattern can be useful for typing cyanobacteria.

The RAPD technique has been optimised for cyanobacterial DNA from the laboratory strains tested. It is important to note that reaction conditions, including the template DNA concentration, the magnesium ion concentration, annealing temperature, primer concentration and DNA polymerase, all affected the relative intensity and presence of the amplified bands. This is consistent with results of Williams and co-workers (1993) with the exception of the primer concentration, where no effect of primer concentration on the relative intensities of bands was found. Therefore, careful choice of conditions and of the thermostable enzyme used are required to obtain consistent results. For reproducibility of the RAPD technique, five PCR programs and two PCR thermal cyclers were tested. The results showed that the different programs and thermal cyclers, both affected the RAPD patterns. Clear and distinct patterns were obtained from program 4. Programs 3 and 5 were least reliable probably due to the holding time (5 s for program 3 and 1 s for program 5) at the denaturation temperature being too short. Programs 1 and 2 gave similar results suggesting that a shorter holding time of 30 s can be used without affecting the amplified DNA patterns. Setting an exact transition interval time

(program 4) provided more reliable results when the two thermal cyclers were used. This supports the findings of Schweder and co-workers (1995) that the transition interval between the denaturation and annealing temperatures appears to have an important impact on RAPD analyses. In order to obtain reliable and consistent results, the conditions of the reaction must be reproduced exactly, which may require accurate recording of transition interval set points as well as temperature set points. Variable ramp characteristics of a thermal cycler during a reaction and between reactions, depending, for instance, on ambient conditions, may help explain why RAPD analyses are so precarious, particularly between different laboratories when analysing template DNA from a single source with identical primers (Penner *et al.*, 1993). So results from this analysis can only be compared with results of RAPDs produced in other laboratories if the same conditions are used.

The choice of primers has proven to be useful for optimising the RAPD reaction. Figure 5.11 shows that specific primers can alter the effect produced by changing magnesium ion concentration and annealing temperature. The primer sequence is very important for successful amplification. In this study, most of the 10-mer oligonucleotides from Kit ABA and a few from Kit AB1 gave successful amplification of the cyanobacterial DNAs tested. In some cases, the different genera of cyanobacteria required different primers for amplification. Other researchers, for example Eskew (1993) and Neilan (1995) also showed that some of these primer sequences could amplify several genera of cyanobacterial DNA. According to Neilan (1995) multiplex PCR increased reproducibility of the RAPD profile, primarily due to the use of pairs of random 10-mer oligonucleotide primers. The products of the multiplex RAPD method, under more stringent conditions, were reproducible across a range of varied parameters, such as enzyme preparation, type of thermal cycler,

and quantity and quality of template DNA. In this study, experiments comparing single- and multiple-primer reactions indicated that most bands derived from the single priming reaction were retained, although some new products were additionally synthesised, as a result of multiplexing. However, no clear evidence was provided to show that multiple-primer reactions provided more reliable results than single-primer. Varying parameters such as enzyme preparation, type of thermal cycler, type of PCR-program, quality and concentration of template DNA, the magnesium ion concentration, annealing temperature, and primer concentration also affected the relative intensity of the amplified multiplex bands. Figure 5.12 indicated that the single primer, ABA-10, provided more reliable results than the combination of primers ABA-9+10. Thus selection of the correct primer is crucial for optimising the RAPD reaction, whether it is a single-primer or multiple-primer reaction. This contrasts with the results of Neilan (1995) where the combination of two primers in a single reaction has proven to be useful for optimising RAPD reaction as has 'the correct selection of primer'.

Primers ABA-7 and 10 were chosen here for generating RAPDs of cyanobacteria, because primer ABA-10 could generate patterns to separate different species in the same genus, whilst primer ABA-7 could generate patterns to distinguish between different strains of the same species. Furthermore, they could both amplify DNA from all the cyanobacteria tested and generate clear reproducible patterns.

Genomic DNA of fifty different laboratory strains and isolates of cyanobacteria was amplified successfully by using primer ABA-7 or ABA-10. For strains of the same species, the same RAPD pattern was obtained by using primer

ABA-10. Different species and genera had different patterns, with the exception of *Nostoc ellipsospora, Anabaena* PCC 7120 and *Anabaena* ATCC 27892. The highly related branches in the distance tree (Fig. 5.16 C) and the similarity indices (Table 5.1) of 80%-90%, for *Nostoc ellipsospora, Anabaena* PCC 7120, and *Anabaena* PCC 7118 indicate that they may be different strains in the same species group. Furthermore, Figure 5.16 C shows that they are close to *Nostoc linckia* D0734. This supports the findings of Lachance (1981), based on DNA-DNA hybridisation data, that *Anabaena* PCC 7120, and *Anabaena* PCC 7118 are more closely related to *Nostoc strains* than to other *Anabaena* strains.

There were limitations to the application of the RAPD technique to the identification of cyanobacteria, where highly diverse strains were analysed. Particular strains of one genus may group together with some strains in other genera when they are present (Fig. 5.16 E). For strains in the same genus (Fig. 5.16 B) or particular strains (Fig. 5.16 D, *Chlorogloeopsis fritschii* and *Fisherella musicola*) from different genera in the same taxonomic order, RAPD-PCR can prove useful for the identification of cyanobacteria. However the results confirm the findings of Neilan (1995) that the use of a single, multiple-primer RAPD-PCR may not be suitable for inferring an accurate phylogeny for all taxonomic groups. In addition, the RAPD-PCR generated too many bands (2-15 bands per strain, per primer) to analyse the combined data from all the separate gels. The RAPDistance package is limited to use with less than 200 bands. So RAPD-PCR analysis can only be applied to about 20 cyanobacterial strains.

The application of RAPD analysis demands that the cultures are axenic, because the presence of any contaminating microorganisms would alter the RAPD

profile. Thus for the technique to be applicable to environmental isolates, the samples must first be purified by selective culturing or by using specific ultra-centrifugation, in order to remove contaminating microorganisms. The RAPD technique has been applied to axenic cultures of newly isolated cyanobacteria. The genetic relatedness among the isolates of cyanobacteria, deduced from the RAPD profiles, was supported by the morphological data (see Fig. 5.16 A and B and compare with the morphological results of Fig. 3.22). In Figure 5.16 E, the larger distance between two *Leptolyngbya* strains suggests that they possibly should be separated into different genera, in line with their morphological differences in constriction at the cross walls and sheath formation, which are morphological characters for separation of simple filamentous cyanobacteria at the genus level.

Results from this study thus indicate that distinct RAPD profiles can be produced for a number cyanobacteria. The technique used was performed without the need for radioactive labelling of the RAPD-PCR products or previous knowledge of the cyanobacterial gene sequences. Therefore, RAPD analysis, which utilises the entire genome as the PCR template, provides a rapid, technically easy approach to the genetic typing of axenic cultures of cyanobacteria. Results are comparable with those achieved by more traditional methods. The finding that certain primers were specific to particular genera of cyanobacteria may be useful for identifying cyanobacteria at the level of genus. Thus RAPDs should provide suitable markers for the identification and classification of cyanobacteria, once a suite of RAPD profiles has been established for different cyanobacteria.

Chapter 6

Identification of cyanobacteria by polymorphisms of PCR-amplified ribosomal DNA spacer region

6.1 Introduction

The limitations of traditional techniques have prompted the development of molecular biological techniques for identification of cyanobacteria. The random amplified polymorphic DNA (RAPD) technique, in conjunction with the polymerase chain reaction (PCR), has been employed to identify many organisms to the strain level of classification (Welsh and McClelland, 1990; 1991). Indeed the technique has been used to identify cyanobacteria in pure culture and cyanobacterial symbionts (Eskew *et al.*, 1993; Neilan, 1995; chapter 5, this study). The approach has enabled discrimination between different strains of cyanobacteria. However, the requirement for axenic cultures restricts the applicability of the RAPD technique.

The use of rRNA genes is well established for identifying evolutionary relationships between organisms, as rRNA genes show a high degree of functionally constancy, which assures relatively good clock-like behaviour (Woese, 1982, 1987). They are essential for the survival of all organisms and are highly conserved in the Bacteria, Archaea and Eukarya (Lane *et al.*, 1985; Woese, 1987). The rRNA operon is transcribed as a pre-rRNA transcript that contains the following components in the order (5' to 3'): 16S rRNA, spacer, tRNA, spacer, 23S rRNA, spacer and 5S rRNA gene sequences (Watson *et al.*,1987; see Fig 1.1). Characterisation of the 16S rRNA gene has proved useful in the identification of species, genera and families of bacteria (Woese, 1987; Amann *et al.*, 1995) and cyanobacteria (Wilmotte, 1994). This is reflected in the large amount of sequence information that has been accumulated for the phylogenetic analysis of microorganisms using this gene (Olsen *et al.*,1991). In contrast, relatively few 23S gene sequences have been reported, and neither this gene nor the 5S gene have been widely used for phylogenetic purposes (Wilmotte,

1994). Sequence information has also become available on the 16S-23S rRNA spacer region and suggests that considerable variation can occur between species in both the length and the sequence of this region (Gurtler and Stanisich, 1996). Such variation together with the fact that many bacteria possess multiple copies of the rRNA operon, provides scope for using this region to identify and type bacteria.

Systematic studies of spacer region variation would require amplification of the region by PCR (Mullis and Faloona, 1987) and hence the availability of conserved sequences in flanking 16S rRNA, tRNA and 23S rRNA genes to serve in primer recognition. In cyanobacteria a variety of primers has already been used for PCR amplification or sequencing purposes (Wilmotte *et al.*, 1993), but the appropriateness of the choices, particularly for those based on the 23S rRNA gene, has been limited by the paucity of sequenced cynaobacterial 23S rRNA genes. Only two 23S rRNA genes from *Synechococcus* sp. strain PCC 6301 and *Synechococystis* strain PCC6803 (cited in Wilmotte, 1994) have been sequenced. The 16S-23S spacer region or internal transcribed spacer (ITS) region of cyanobacteria typically contains the following components in the order (5' to 3'): spacer, $tRNA^{ile}$, spacer, $tRNA^{ala}$, spacer (see Fig 6.1), although some cyanobacteria have only the $tRNA^{ile}$ gene. For PCR amplification of the entire or partial ITS region, several primers are available (Wilmotte *et al.*, 1993). Variation within this region may be detected by a number of techniques, including restriction fragment length polymorphisms (RFLPs) or DNA sequencing.

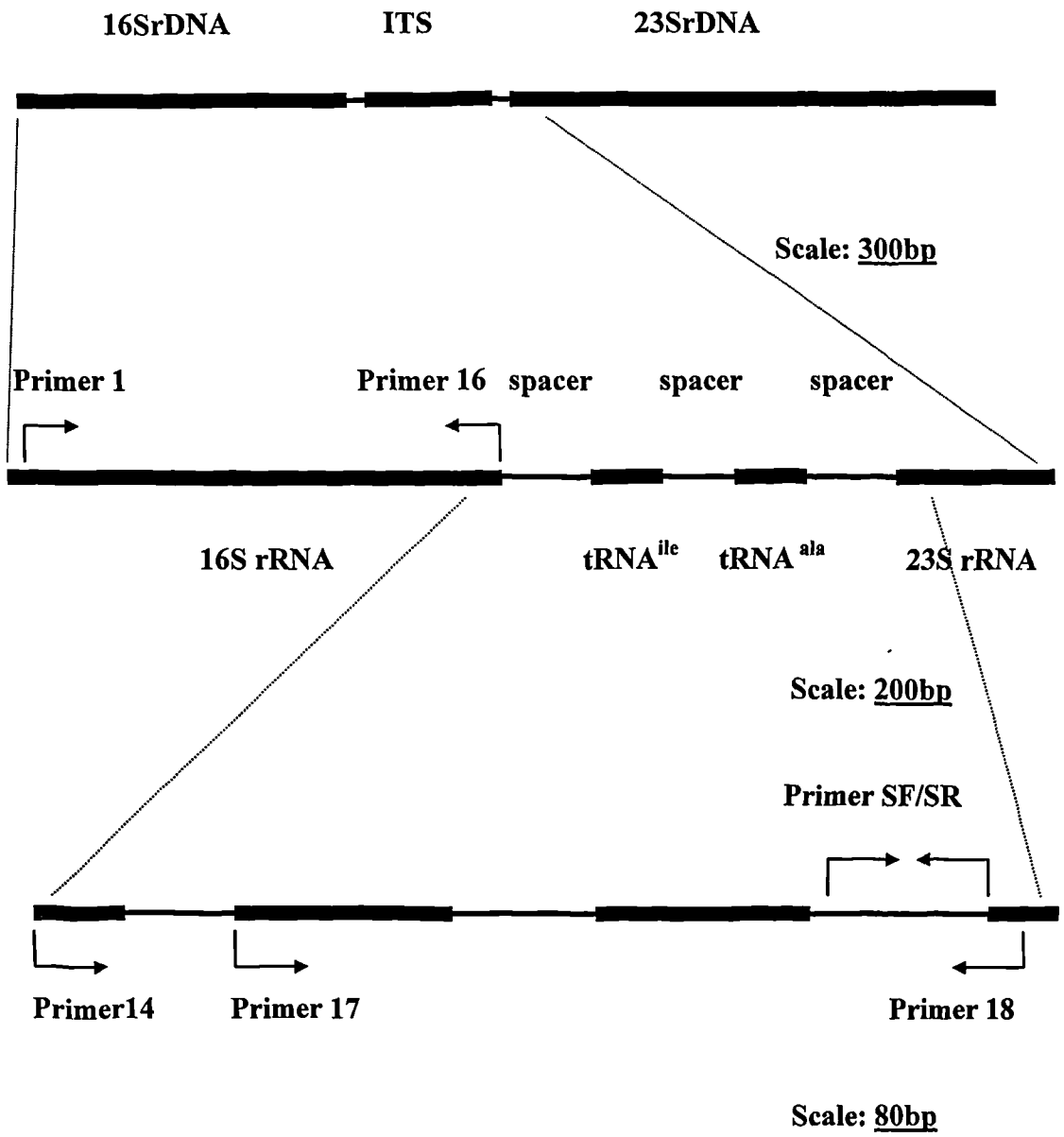


Figure 6.1. Schematic diagram of ribosomal DNA (rDNA) repeat showing internal transcribed spacer region (ITS). Transcription of the operon is from left to right. Some cyanobacteria have two transfer RNA (tRNA) genes as shown; others just have $tRNA^{ile}$. The arrowed bars show the location of primers used in this study.

SF/SR, cyano-specific forward/reverse primers developed in this study; $tRNA^{ala}$, alanine tRNA; $tRNA^{ile}$, isoleucine tRNA.

6.1.1 RFLP-PCR of rDNA spacer

PCR technology has been used in several recent studies as a rapid means of amplifying the 16S-23S spacer region for the specific purpose of detecting heterogeneity between and within species (Barry *et al.*,1991; Jensen *et al.*, 1993). Such heterogeneity has been found in terms of both the number and the length of the spacer, and raises the possibility that this methodology has broad applicability as a rapid, possibly automatable, general method for bacterial identification (Gurtler and Stanisich, 1996). The polymorphic character of the PCR-amplified product can be revealed by digesting with restriction enzymes. Resulting fragments are resolved electrophoretically and patterns can be indicative of a particular species. Furthermore, size of the PCR- amplified products themselves can be diagnostic. In cyanobacteria, Neilan and co-workers (1995) developed primers to amplify the phycocyanin operon (*cpc* gene), and then used restriction fragment length polymorphisms of PCR products to infer the genetic relatedness and evolution of toxic and bloom-forming cyanobacteria. Genetic characterisation of strains of cyanobacteria has also been undertaken using restriction fragment length polymorphisms of the intergenic spacer of the genes encoding phycobilisome subunits (*cpc BA*) (Bolch *et al.*, 1996).

Whilst RFLP analysis can be useful for rapid screening and for distinguishing between different genera and species, it only detects a small amount of the variation that may be present in the spacer region. More information can be obtained from DNA sequencing.

6.1.2 DNA Sequencing of rDNA spacer

Comparative studies of the nucleotide sequences of rRNA genes provide a means of analysing phylogenetic relationships over a wide range of taxonomic levels (e.g. Woese and Olsen 1986; Wilmotte, 1994; Wilmotte *et al.*, 1992,1993,1994; Neilan *et al.*, 1994; Neilissen *et al.*, 1994, 1996). Numerous sequences of rRNA genes have been obtained, primarily by isolating and sequencing individual cloned genes (Medlin *et al.*,1988). Direct rRNA sequencing (Lane *et al.*, 1985) has also been used to obtain sequence data rapidly. However, this method requires relatively large amounts of RNA and is prone to errors, since only one strand is sequenced.

The polymerase chain reaction (PCR) and direct DNA sequencing provide several advantages over cloning and direct rRNA sequencing. These include: (1) the method utilises relatively crude preparations of total DNA, such as those from minipreps, or can be performed directly using whole cells, without further DNA preparation and purification (see section 2.2.14.3); (2) only small amounts of DNA are required, about 0.1 to 10 ng per amplification; (3) both strands of the gene can be sequenced, which reduces errors; (4) the method is compatible with automated DNA sequencing instruments that utilise fluorescently-labelled dideoxynucleotide triphosphates or primers (White *et al.*, 1990).

16S rRNA gene sequences are probably too conserved to investigate intra-species variability (Ward *et al.*, 1992). The sequence of the ITS region (16S-23S rDNA spacer), may be more suitable for studying such variability. Gurtler and Stanisich (1996) reviewed and aligned 33 different 16S-23S spacer sequences from 13 species of bacteria. Despite the limited scope of the analysis and the occurrence

of gaps in the DNA alignments, due to the variable length of the spacers and the relative positions of the tRNA genes, no highly conserved regions were found. The high inter-specific variability reported for the spacer makes it an appropriate candidate for restriction fragment length polymorphisms (RFLPs). These workers suggested that homology searches based on very short regions (20 bp or less) might reveal inter-species homologies; such regions would need to be avoided when designing species-specific primers. However, they could be of great use in the design of genera-specific primers.

Analysing phylogenetic relationships is a very complex and controversial issue and a review of the different methodologies is provided by Swofford and Olsen (1990). Distance methods and parsimony analysis are most often used to infer phylogenetic trees. Distance methods belong to the phenetic techniques and are based on the calculation of dissimilarities for each pair of organisms. These dissimilarity values are corrected for multiple mutations and entered in a matrix. A tree topology, in which the distances between organisms in the tree will be as close as possible to the matrix values, is constructed by an algorithm. Parsimony analysis is based on the cladistic approach. It considers that only the similarity due to the possession of new (derived) characters is phylogenetically informative. Thus, only the variable positions, where at least two organisms share the same derived nucleotide, are used in the calculation of the tree topology. Ancestral sequences are re-constructed at each node of the tree. The final tree topology is the one requiring the minimal number of evolutionary changes. A statistical evaluation of tree topologies can be carried out by bootstrap analysis (Felsenstein, 1985). New sequence alignments are generated by random sampling of the positions with replacement. For each re-sampled data set, a new tree is constructed. The bootstrap value for each node is the number of re-sampled trees in which the same organisms are

clustered together. Only the grouping of strains is considered, not the topology and lengths of the branches that diverge after the node.

This chapter reports the development of a PCR-based technique that requires only small quantities of relatively impure DNA, and the use of the variability of the length and sequence of the 16S-23S rDNA spacer region to distinguish between various genera and species of cyanobacteria. The approach used was to amplify the 16S-23S rDNA spacer from cyanobacteria by PCR, and test various restriction enzymes to find those producing RFLPs useful in identification. In addition, the extent of sequence similarity in the rDNA spacer of various cyanobacterial strains was established by direct DNA sequencing of PCR products and by cloning and plasmid sequencing, followed by sequence alignment. Oligonuleotide sequences selected from conserved regions were evaluated as cyano-specific primers, for amplification of cyanobacterial DNA from laboratory cultures and environmental samples.

6.2 Results

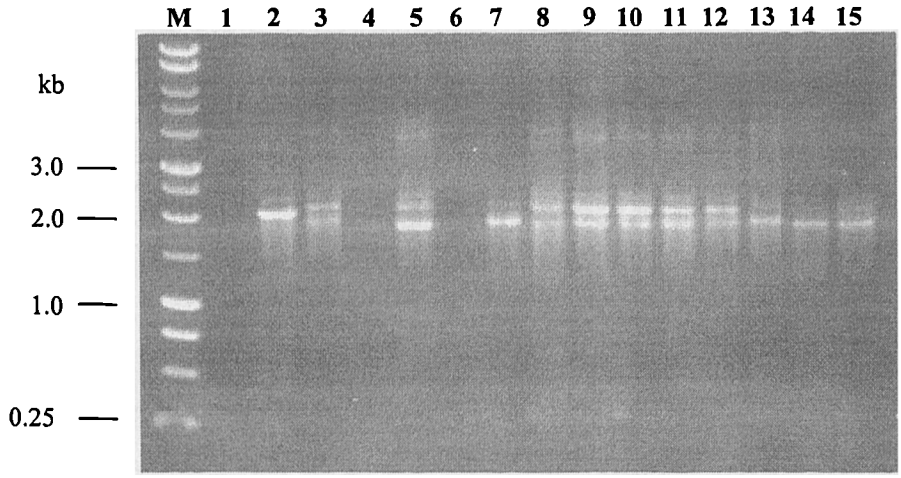
6.2.1 RFLP-PCR

A number of primer pairs was tested for amplification of the 16S-23S rDNA spacer with a view to generating products useful for discriminating between cyanobacterial strains. The priming sites were selected from regions of highly conserved sequences found in the 16S rDNA, tDNA and 23S rDNA regions adjacent to the intervening spacer region (see Fig. 6.1). The PCR procedure was optimised for the primers used and was either performed directly on cyanobacterial cultures or on DNA extracted from them (see section 2.2.20). In each case identical results were obtained.

The choice of primers was critical to the use of spacer variation for species identification. Primer pair 1/18 (which amplifies 16S rDNA, ITS region, $tDNA^{ile}$ [or $tDNA^{ile}$ and $tDNA^{ala}$] and 5'end 23S rDNA) and 14/18 (which amplifies 3'end of 16S rDNA, ITS region, $tDNA^{ile}$ [or $tDNA^{ile}$ and $tDNA^{ala}$] and 5'end of 23S rDNA) amplified the rDNA to give multiple DNA bands of different intensities, for a number of the species tested. The intense and reproducible bands have been classified as primary products, the weaker, variable bands as secondary products (Table 6.1 & Fig. 6.2 a, b). Weak bands were common in the heterocystous strains, especially in the genera *Nostoc* and *Anabaena*. The secondary amplification products could be heteroduplexes containing conserved homologous sequences at the 3' and 5' ends, separated by highly variable ITS sequences. These heteroduplexes would migrate more slowly in gels than the expected homoduplexes because of the single-stranded regions. In order to test for heteroduplex formation, susceptibility of amplification products to the single-strand specific endonulease, S1, was determined. Figure 6.3 shows that when amplified products (using primer pair 14/18) were digested with S1 nuclease, the most intense bands remained. Whilst the less intense and weak bands (normally >780 bases) were removed. A number of new, fainter fragments in the size range of 120-400bp could be seen. These smaller fragments are probably residual double-stranded components, remaining after digestion of single-stranded positions of the heteroduplex DNA structures. The undigested bands that remained are presumably the homoduplex 16S/23S spacer amplification products. The sizes of these products are also indicated in Table 6.1. The variation in heteroduplex and homoduplex 16S-23S spacer amplification products may be useful in identifying heterocyst-forming filamentous cyanobacteria, by using single-strand conformation polymorphisms (SSCP). For the unicellular and non-heterocyst-forming filamentous cyanobacteria a single amplified rDNA product was

Figure. 6.2 Agarose electrophoresis gels of amplified rDNA spacer products of selected cyanobacterial strains. (A) PCR-amplification using primer 1/18; (B) PCR-amplification using primer 14/18; (C) PCR-amplification using primer 17/18; M, 1kb DNA ladder; 1. *Nostoc MAC* R1; 2. *Nostoc MAC* PCC8009; 3. *Nostoc commune*; 4. *Nostoc planctonicum*; 5 . *Nostoc piscinale* D0201; 6. *Nostoc* sp. D0740; 7. *Nostoc linckia* D0734; 8. *Nostoc muscorum*; *9. Nostoc ellipsospora*; 10. *Anabaena* PCC 7120; 11. *Anabaena* PCC 7118; 12. *Anabaena* PCC 7118 R3.2; 13. *Anabaena cylindrica*; 14. *Anabaena flos-aquae* D0848; 15. *Anabaena* sp. D0697; 16. *Synechococcus* PCC 7942; 17. *Synechococcus* CCAP 1479/7; 18. *Synechococcus* WH 7803; 19. *Synechocystis* PCC 6803; 20. *Microcystis aeruginosa;* 21. *Gloeocapsa* sp.; 22. *Chroococcus prescottii;* 23. *Pseudanabaena* sp. LJM001; 24 *Oscillatoria animalis*; 25. *Phormidium autumnale;* 26. *Gloeotrichia* sp.; 27. *Aphanizomenon gracile*; 28. *Tolypothrix distorta;* 29. *Chlorogloeopsis fritschii*; 30. *Fisherella musicola.*

A.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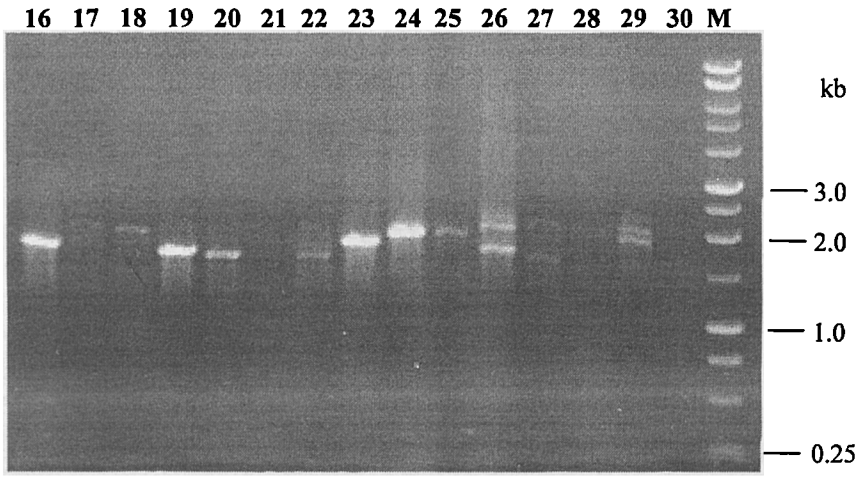
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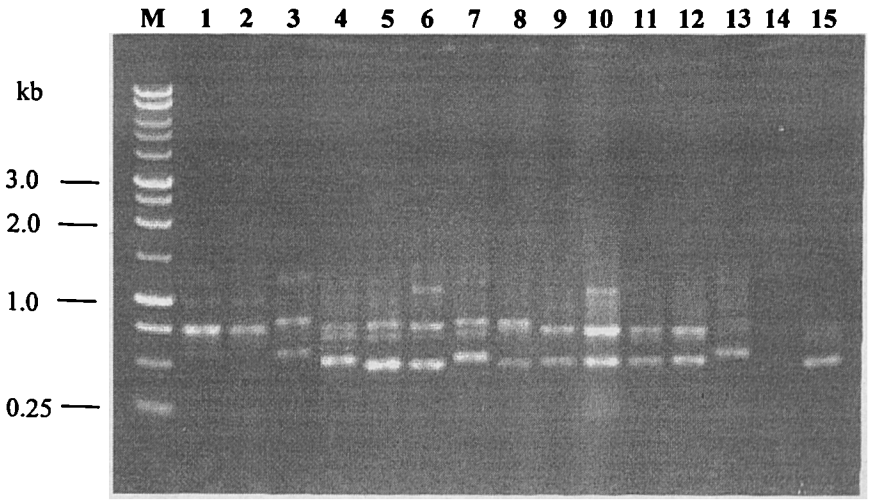
B.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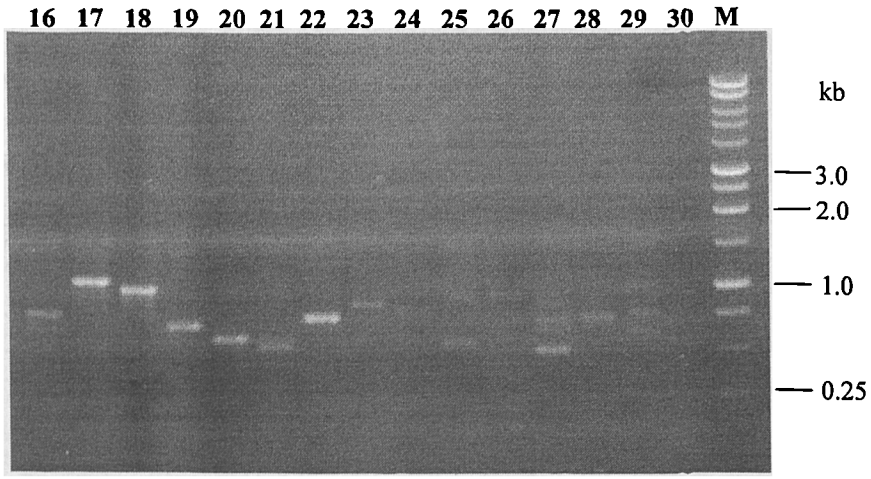
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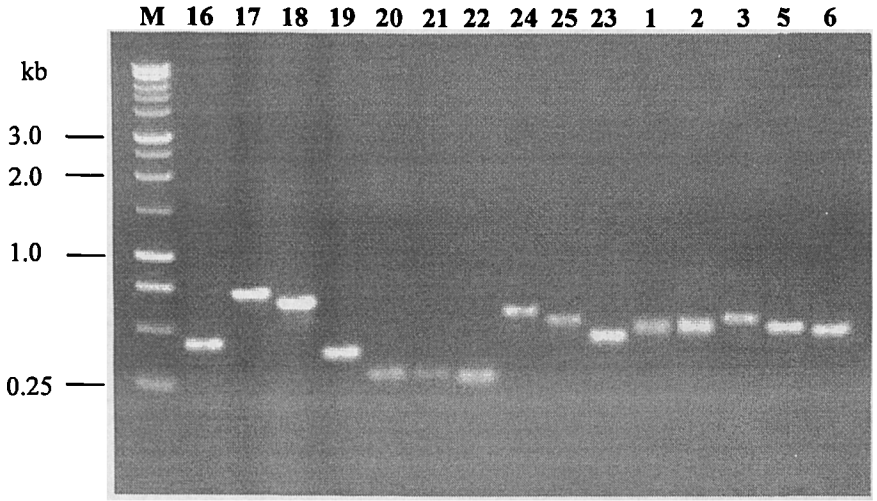
C.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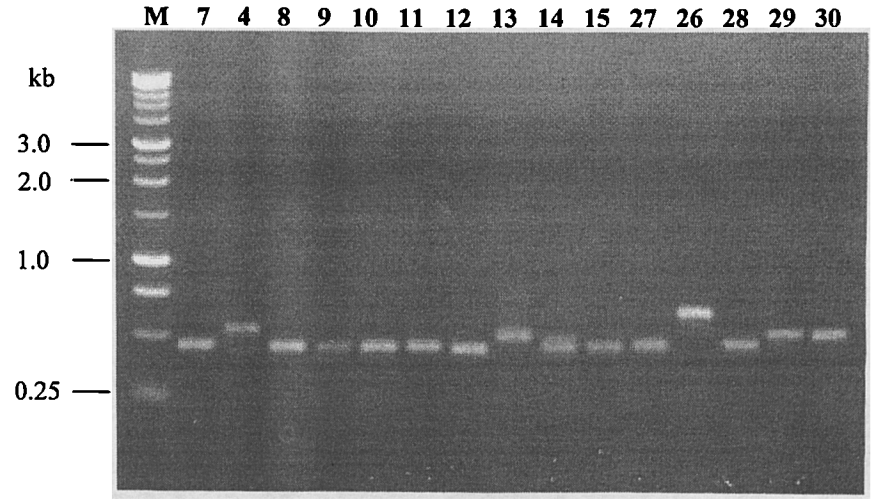
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kb
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Table 6.1. Cyanobacteria used and the sizes of rDNA fragments amplified by primer pairs 1/18, 14/18 and 17/18.

Strain	Source[a]	amplified rDNA (bp)[b]		
		primer 1/18	primer 14/18[c]	primer 17/18
Unicellular				
Chroococcus prescottii	BSM	1865	555	310
Gloeocapsa sp.	BSM	1910	560	322
Microcystis aeruginosa	BSM	1905	585	320
Synechococcus PCC7942	LJMU	2100	735	425
Synechococcus CCAP1479/7	LU	2400	1010	665
Synechococcus WH7803	LU	2300	930	625
Synechocystis PCC6803	UW	1940	665	390
Filamentous non-heterocystous				
Oscillatoria animalis	BSM	2200	840	575
Phormidium autumnale	BSM	2200	800	535
Pseudanabaena liv JM 001	LJMU	2075	735	450
Filamentous heterocystous				
Anabaena ATCC27892	LU	1875,2100(2300,3642)	515,730(665,857,960,1100)	435
Anabaena ATCC27892 R3.2	LU	1875,2100(2300,3642)	515,730(665,857,960,1100)	435
Anabaena cylindrica	UW	1890(2150,2230)	575(723,767,948,1036,1217)	485
Anabaena cylindrica (AN 23)	LJMU	1890(2150,2230)	575(723,767,948,1036)	485
Anabaena flos-aquae D0848	UD	1836(2065)	535(767,887,995)	430
Anabaena PCC 7120	UW	1875,2100(2300,3642)	515,730(665,857,960,1100)	435
Anabaena sp. D0697	UD	1836(2065,2230)	530(695,750,887,995)	430
Anabaena sp. D0746	UD	1836(2165,2330)	498,745(670,930,1180)	460
Anabaena sp. D0752	UD	1870(2120,2285)	515,740 (675,867,950)	478
Aphanizomenon gracile	BSM	1780,2350	450,858(329)	440

Filamentous heterocystous continued.....

Chlorogloeopsis(Chlorogloea) fritschii	LJMU	2018,2185(2300)	593(730,980)	480
Fischerella musicola	BSM	2018(2105,2340)	530(730,930)	475
Gloeotrichia sp.	UW	1940,2300(2465)	494,845(1029,1170)	586
Nostoc commune	BSM	1970,2000,2190(2460,3943)	560,790(705,985,1230)	535
Nostoc commune D0800	UD	1970,2000,2190(2460,3943)	560,790(705,985,1230)	535
Nostoc ellipsospora	UW	1875,2100(2300,3642)	515,730(665,857,960,1100)	435
Nostoc linckia D0734	UD	1930(2190,2400)	530,686,790(930,1050,1182)	445
Nostoc MAC PCC8009	UW	2090 (1979, 2320)	710 (630, 960)	495
Nostoc MAC R1	LU	2090 (1979,2320,1876)	710 (630,960,540)	495
Nostoc muscorum	BSM	1875,2195(3642)	780(510,675,857,960,1100)	435
Nostoc piscinale D0201	UD	1890,2190(2410,3743,4133)	490,675,760(880,985,1182)	490
Nostoc planctonicum	BSM	1875(2050)	515(675,740,867)	520
Nostoc sp. D0740	UD	1890,(2190,2500)	490,745(675,930,1100)	445
Nostoc sp. D0766	UD	330,1975(1025)	508,790(660,1010)	460
Tolypothrix distorta	BSM	2175(1821)	682(602)	440

a, LJMU, UW, BSM, LU, UD, Liverpool John Moores University, University of Warwick, Biobred Sciento Manchester, Lancaster University, University of Durham respectively.

b, Data are the means of three determinations. Figures in brackets are secondary fragments (low intensity bands).

c, Products not susceptible to endonuclease S1, presumed homoduplex products, are underlined, other secondary fragments susceptible to endonuclease S1 are heteroduplexes or single-stranded DNA.

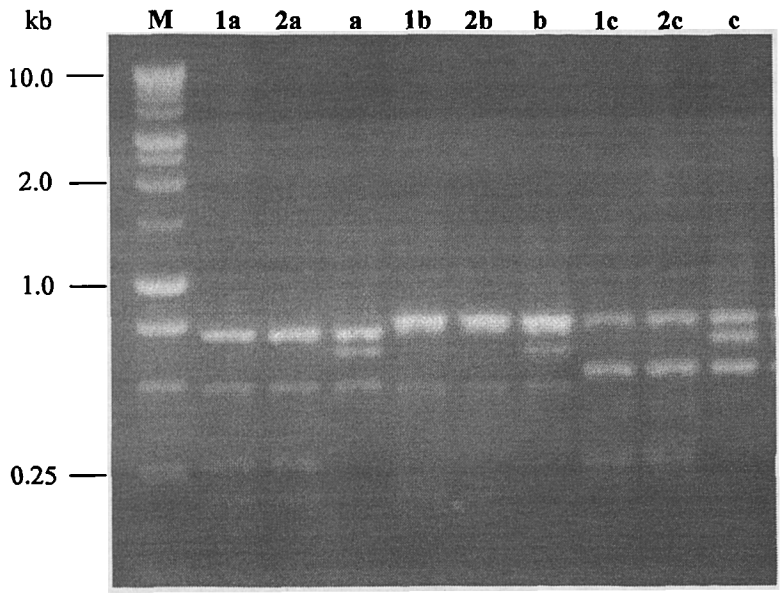


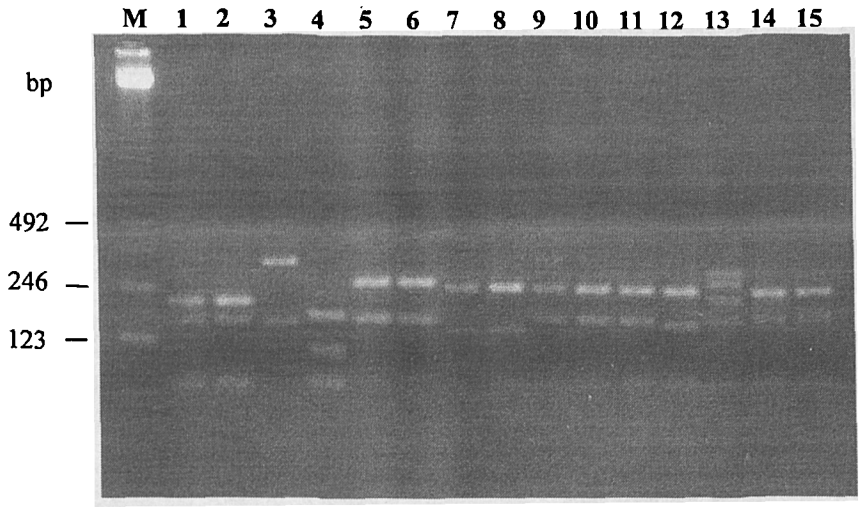
Figure 6.3. Agarose gel electrophoresis of single-strand conformation polymorphisms (SSCP) patterns of selected cyanobacterial strains. 1 and 2, SSCP patterns of rDNA spacer amplified using primers 14 and 18 and digested with the single-strand specific endonuclease, S1, 40 units and 80 units respectively; a, *Anabaena* PCC 7120; b, *Nostoc muscorum*; *c. Anabaena cylindrica*; M, 1kb marker.

always obtained using either primer pair 14/18 or 1/18.

The pattern of amplified products with primer pair 14/18 enabled clearer discrimination between species than that with primer pair 1/18. Amplification with primer pair 1/18 generated larger fragments (containing 1.5 kb highly conserved 16S rDNA) making it more difficult to resolve the variability between amplified products, than with primer pair 14/18. The pattern and length heterogeneity of the amplified bands was generally sufficient to resolve the different genera and species with the exception of *Nostoc ellipsospora, Anabaena* PCC7120, *Anabaena* ATCC27892 and *Anabaena* ATCC27892 R.32 (which carries cyanophage AN23 DNA), which all shared the same sized fragments. However, a different RAPD DNA profile was generated in each case (Fig.5.14). The same primary fragment of 2090 bp (with primer 1/18) or 710 bp (with primer 14/18) was generated for both strains of *Nostoc MAC*, with two weak secondary fragments in each case. However, strain R1 showed a further weak fragment, using either primer pair. The same primary and secondary fragments were also generated from both strains of *Nostoc commune* using either primer pair.

Analysis of length polymorphism and RFLPs of the amplified rDNA spacer using primer pair 17/18 has generally permitted discrimination between the cyanobacteria tested to genus and species level. This primer pair (which amplifies $tDNA^{ile}$ [or $tDNA^{ile}$ and $tDNA^{ala}$], ITS region and 5'end of 23S rDNA) amplified rDNA from all the strains tested generating a single band of different sizes (Table 6.1 & Fig. 6.2 c). This amplified DNA was digested with twenty different restriction enzymes to identify RFLPs. Most of the enzymes did not cut the DNA, or only very small fragments were cut from the ends. However, *Hin*fI, *Dde*I, *Alu*I and *Taq*I showed potentially useful discrimination among the different genera and species (Fig. 6.4 & Fig. 6.5).

A.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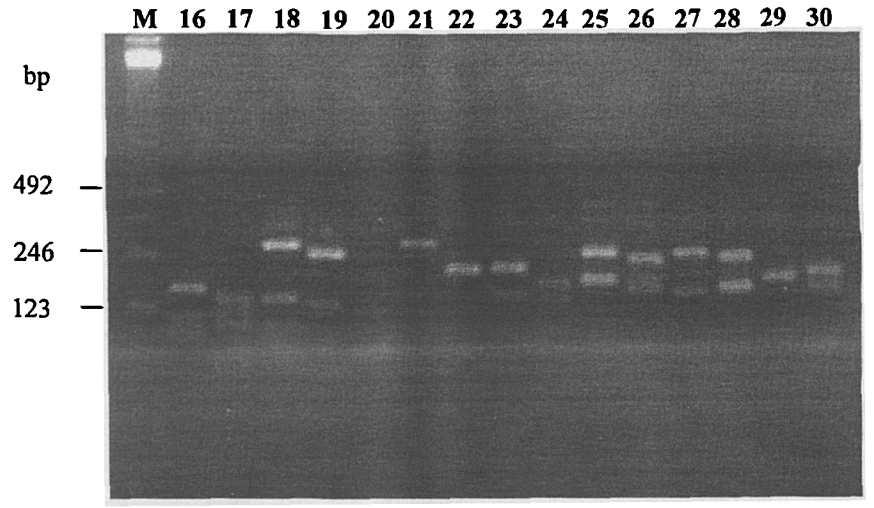
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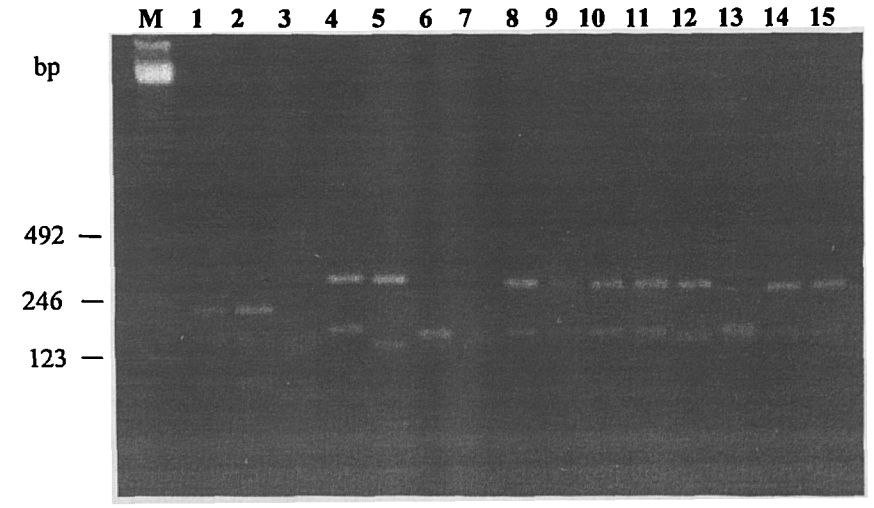
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bp
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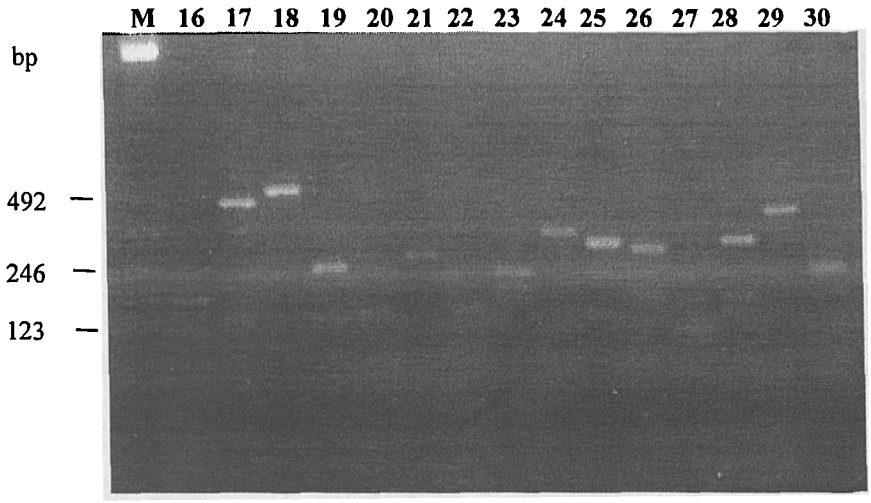
246
123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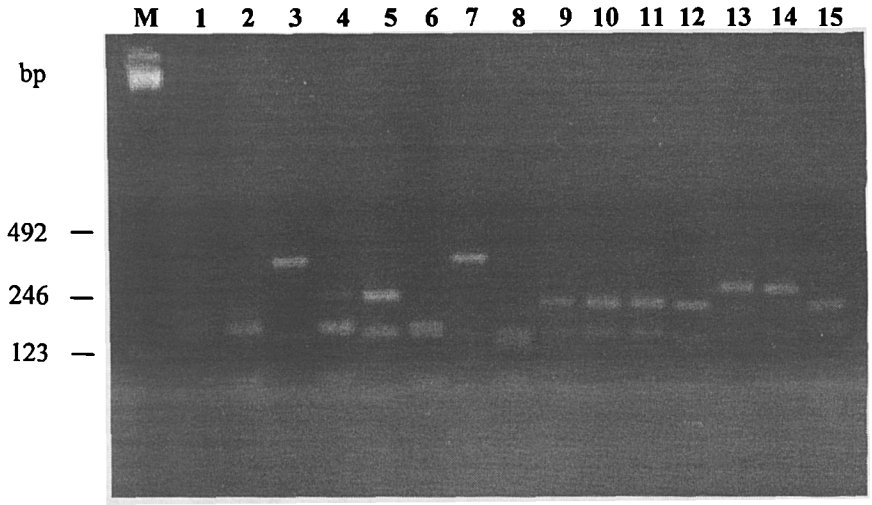
bp
492
246
123

C.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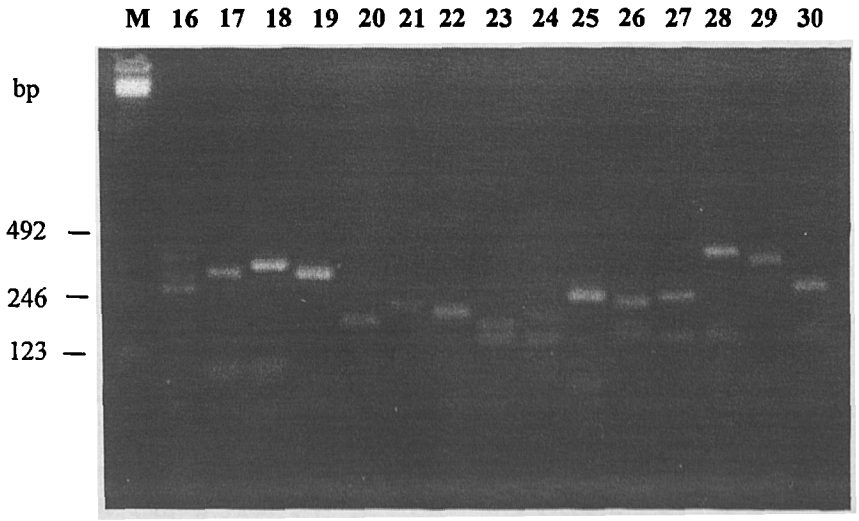
bp
492
246
123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bp
492
246
123

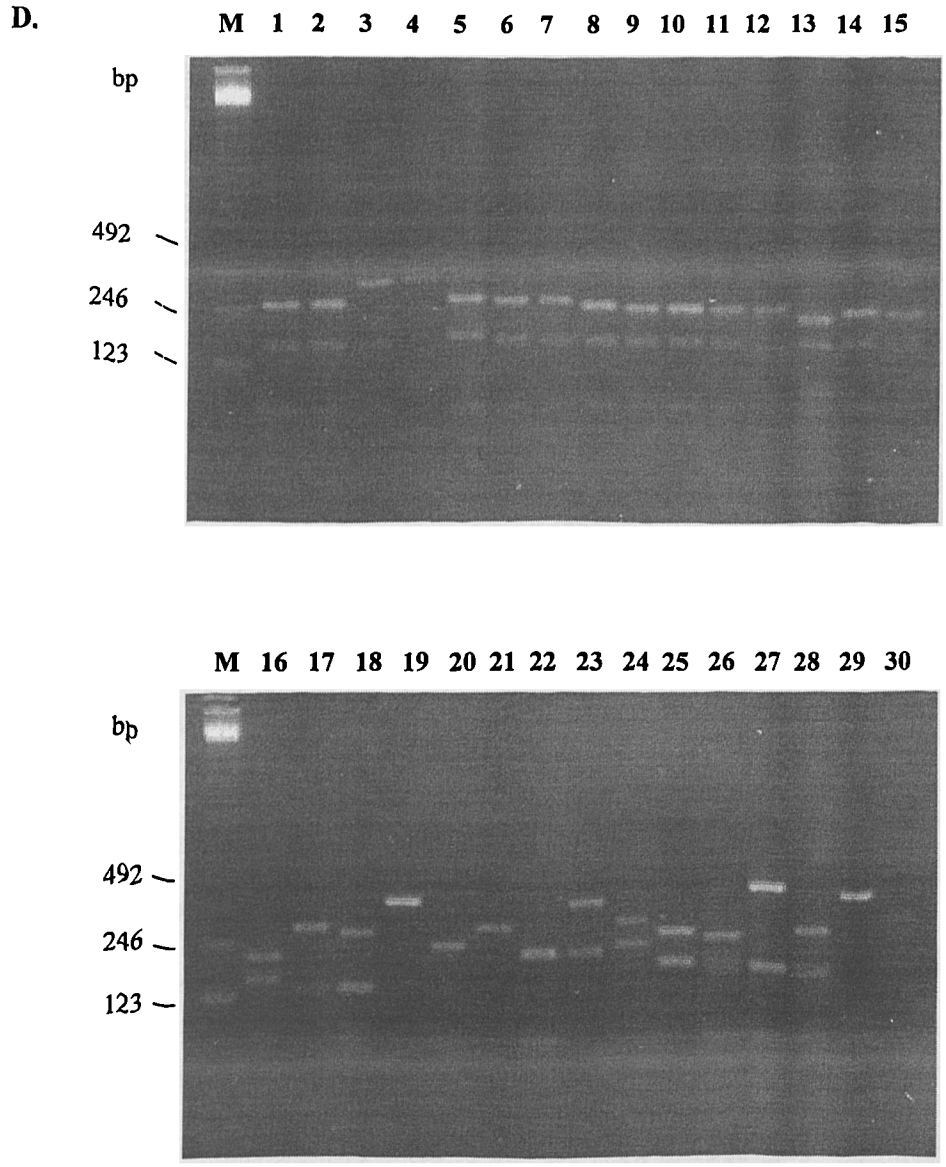
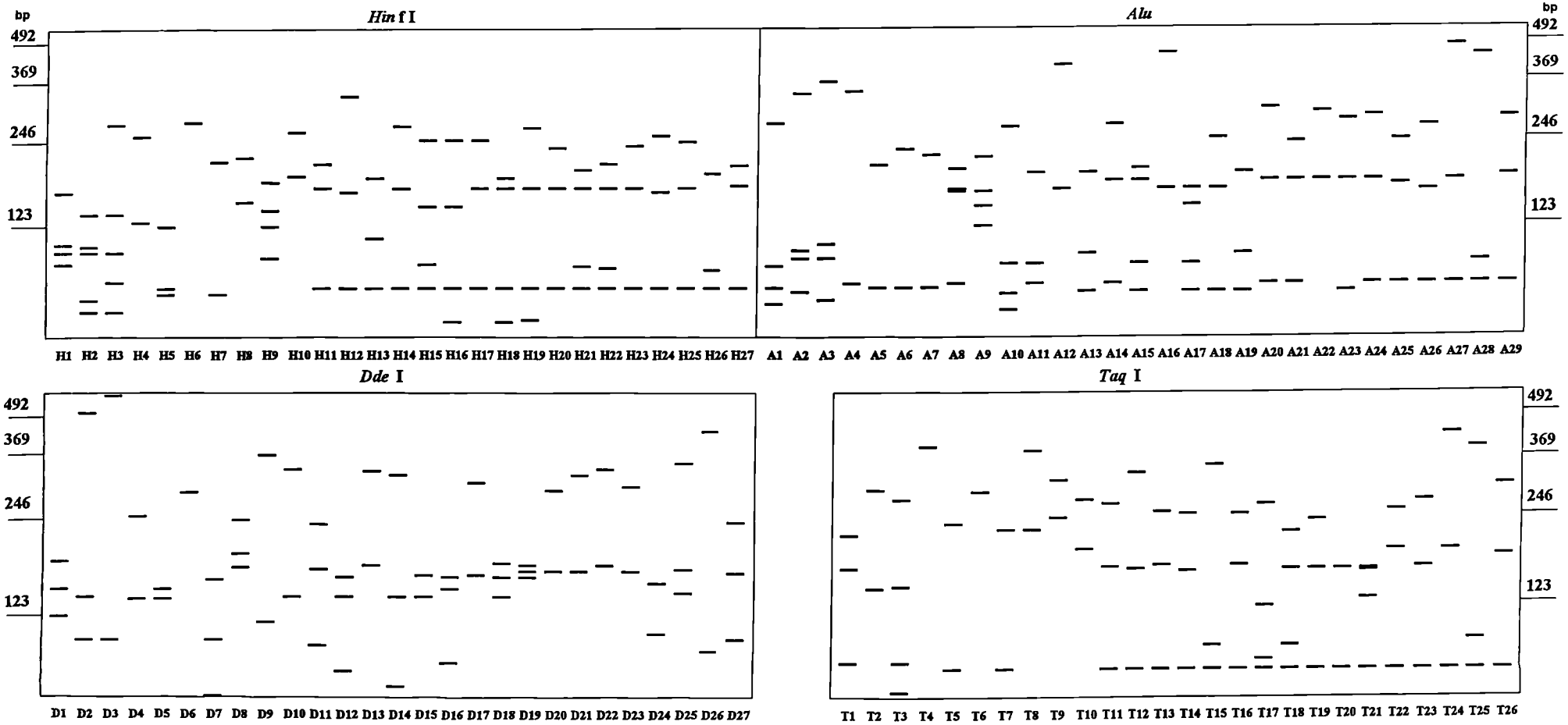


Figure 6.4. Agarose electrophoresis gels of RFLP patterns of selected cyanobacterial strains. (A) RFLP patterns of rDNA spacer amplified using primers 17 and 18 and digested with the restriction enzyme *Hinf* I; (B) RFLP patterns of rDNA spacer amplified using primers 17 and 18 and digested with the restriction enzyme *Dde* I; (C) RFLP patterns of rDNA spacer amplified using primers 17 and 18 and digested with the restriction enzyme *Alu* I; (D) RFLP patterns of rDNA spacer amplified using primers 17 and 18 and digested with the restriction enzyme *Taq* I; M, 123bp DNA ladder; 1. *Nostoc MAC* R1; 2. *Nostoc MAC* PCC8009; 3. *Nostoc commune*; 4. *Nostoc planctonicum*; 5. *Nostoc piscinale* D0201; 6. *Nostoc* sp. D0740; 7. *Nostoc linckia* D0734; 8. *Nostoc muscorum*; *9. Nostoc ellipsospora*; 10. *Anabaena* PCC 7120; 11. *Anabaena* PCC 7118; 12. *Anabaena* PCC 7118 R3.2; 13. *Anabaena cylindrica*; 14. *Anabaena flos-aquae* D0848; 15. *Anabaena* sp. D0697; 16. *Synechococcus* PCC 7942; 17. *Synechococcus* CCAP 1479/7; 18. *Synechococcus* WH 7803; 19. *Synechocystis* PCC 6803; 20. *Microcystis aeruginosa;* 21. *Gloeocapsa* sp.; 22. *Chroococcus prescottii;* 23. *Oscillatoria animalis*; 24. *Phormidium autumnale*; 25. *Pseudanabaena* sp. LJM001; 26. *Aphanizomenon gracile*; 27. *Gloeotrichia* sp.; 28. *Tolypothrix distorta;* 29. *Chlorogloeopsis fritschii*; 30. *Fisherella musicola.*

Figure 6.5. Diagramatic representation of RFLP patterns of amplified rDNA products. RFLP patterns of amplified rDNA products (using primers 17 and 18) obtained from selected strains of cyanobacteria with the restriction enzymes *Hin*fI, *Dde*I, *Alu*I and *Taq*I. The numbers below the tracks correspond to the RFLP patterns in Table 6.2.


bp
492
369
246
123
Hin f I
H1 H2 H3 H4 H5 H6 H7 H8 H9 H10 H11 H12 H13 H14 H15 H16 H17 H18 H19 H20 H21 H22 H23 H24 H25 H26 H27
Alu
A1 A2 A3 A4 A5 A6 A7 A8 A9 A10 A11 A12 A13 A14 A15 A16 A17 A18 A19 A20 A21 A22 A23 A24 A25 A26 A27 A28 A29
Dde I
D1 D2 D3 D4 D5 D6 D7 D8 D9 D10 D11 D12 D13 D14 D15 D16 D17 D18 D19 D20 D21 D22 D23 D24 D25 D26 D27
Taq I
T1 T2 T3 T4 T5 T6 T7 T8 T9 T10 T11 T12 T13 T14 T15 T16 T17 T18 T19 T20 T21 T22 T23 T24 T25 T26

In most cases, a different RFLP pattern of amplified 16S-23S rDNA spacer was generated (generally of 3 bands) for each species tested, with each restriction enzyme used. The organism could be assigned an RFLP pattern-type on the basis of the four enzymes used (Table 6.2). However, for strains of the same species the same length of amplified product and the same RFLP pattern-type were obtained. For example, the two *Nostoc MAC* strains exhibited an identical length (495 bp) and RFLP pattern-type (U), and the two *Nostoc commune* strains exhibited an identical length (535 bp) and RFLP pattern-type (S). Different species and genera had different pattern types, with the exception of *Nostoc ellipsospora, Anabaena* PCC 7120, *Anabaena* ATCC 27892 and *Anabaena* ATCC 27892 R3.2. An identical length (435 bp) and RFLP pattern-type (K) were obtained for these cyanobacteria, indicating that they may be in the same species group. This is consistent with results using primer pairs 1/18 and 14/18, where identical product profiles were obtained for these strains (Table 6.1). In addition, pattern-type K showed very high similarity to pattern-type V for *Nostoc muscorum*, and pattern-type M for *Anabaena flos-aquae* D0848 showed very high similarity to pattern-type N for *Anabaena* sp. D0697. This suggests that these strains are closely related. Furthermore, Figure 6.5 shows that all heterocystous cyanobacteria share a band of identical length (65bp) using the restriction enzymes *Hinf* I and *Taq* I, which is not found in the unicellular and non-heterocystous cyanobacteria tested, with the exception of *Microcystis aeruginosa* and *Chroococcus prescottii*. This indicates that these heterocystous strains share common recognition sites for the enzymes and therefore similar sequences.

Table 6.2. RFLP patterns and pattern-types obtained for selected cyanobacterial strains.

Strain	Pattern-type[a]	*Hinf* I	*Dde* I	*Alu* I	*Taq* I
Unicellular					
Chroococcus prescottii	A	H7	D7	A7	T7
Gloeocapsa sp.	B	H6	D6	A6	T6
Microcystis aeruginosa	C	H5	D5	A5	T5
Synechococcus PCC 7942	D	H1	D1	A1	T1
Synechococcus CCAP 1479/7	E	H2	D2	A2	T2
Synechococcus WH7803	F	H3	D3	A3	T3
Synechocystis PCC 6803	G	H4	D4	A4	T4
Filamentous non-heterocystous					
Oscillatoria animalis	H	H8	D8	A8	T8
Phormidium autumnale	I	H9	D9	A9	T9
Pseudanabaena liv JM 001	J	H10	D10	A10	T10
Filamentous heterocystous					
Anabaena ATCC27892	K	H17	D17	A18	T16
Anabaena ATCC27892 R 3.2	K	H17	D17	A18	T16
Anabaena PCC 7120	K	H17	D17	A18	T16
Anabaena cylindrica	L	H19	D19	A20	T18
Anabaena cylindrica (AN 23)	L	H19	D19	A20	T18
Anabaena flos-aquae D0848	M	H20	D20	A22	T19
Anabaena sp. D0697	N	H20	D20	A21	T19
Anabaena sp. D0746	O	H21	D21	A23	T20
Anabaena sp. D0752	P	H22	D22	A24	T21

Filamentous heterocystous continued.....

Aphanizomenon gracile	Q	H23	D23	A25	T22
Chlorogloeopsis(Chlorogloea) fritschii	R	H26	D26	A28	T25
Fischerella musicola	S	H27	D27	A29	T26
Gloeotrichia sp.	T				
Nostoc commune (BSM)	U	H12	D12	A12	T12
Nostoc commune D0800 (UD)	U	H12	D12	A12	T12
Nostoc ellipsospora	K	H17	D17	A18	T16
Nostoc linckia D0734	V	H14	D15	A15	T14
Nostoc MAC PCC 8009	W	H11	D11	A11	T11
Nostoc MAC R1	W	H11	D11	A11	T11
Nostoc muscorum	X	H16	D17	A17	T16
Nostoc piscinale D0201	Y	H13	D13	A13	T13
Nostoc planctonicum	Z	H15	D16	A16	T15
Nostoc sp. D0740	AA	H14	D14	A14	T14
Nostoc sp. D0766	AB	H18	D18	A19	T17
Tolypothrix distorta	AC	H24	D24	A26	T23

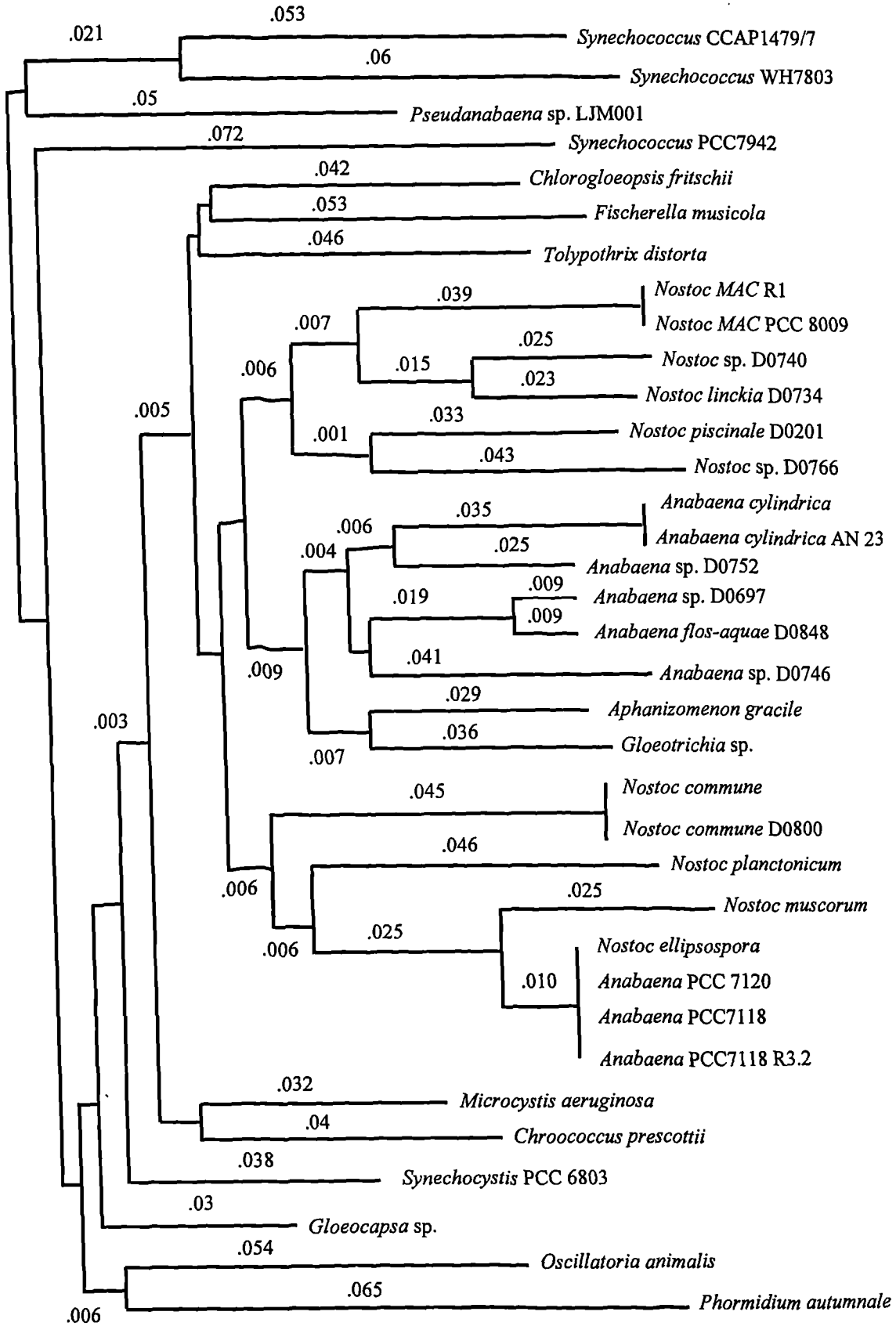
a, Details of the patterns (H1-H24, D1-D24, A1-A26 and T1-T23) for amplified (primer pair 17/18) rDNA digests are given in Fig. 6.5. Pattern-type derives from the pattern combination with the four test restriction enzymes.

A dendrogram (Fig.6.6) constructed on the basis of pair-wise genetic distances using the combined RFLP data from the four restriction enzymes, further indicates the similarity between *Anabaena* and *Nostoc* strains. In addition, it reveals a low similarity between the freshwater *Synechococcus* PCC 7942 and the marine *Synechococcus* WH 7803, and shows the marine *Synechococcus* WH 7803 more related to brackish water *Synechococcus* CCAP 1479/7 than *Synechococcus* PCC 7942. It is noteworthy that all the heterocystous strains form a cluster excluding all the other strains.

Heterogeneity of length and RFLP pattern-type of the rDNA spacer can also be used to detect more than one cyanobacterial species in a mixed culture (Fig. 6.7 a). Both size and intensity of bands need to be considered for discrimination between the species (Fig. 6.7 b). However, the method would probably not be suitable for distinguishing between a large number of species, due to the multiplicity of bands generated.

In addition to amplifying the 16S-23S rDNA spacer, the 16S rDNA was amplified by using primer pair 1/16 (which in the case of *Synechococcus* PCC 6301, amplifies 16S rDNA from the 8th base at the 5' end to base 1486) for a number of strains. A similar sized amplified product of about 1.48 kbp was generated in each case. The amplified products were digested using the same restriction enzymes as those used for the spacer region (Fig. 6.8). Highly similar patterns were obtained for all strains tested. However, the sizes of the restriction fragments were not consistent with those expected from the size of the original amplified product.

Figure 6.6. Dendrogram indicating genetic distances among cyanobacterial strains. The genetic distance from the node, based on the RFLP profiles of 35 strains, is indicated above each branch. The less closely related the strains are the greater the genetic distance.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.021
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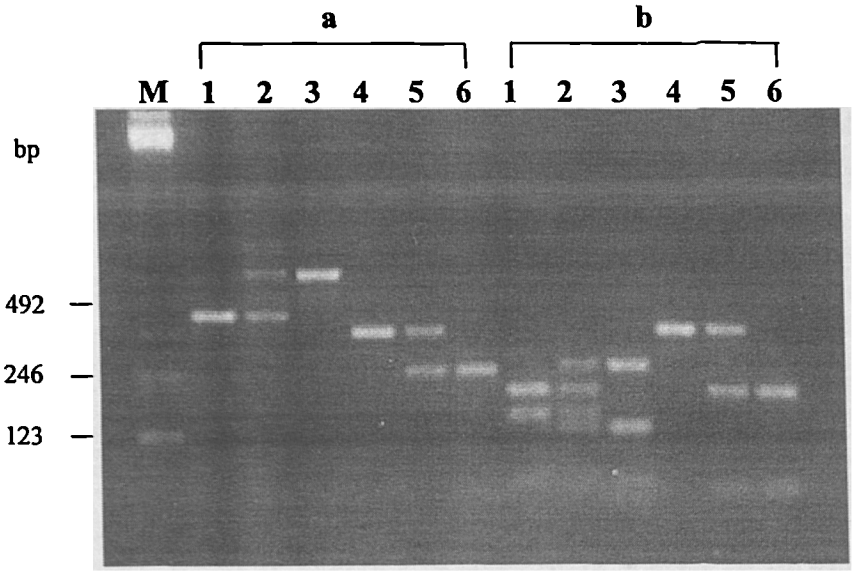
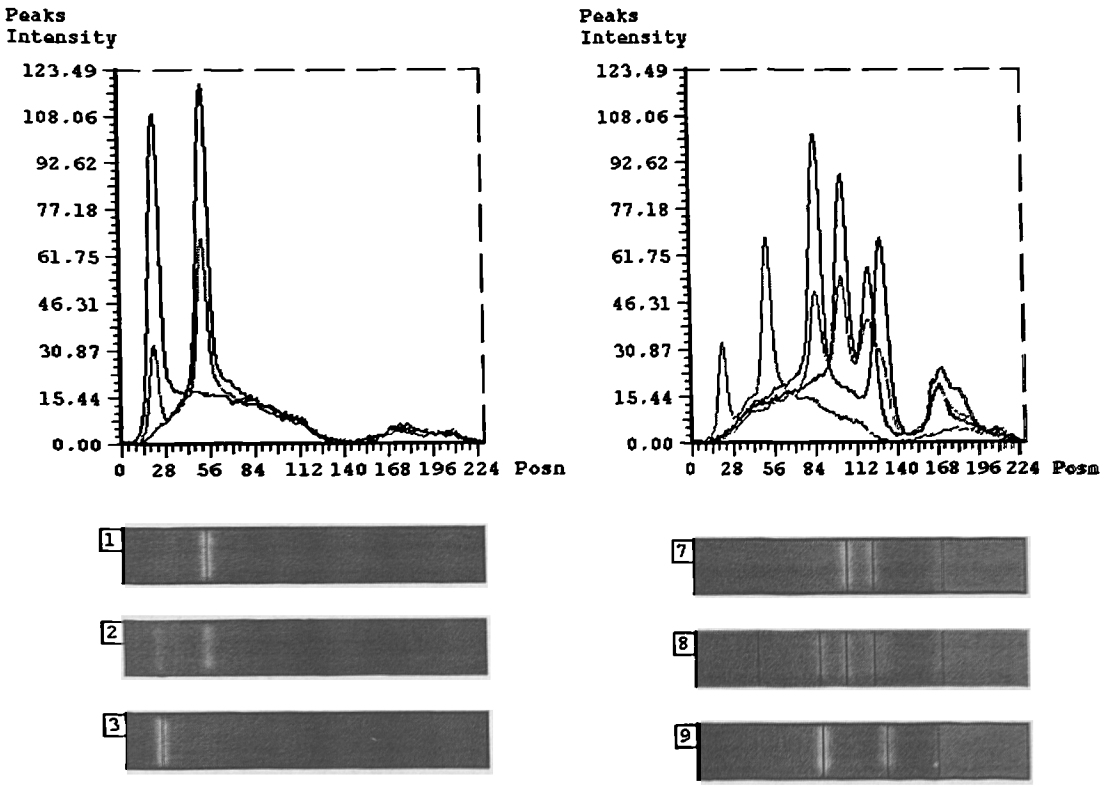
.06
Synechococcus WH7803
.05
Pseudanabaena sp. LJM001
.072
Synechococcus PCC7942
.042
Chlorogloeopsis fritschii
.053
Fischerella musicola
.046
Tolypothrix distorta
.039
Nostoc MAC R1
.007
Nostoc MAC PCC 8009
.025
.006
.015
Nostoc sp. D0740
.023
Nostoc linckia D0734
.005
.033
.001
Nostoc piscinale D0201
.043
Nostoc sp. D0766
.035
Anabaena cylindrica
.006
Anabaena cylindrica AN 23
.004
.025
Anabaena sp. D0752
.009
.019
Anabaena sp. D0697
.009
Anabaena flos-aquae D0848
.009
.041
Anabaena sp. D0746
.029
Aphanizomenon gracile
.003
.036
Gloeotrichia sp.
.007
.045
Nostoc commune
Nostoc commune D0800
.046
Nostoc planctonicum
.006
.025
Nostoc muscorum
.025
.006
Nostoc ellipsospora
.010
Anabaena PCC 7120
Anabaena PCC7118
Anabaena PCC7118 R3.2
.032
Microcystis aeruginosa
.04
Chroococcus prescottii
.038
Synechocystis PCC 6803
.03
Gloeocapsa sp.
.054
Oscillatoria animalis
.065
.006
Phormidium autumnale

A


a
b
M 1 2 3 4 5 6 1 2 3 4 5 6
bp
492
246
123

B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108.06
92.62
77.18
61.75
46.31
30.87
15.44
0.00
0 28 56 84 112 140 168 196 224 Posn
Peaks Intensity
123.49
108.06
92.62
77.18
61.75
46.31
30.87
15.44
0.00
0 28 56 84 112 140 168 196 224 Posn
1
2
3
7
8
9

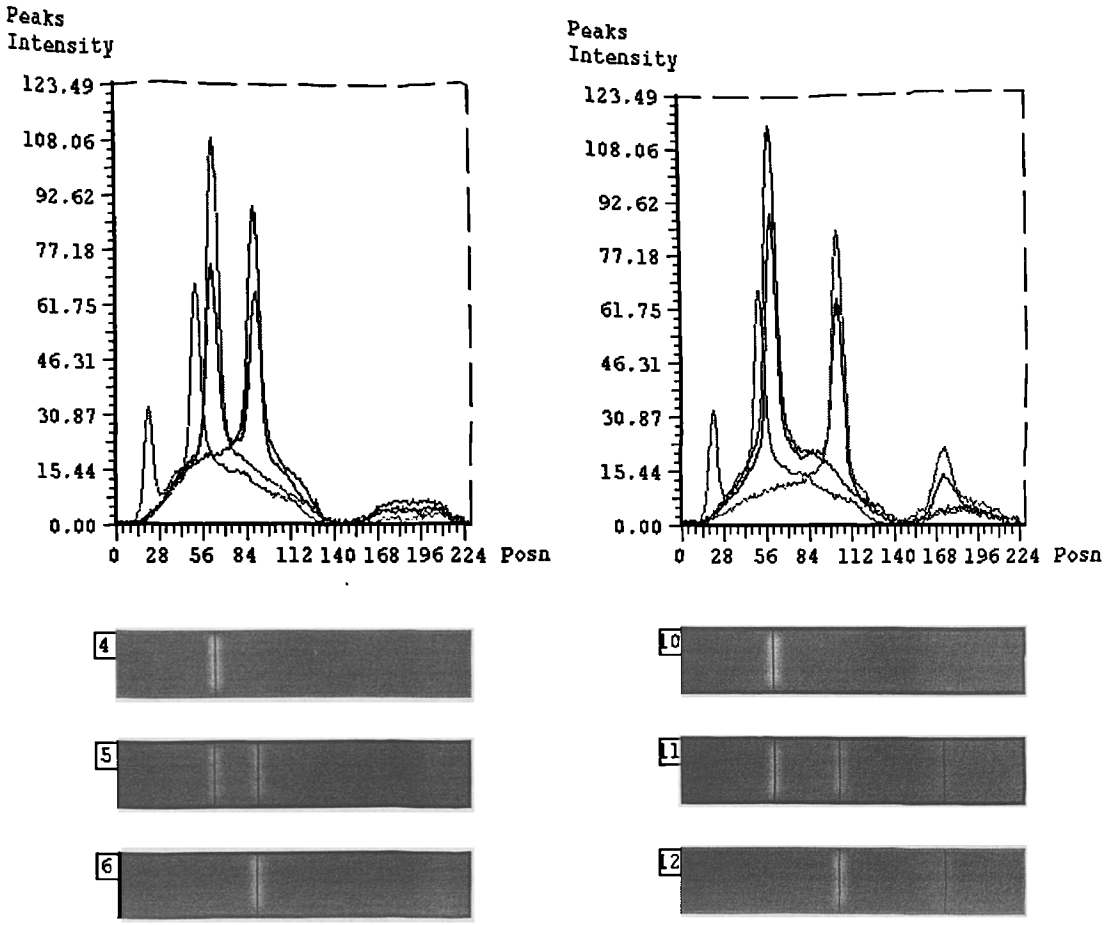
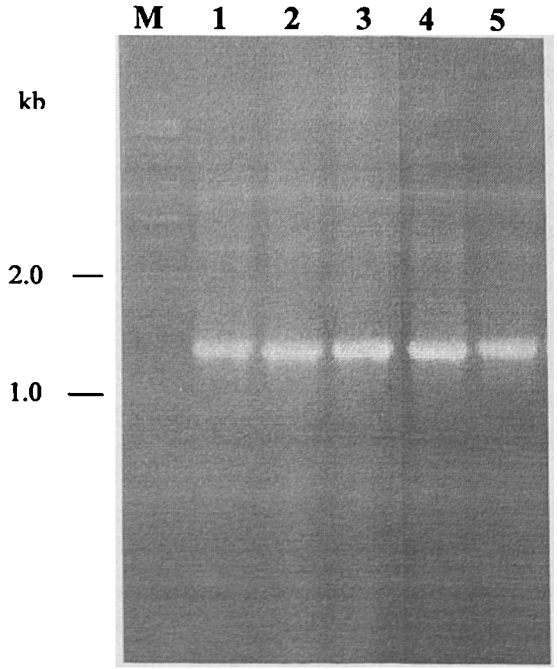


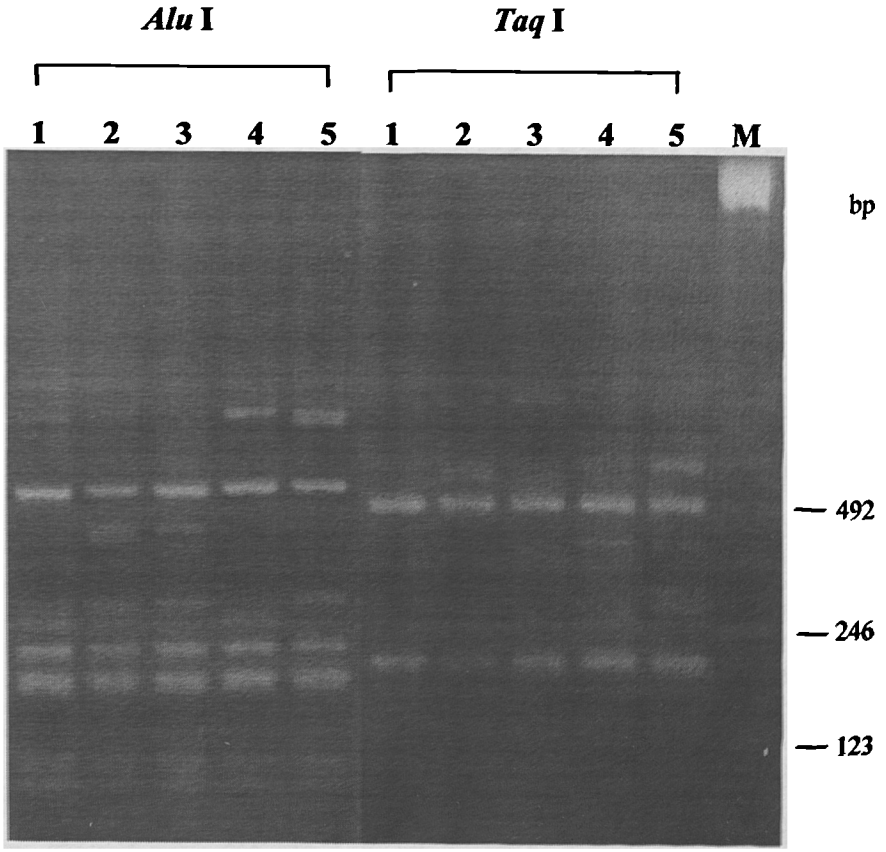
Figure 6.7. Agarose electrophoresis gel of amplified rDNA spacer products and RFLP patterns of selected cyanobacterial strains. (A) a, PCR-amplification using primer 17/18. b, RFLP patterns of the amplified rDNA spacer digested with the restriction enzyme *Taq* I. 1. *Synechococcus* PCC 7942; 2. *Synechococcus* PCC 7942 + *Synechococcus* WH 7803; 3. *Synechococcus* WH 7803; 4. *Synechocystis* PCC 6803; 5. *Synechocystis* PCC 6803 + *Chroococcus prescottii*; 6. *Chroococcus prescottii*. M, 123bp DNA ladder. (B) Analysis of results of agarose electrophoresis gel by using the Phoretix ID (version 2.51).

Figure 6.8. Agarose electrophoresis gels of amplified partial 16S rDNA products and RFLP patterns of selected cyanobacterial strains. (A) PCR- amplification using primer 1/16; (B) RFLP patterns of 16S rDNA amplified using primers 1/16 and digested with the restriction enzymes *Alu* I or *Taq* I. M, 1kb DNA ladder; 1, *Aphanizomenon gracile*; 2, *Anabaena* sp. D0752; 3, *Nostoc linckia* D0734; 4, *Nostoc* sp. D0766; 5, *Nostoc commune.*

A



B



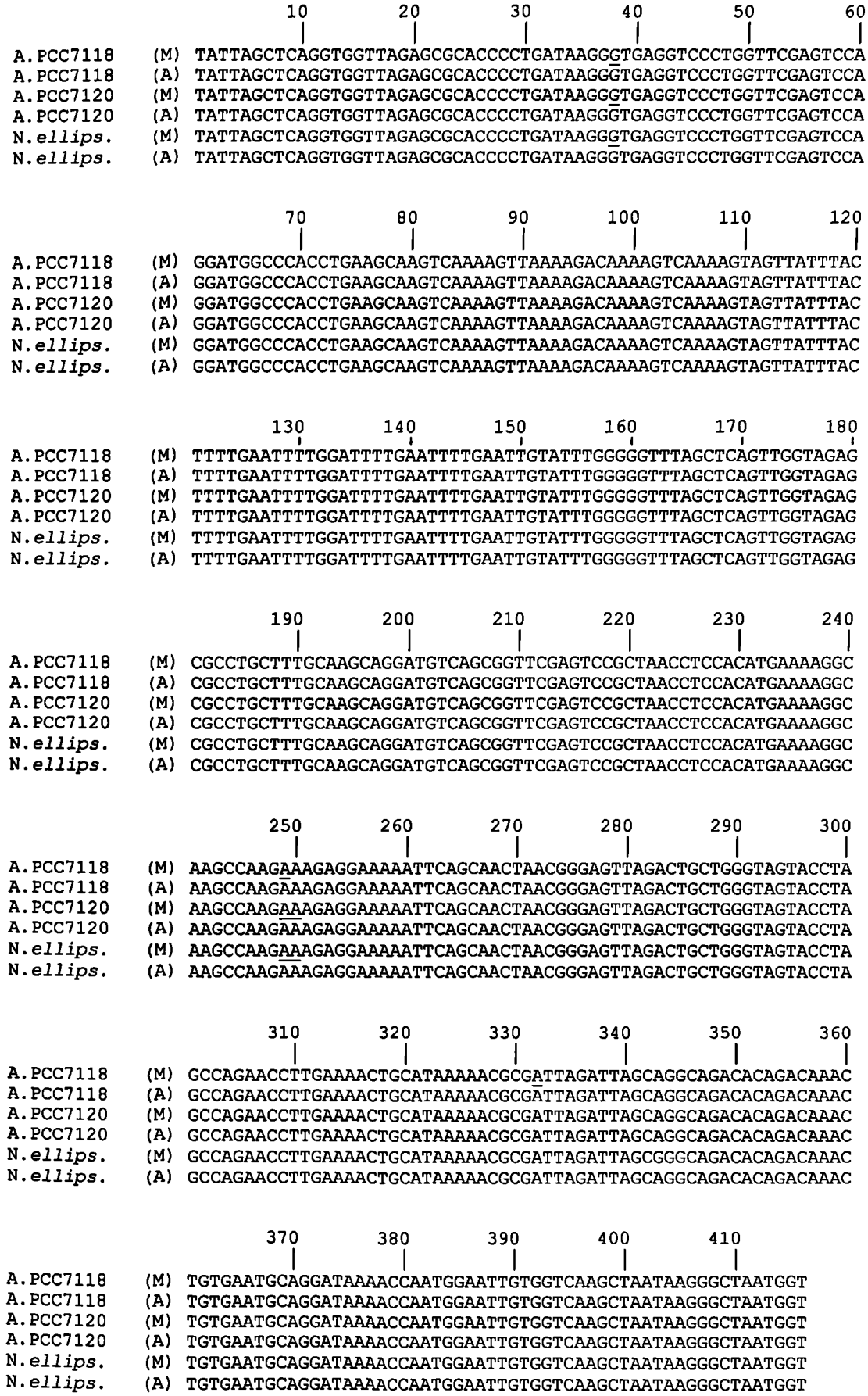
6.2.2 16S-23S rDNA spacer sequencing

The high inter-specific variability found for the 16S-23S rDNA spacer, based on RFLP analysis, makes it a potentially useful region for revealing sequence heterogeneity that may be used for typing cyanobacteria. Sequences of the rDNA spacer of various cyanobacterial strains have therefore been determined and analysed. Moreover, by establishing the extent of sequence similarity, any highly conserved regions could be identified that may be useful in designing cyano-specific primers or probes.

PCR products of the partial 16S-23S rDNA spacer, amplified by using primer 17/18 and DyNAZyme II (see section 2.2.20), were purified by using spin columns (see section 2.2.24), and sequenced using both manual and automatic techniques. For manual sequencing, PCR products were cloned into the pGEM-T vector (Promega) or pCR II vector (Invitrogen) (see section 2.2.24), and recombinant plasmid DNA was isolated and purified (section 2.2.25), prior to sequencing. Results showed that 100% of the recombinant pCR II plasmid DNA (isolated from different white clones) contained the correct sized PCR products, compared with only 30-40% of recombinant pGEM-T plasmid DNA. Thus the pCR II vector was a more suitable choice for subsequent cloning experiments. For automated sequencing, amplified products from separate PCR-amplification reactions were sequenced directly.

The sequences of three strains, *Anabaena* PCC 7118, *Anabaena* PCC 7120 and *Nostoc ellipsospora*, each from three individual clones were determined by using both automated and manual sequencing (see section 2.2.26) to check the reliability of the two methods. The sequences were aligned, and were found to be almost identical for any particular species, using either method (Fig. 6.9). For manual sequencing, compression

Figure 6.9 Alignment of 16S-23S rDNA spacer sequences from *Anabaena* PCC 7118, *Anabaena* PCC 7120 and *Nostoc ellipsospora* starting from the 5' end of $tRNA^{ile}$ to the beginning of 23S rRNA gene. Alignment was obtained using the CLUSTAL W program version 1.6 (http://www.ibcp.fr/clustalw.html). (M), manual sequencing data; (A), automated sequencing data. A-Adenine, T-Thymine, G-Guanine, C-Cytosine. Numbers represent base positions. Differences that appeared between upper and lower strands from manual sequencing data are underlined. Data are from three repeats. For manual sequencing both upper and lower strands were sequenced. The sequences of the upper strands are shown (5'→ 3').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A.PCC7120 (M) TATTAGCTCAGGTGGTTAGAGCGCACCCCTGATAAGGGTGAGGTCCCTGGTTCGAGTCCA
A.PCC7120 (A) TATTAGCTCAGGTGGTTAGAGCGCACCCCTGATAAGGGTGAGGTCCCTGGTTCGAGTCCA
N.ellips. (M) TATTAGCTCAGGTGGTTAGAGCGCACCCCTGATAAGGGTGAGGTCCCTGGTTCGAGTCCA
N.ellips. (A) TATTAGCTCAGGTGGTTAGAGCGCACCCCTGATAAGGGTGAGGTCCCTGGTTCGAGTCCA
70 80 90 100 110 120
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A.PCC7118 (A) GGATGGCCCACCTGAAGCAAGTCAAAAGTTAAAAGACAAAAGTCAAAAGTAGTTATTTAC
A.PCC7120 (M) GGATGGCCCACCTGAAGCAAGTCAAAAGTTAAAAGACAAAAGTCAAAAGTAGTTATTTAC
A.PCC7120 (A) GGATGGCCCACCTGAAGCAAGTCAAAAGTTAAAAGACAAAAGTCAAAAGTAGTTATTTAC
N.ellips. (M) GGATGGCCCACCTGAAGCAAGTCAAAAGTTAAAAGACAAAAGTCAAAAGTAGTTATTTAC
N.ellips. (A) GGATGGCCCACCTGAAGCAAGTCAAAAGTTAAAAGACAAAAGTCAAAAGTAGTTATTTAC
130 140 150 160 170 180
A.PCC7118 (M) TTTTGAATTTTGGATTTTGAATTTTGAATTGTATTTGGGGGTTTAGCTCAGTTGGTAGAG
A.PCC7118 (A) TTTTGAATTTTGGATTTTGAATTTTGAATTGTATTTGGGGGTTTAGCTCAGTTGGTAGAG
A.PCC7120 (M) TTTTGAATTTTGGATTTTGAATTTTGAATTGTATTTGGGGGTTTAGCTCAGTTGGTAGAG
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190 200 210 220 230 240
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A.PCC7120 (A) TGTGAATGCAGGATAAAACCAATGGAATTGTGGTCAAGCTAATAAGGGCTAATGGT
N.ellips. (M) TGTGAATGCAGGATAAAACCAATGGAATTGTGGTCAAGCTAATAAGGGCTAATGGT
N.ellips. (A) TGTGAATGCAGGATAAAACCAATGGAATTGTGGTCAAGCTAATAAGGGCTAATGGT

of the bands was sometimes found where there were runs of certain bases, and all nucleotides could occasionally be found at a particular position in the sequencing gel, making reading of some gels a little difficult. However, these technical discrepancies were eliminated by repeating the sequencing procedure and by sequencing both strands. For example, differences in sequence of the lower strand were found at base position 38, 249, 250, 332 and 343 by using the M13 forward primer in the sequencing reaction. At position 249 and 250, all four bands A, T, G, C were present at the same position in the gel, for *Anabaena* PCC 7120 and *Nostoc ellipsospora* DNA; whilst for *Anabaena* PCC 7118 DNA there was one very faint band T at position 249 and one strong band T at position 250 (Fig. 6.10). These errors were corrected by sequencing the upper strand by using the reverse M13 primer: a single band 'A' being found on the gel at both position 249 and 250 for all three strains. At position 343 A or G could be found using *Nostoc ellipsospora* DNA (Three cloned *Nostoc ellipsospora* DNA fragments were manually sequenced, two of them indicated as G in that position, one of them indicated as A, which may be due to microheterogeneities or misincorporations by the *Taq* polymerase).

Problems with resolving bands on the gels did not occur with the automated sequencing, where output was in a machine readable form, so that the sequence could be translated as a fluorogram trace. Representative auto-sequencing data from the 16S-23S spacer region of strain *Synechocystis* PCC 6803 and *Microcystis aeruginosa* are shown in machine output format in Fig. 6.11. The sequences can be automatically and easily determined from the peaks: A, T, G, C. Figure 6.12 shows the partial sequence of the 16S-23S rDNA spacer of strain *Synechocystis* PCC 6803 by automated sequencing, compared with the published sequence from Genbank data (Accession D 90916, upper strands sequenced). The sequences were identical. Table 6.3 compares manual and automated sequencing techniques for analysis of the 16S-23S rDNA spacer region.

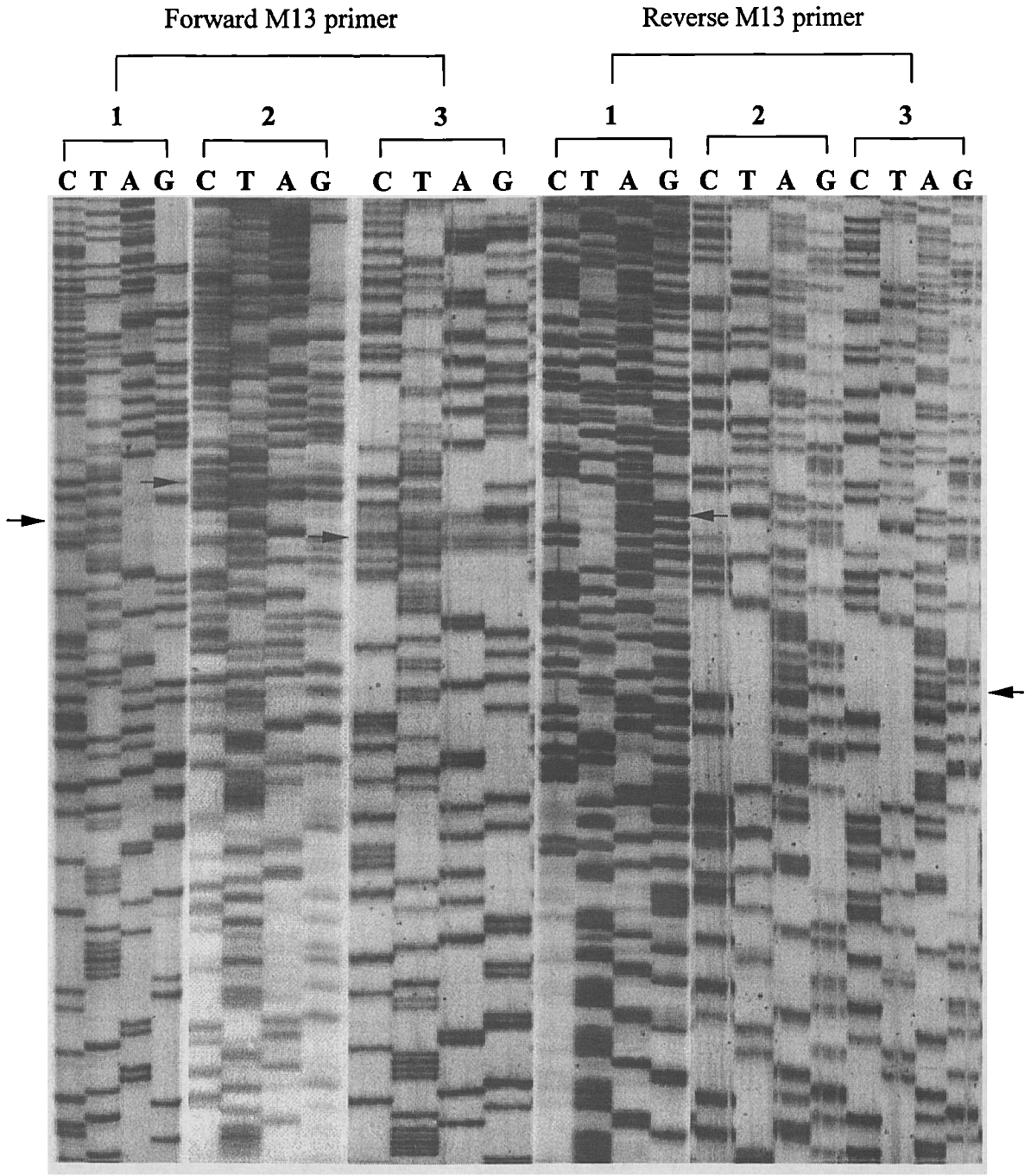
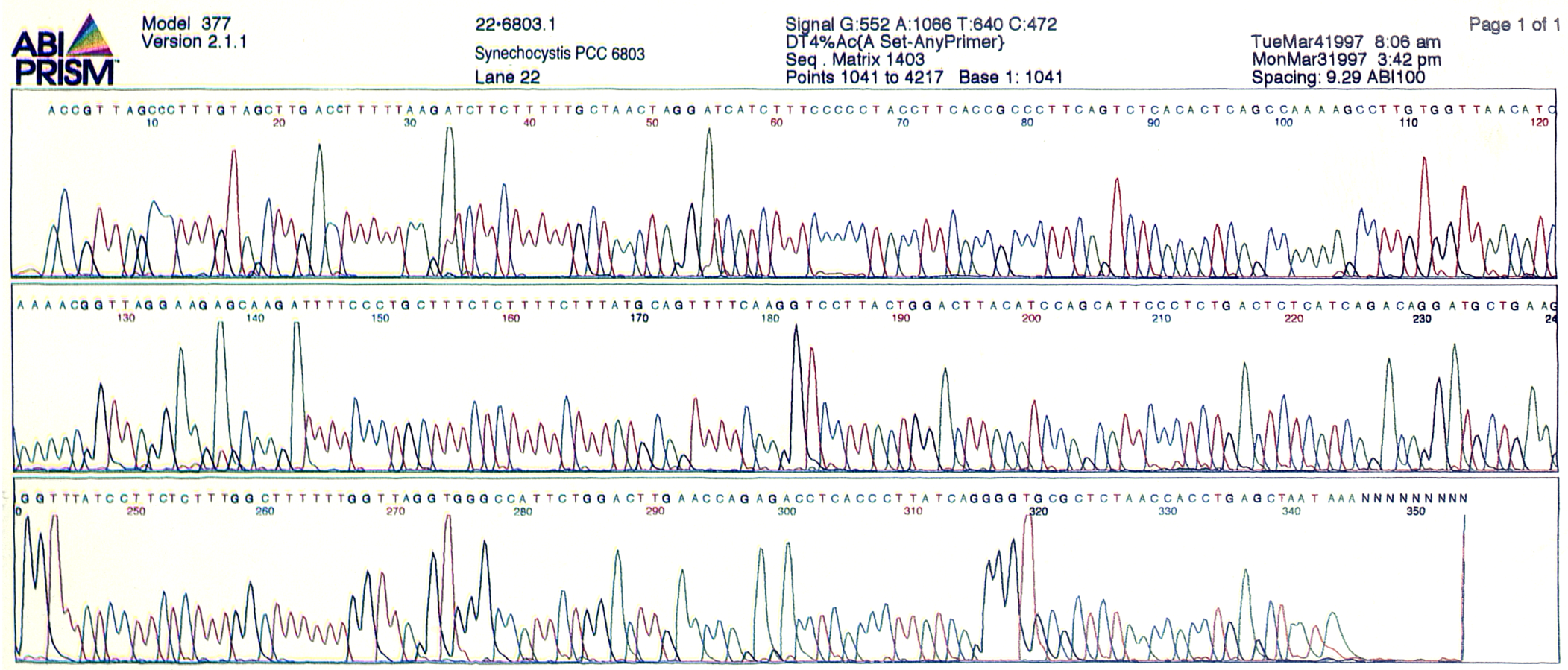


Figure. 6.10 Sequencing electrophoresis gels of amplified products of partial 16S-23S rDNA spacer of selected cyanobacterial strains. The forward M13 primer, reverse M13 primer and the Sequenase 2.0 sequencing kit (see section 2.1.1) were used; 1. *Anabaena* PCC 7118; 2. *Anabaena* PCC 7120; 3. *Nostoc ellipsospora*. The positions of the arrow bars (→) correspond to base position 250 from Figure 6.9.

Figure 6.11. Fluorogram trace of automated sequencing data. The data from the 16S-23S spacer region of strain *Synechocystis* PCC 6803 and *Microcystis aeruginosa* in machine output format from the ABI PRISM 377 automated DNA sequencer.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Model 377
Version 2.1.1
22•6803.1
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Lane 22
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DT4%Ac{A Set-AnyPrimer}
Seq . Matrix 1403
Points 1041 to 4217 Base 1: 1041
TueMar41997 8:06 am
MonMar31997 3:42 pm
Spacing: 9.29 ABI100
Page 1 of 1
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AAAACGGTTAGGAAGAGCAAG ATTTTCCCTGCTTTCTCTTTTCTTTATGCAGTTTTCAAGGTCCTTACTGGACTTACATCCAGCATTCCCTCTGACTCTCATCAGACAGGATGCTGAAG
130 140 150 160 170 180 190 200 210 220 230
GGTTTATCCTTCTCTTTGGCTTTTTTGGTTAGGTGGGCCATTCTGGACTTGAACCAGAGACCTCACCCTTATCAGGGGTGCGCTCTAACCACCTGAGCTAAT AAANNNNNNNNN
0 250 260 270 280 290 300 310 320 330 340 350

ABI PRISM™

Model 377
Version 2.1.1

34•MA.1

Microcystis aeruginosa
Lane 34

Signal G:246 A:303 T:197 C:158
DT4%Ac{A Set-AnyPrimer}
Seq . Matrix 1403
Points 957 to 2977 Base 1: 957

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FriJan241997 3:39 pm
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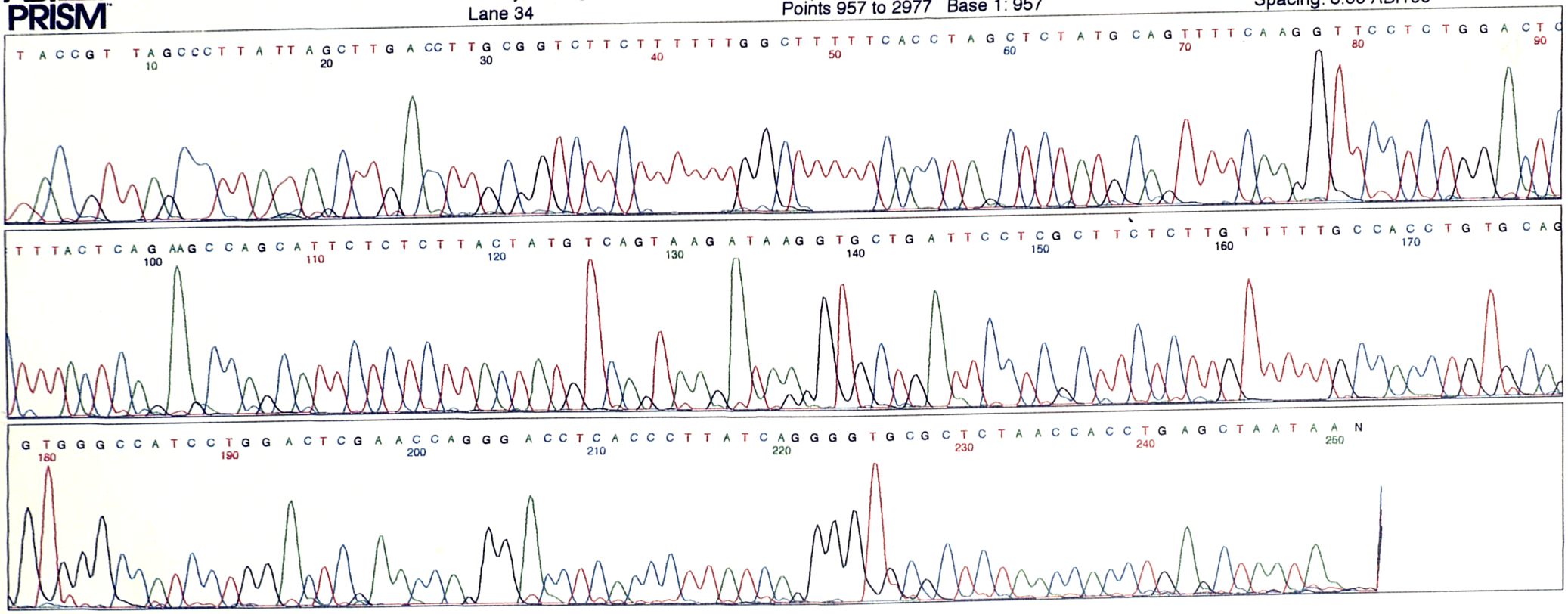

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            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
G: 3326689 TATTAGCTCAGCTGGTTAGAGCGCACCCCTGATAAGGGTGAGGTCTCTGGTTCAAGTCCAGAATGGCCCACCTAA 3326763

S:      269 CCAAAAAAGCCAAAGAGAAGGATAAACCCTTCAGCATCCTGTCTGATGAGAGTCAGAGGGAATGCTGGATGTAAG 195
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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S:      194 TCCAGTAAGGACCTTGAAAACTGCATAAAGAAAAGAGAAAGCAGGGAAAATCTTGCTCTTCCTAACCGTTTTGAT 120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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S:      119 GTTAACCACAAGGCTTTTGGCTGAGTGTGAGACTGAAGGGCGGTGAAGGTAGGGGGAAAGATGATCCTAGTTAGC 45
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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S:       44 AAAAAGAAGATCTTAAAAAGGTCAAGCTACAAAGGGCTAACGGT 1
            |||||||||||||||||||||||||||||||||||||||||||
G: 3326989 AAAAAGAAGATCTTAAAAAGGTCAAGCTACAAAGGGCTAACGGT 3327032
```

Figure 6.12 Comparison of partial 16S-23S spacer sequence of *Synechocystis* sp. PCC6803. S, the automated sequencing data (this study) G, the sequence taken from *Synechocystis* sp. PCC6803 the complete genome (3270710-3418851) (Accession number D90916) from Genbank. Numbers indicate the positions of each sequence. Identity is 100% for the 344bp sequences. Sequences are shown 5'-3' upper strand.

Table 6.3 Comparison of manual and automated sequencing techniques.

Criterion	Manual sequencing	Automatic sequencing
DNA requirement	Cloned PCR products by using TA vector	PCR products or cloned PCR products
Number of 500bp sequences resolved on one gel	1-2	9 (using dye- primer); 36 (using dye- terminator)
Sequence reading	Using autoradiogram (36h exposure), read manually or automatically by scanning into computer.	Directly read (real-time) from gel by computer
Number of bases read in one loading	150 - 200 bp	500 - 700 bp (dye - ddNTP); up to 900 bp (dye - primer)
Time taken to obtain results	5 days	2 days
Potential errors	Gaps or all bases A,T,G,C appearing at the same position or compression of bands or leakage of sample from next well leading to mis-reading.	Impure PCR products cause too much background or more than one peak shown in the same position. Also there can be leakage of sample from next well of gel.

A-Adenine, T-Thymine, G-Guanine, C-Cytosine.

Overall the sequencing results indicated that automated sequencing should provide a reliable method for rapidly surveying sequences of cyanobacterial DNA, and the technique has been used here to sequence selected strains. However, it should be noted that PCR products of the partial 16S-23S rDNA spacer, amplified by *Taq* polymerase from Bioline (U.K.), all failed to provide accurate data by automated sequencing. A strong background, in the form of non-specific peaks, appeared on the fluorogram trace when sequencing such amplified products. Clear sequencing data, without background, were provided from PCR products amplified using DyNAZyme II. The error frequency of DyNAZyme II is supposedly two-fold lower than that of *Taq* DNA polymerase (according to Finnzymes Oy instructions). This higher degree of specificity made DyNAZyme II the enzyme of choice for PCR-amplification of the cyanobacterial DNA for automated sequencing, in order to reduce background interference.

Partial cyanobacterial 16S-23S rDNA spacer sequences from amplified PCR products using primers 17/18 (which amplify $tDNA^{ile}$, spacer, $tDNA^{ala}$, spacer and 5' end of 23S rDNA) were recovered, except for an estimated 5 nucleotides at the 5' end of $tDNA^{ile}$, for selected cyanobacteria by automated sequencing. Conserved nucleotide positions were aligned using the CLUSTAL W program for all these laboratory strains, together with *Synechococcus* PCC6301 (Accession No. X00346), *Trichodesmium* NIBB1067 (Accession No. X72871), *E. coli* (Accession No. J01702) and *Chondrus crispus* (red alga) chloroplast (Accession No. Z29521), to determine the extent of sequence homology (Figure 6.13). The two tDNA sequences were found in all cyanobacterial strains, except *Microcystis aeruginosa*, which did not contain $tDNA^{ala}$. Two other conserved regions and one region of high sequence similarity were also present in the cyanobacteria: the two conserved regions are within the variable spacer

Figure 6.13 Alignment of 16S-23S rDNA spacer sequences from various cyanobacterial species. Sequence starting from the 5' end of $tDNA^{ile}$ to the beginning of 23S rDNA. Alignment was obtained for sequences from 15 laboratory strains (this study) together with *Synechococcus* PCC6301 (Accession No. X00346), *Trichodesmium* NIBB1067 (Accession No. X72871), *E. coli* (Accession No. J01702) and *Chondrus crispus* (red alga) chloroplast (Accession No. Z29521) from Genbank, using the CLUSTAL W program version 1.6 (http://www.ibcp.fr/clustalw.html). Alignment across the $tDNA^{ile}$ (position 1-70) and $tDNA^{ala}$ (163-238) sequence was very good, but gaps were present within the spacer region. Some of these gaps were manually removed. A-Adenine, T-Thymine, G-Guanine, C-Cytosine. -, absence of nucleotide. Numbers above the sequences represent base positions. *, heterocystous cyanobacterial strains. The regions of high homology are boxed.

```
                          10        20        30        40        50        60
                          |         |         |         |         |         |
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M.aeruginosa              TATTAGCTCAGGTGGTTAGAGCGCACCCCTGATAAGGGTGAGGTCCCTGGTTCGAGTCCA
S.PCC6301                 TATTAGCTCAGGTGGTTAGAGCGCACCCCTGATAAGGGTGAGGTCCCTGGTTCAAGTCCA
S.PCC7942                 TATTAGCTCAGGTGGTTAGAGCGCACCCCTGATAAGGGTGAGGTCCCTGGTTCAAGTCCA
O.animalis                TATTAGCTCAGGTGGTTAGAGCGCACCCCTGATAAGGGTGAGGTCTCTGGTTCAAATCCA
T.NIBB1067                TATTAGCTCAGGTGGTTAGAGCGCACCCCTGATAAGGGTGAGGTCCCTGGTTCAAGTCCA
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A.D0746 *                 TATTAGCTCAGGTGGTTAGAGCGCACCCCTGATAAGGGTGAGGTCCCTGGTTCGAGTCCA
A.D0697 *                 TATTAGCTCAGGTGGTTAGAGCGCACCCCTGATAAGGGTGAGGTCCCTGGTTCGAGTCCA
A.cylindrica *            TATTAGCTCAGGTGGTTAGAGCGCACCCCTGATAAGGGTGAGGTCCCTGGTTCGAGTCCA
A.PCC7118 *               TATTAGCTCAGGTGGTTAGAGCGCACCCCTGATAAGGGTGAGGTCCCTGGTTCGAGTCCA
A.PCC7120 *               TATTAGCTCAGGTGGTTAGAGCGCACCCCTGATAAGGGTGAGGTCCCTGGTTCGAGTCCA
C.fritschii *             TATTAGCTCAGGTGGTTAGAGCGCACCCCTGATAAGGGTGAGGTCCCTGGTTCGAGTCCA
F.musicola *              TATTAGCTCAGGTGGTTAGAGCGCACCCCTGATAAGGGTGAGGTCCCTGGTTCGAGTCCA
G.sp *                    TATTAGCTCAGGTGGTTAGAGCGCACCCCTGATAAGGGTGAGGTCCCTGGTTCGAGTCCA
N.ellipsospora*           TATTAGCTCAGGTGGTTAGAGCGCACCCCTGATAAGGGTGAGGTCCCTGGTTCGAGTCCA
N.muscorum *              TATTAGCTCAGGTGGTTAGAGCGCACCCCTGATAAGGGTGAGGTCCCTGGTTCGAGTCCA
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C.c.chloroplast           TATTAGCTCAGATGGTTAGAGCGCACCCCTGATAAGGGTGAAGTCCCTGGTTCAATTCCA

                              70        80        90       100       110       120
                              |         |         |         |         |         |
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M.aeruginosa              GGATGGCCCACCTG-------------------------------------------
S.PCC6301                 GGATGGCCCACCTAGC------TTTTTCATGAGAGTGAAGAAGT----------------
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S.PCC7942                 ------------------------------------------GATGTGGGGGTTTAGCTCA
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C.fritschii *             ------------------------------------------GGGGGTATAGCTCA
F.musicola *              TTAA-TTTTTAATTACTAANTTTGAATTTTTAATT----CTTTAGCGGGGGTTTAGCTCA
G.sp *                    AT-TCTTTTGAATTTTTCCTTTAAACTTTTACCTT----GTG-GATGGGGGTTTAGCTCA
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N.muscorum *              GT---TTTTGAATTTTGCATTTTGAATTTTGAATT----GTGTTGGGGGGGTTTAGCTCA
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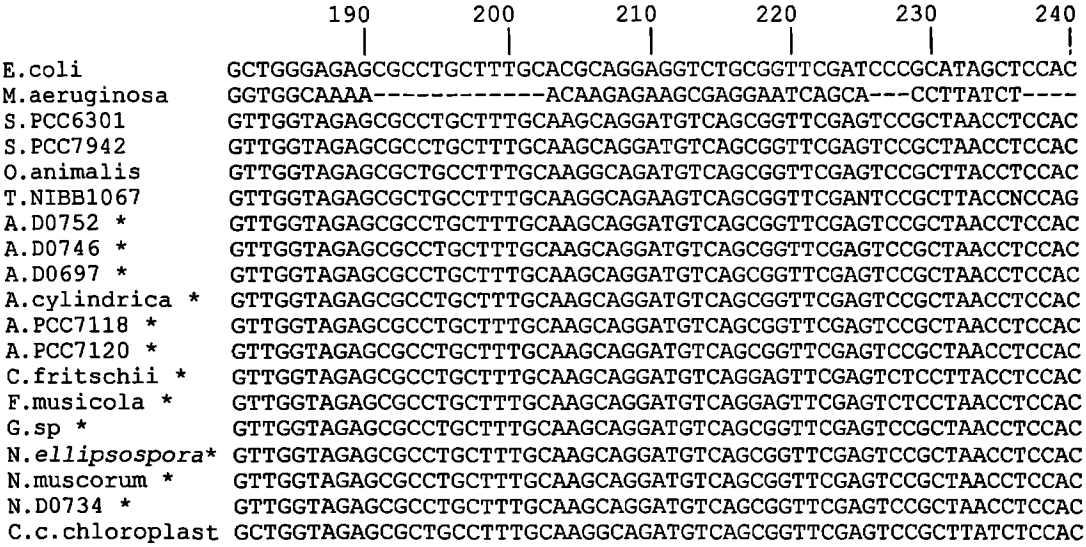
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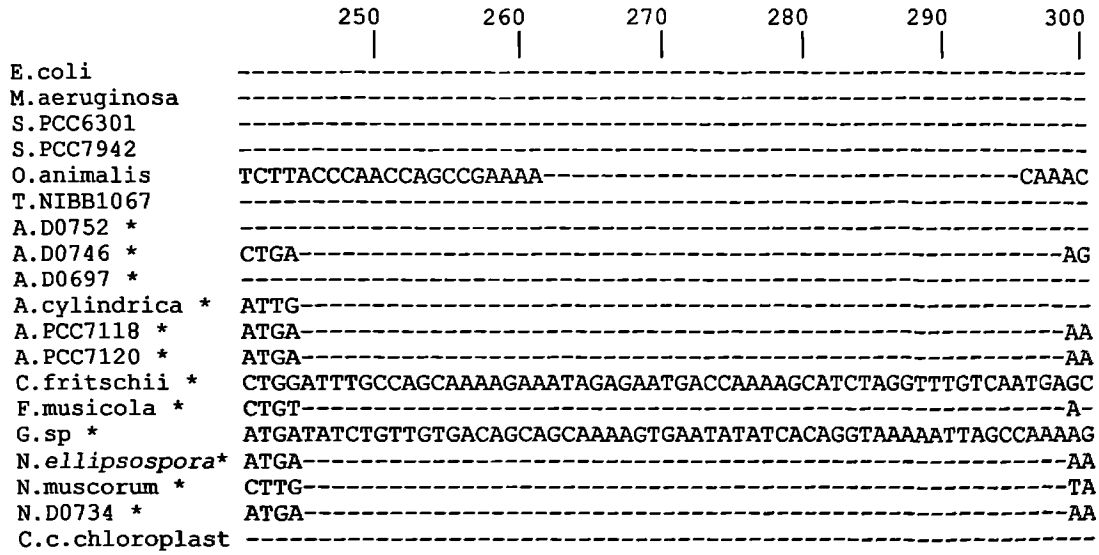
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A.D0752 * ----------
A.D0746 * CTGA----------AG
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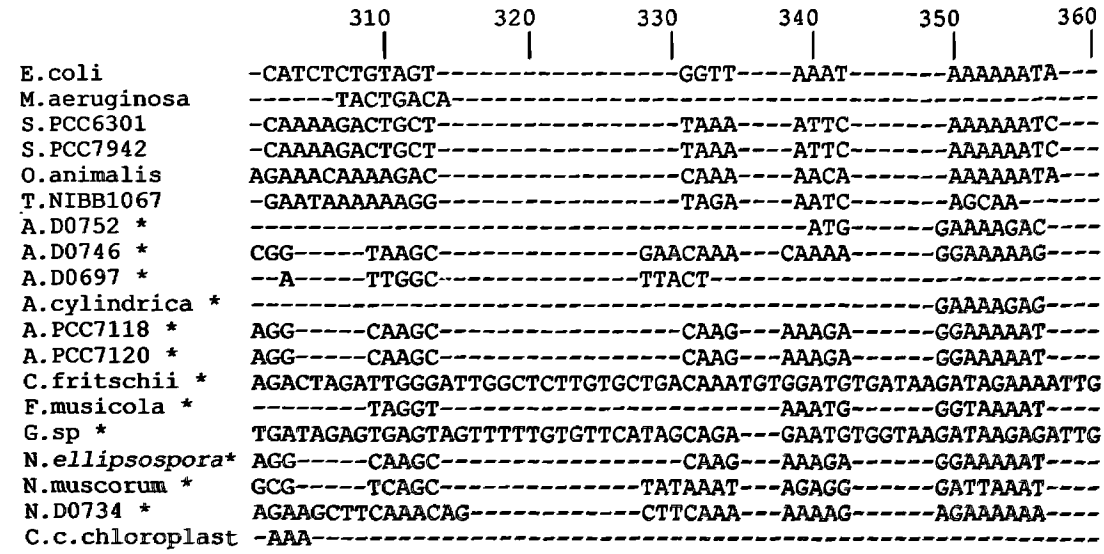
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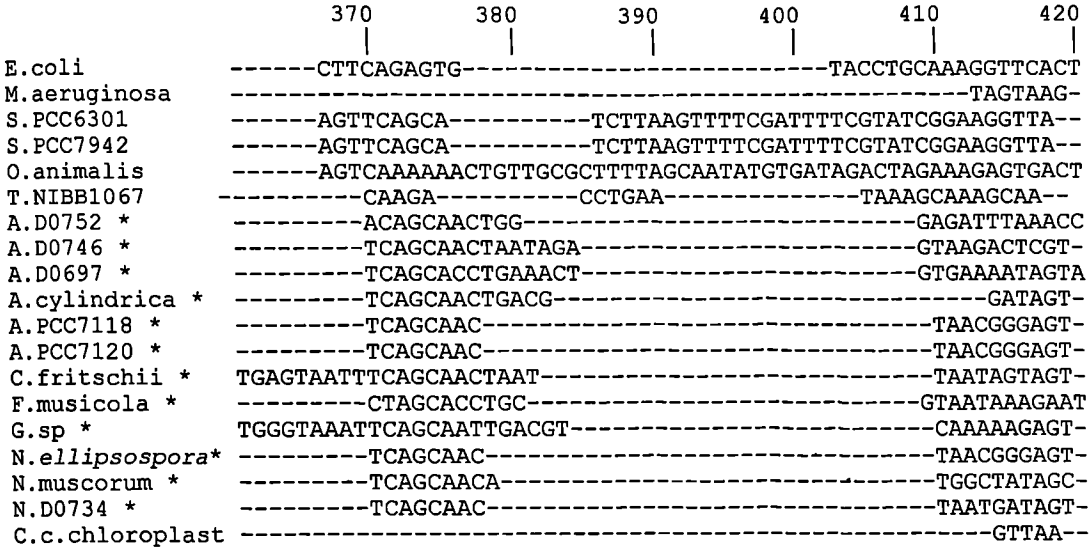
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O.animalis ------AGTCAAAAAACTGTTGCGCTTTTAGCAATATGTGATAGACTAGAAAGAGTGACT
T.NIBB1067 ----------CAAGA----------CCTGAA----------TAAAGCAAAGCAA--
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A.D0746 * ---------TCAGCAACTAATAGA----------GTAAGACTCGT-
A.D0697 * ---------TCAGCACCTGAAACT----------GTGAAAATAGTA
A.cylindrica * ---------TCAGCAACTGACG----------GATAGT-
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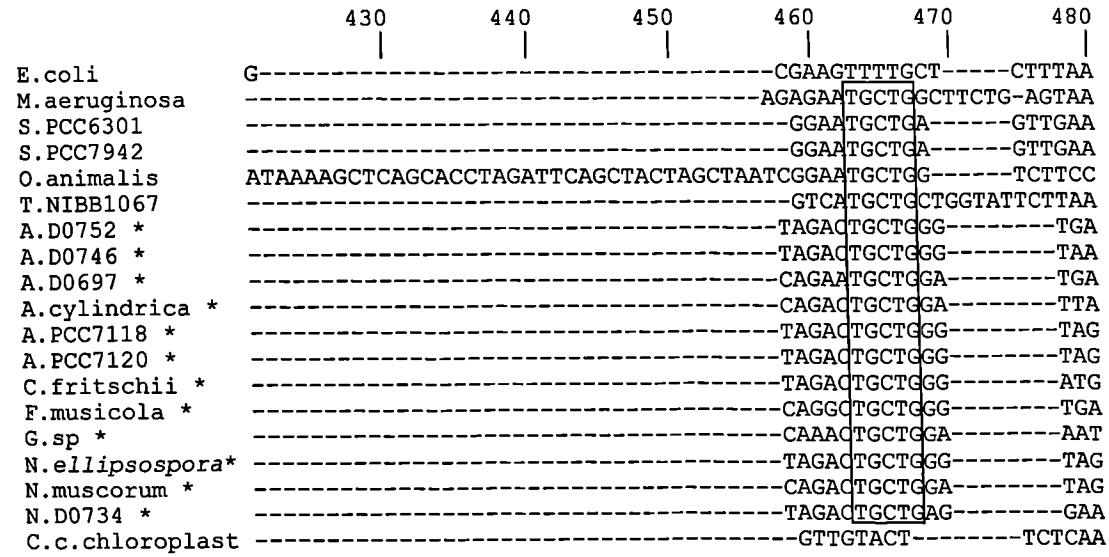
C.fritschii * TGAGTAATTTCAGCAACTAAT----------TAATAGTAGT-
F.musicola * ---------CTAGCACCTGC----------GTAATAAAGAAT
G.sp * TGGGTAAATTCAGCAATTGACGT----------CAAAAAGAGT-
N.ellipsospora* ---------TCAGCAAC----------TAACGGGAGT-
N.muscorum * ---------TCAGCAACA----------TGGCTATAGC-
N.D0734 * ---------TCAGCAAC----------TAATGATAGT-
C.c.chloroplast ----------GTTAA--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M.aeruginosa ----------AGAGAATGCTGGCTTCTG-AGTAA
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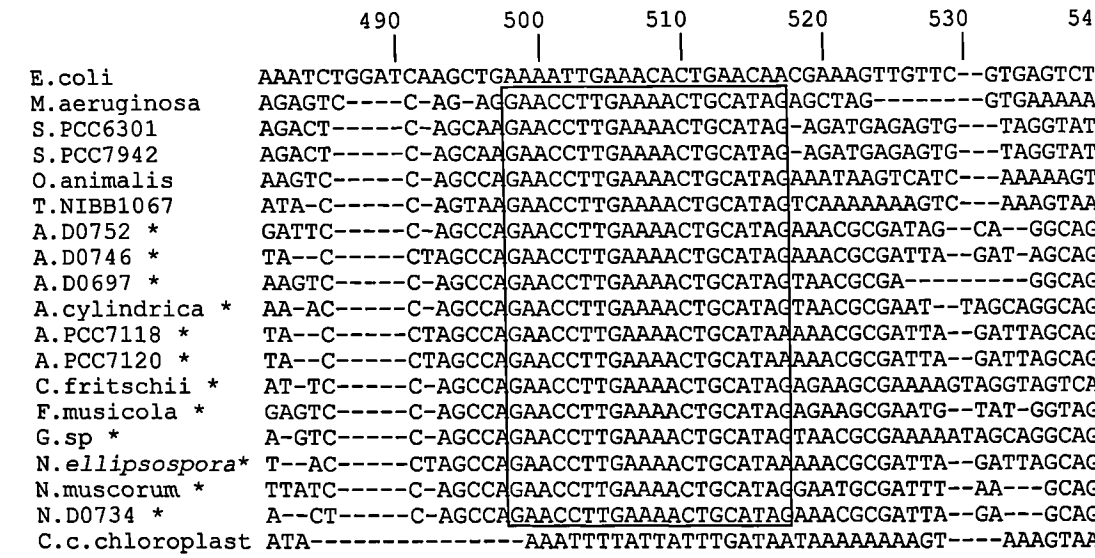
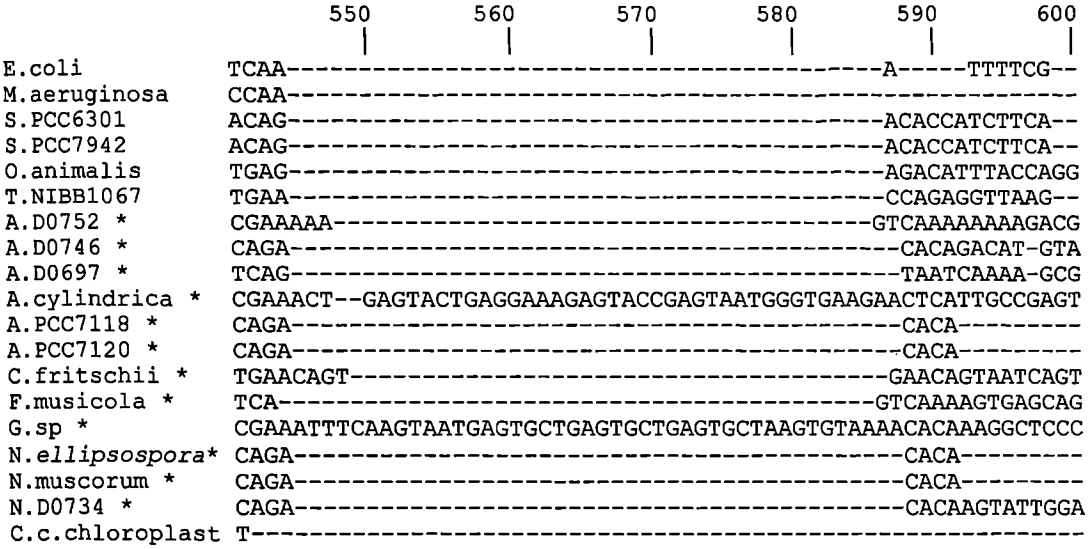
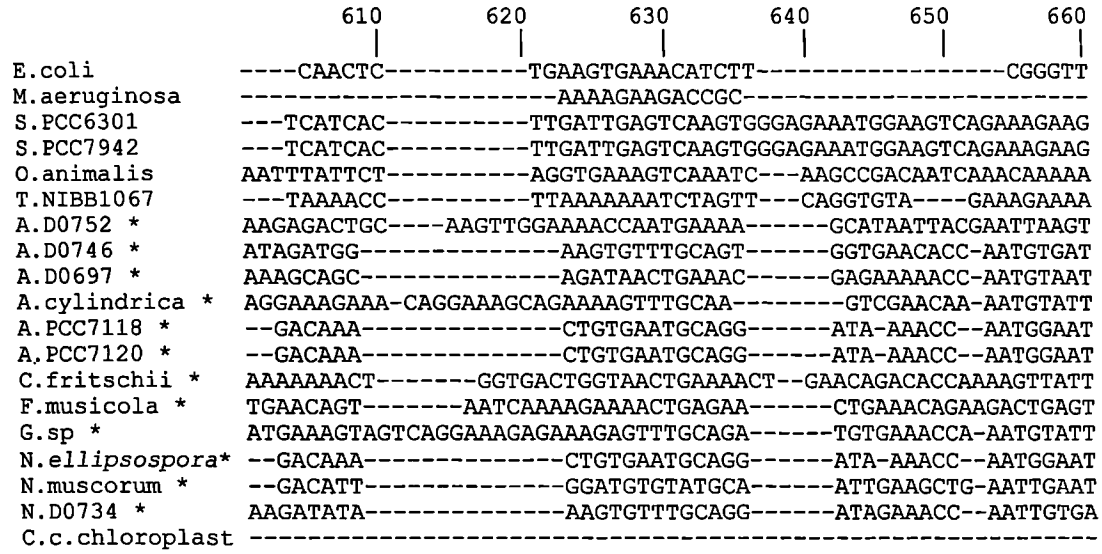
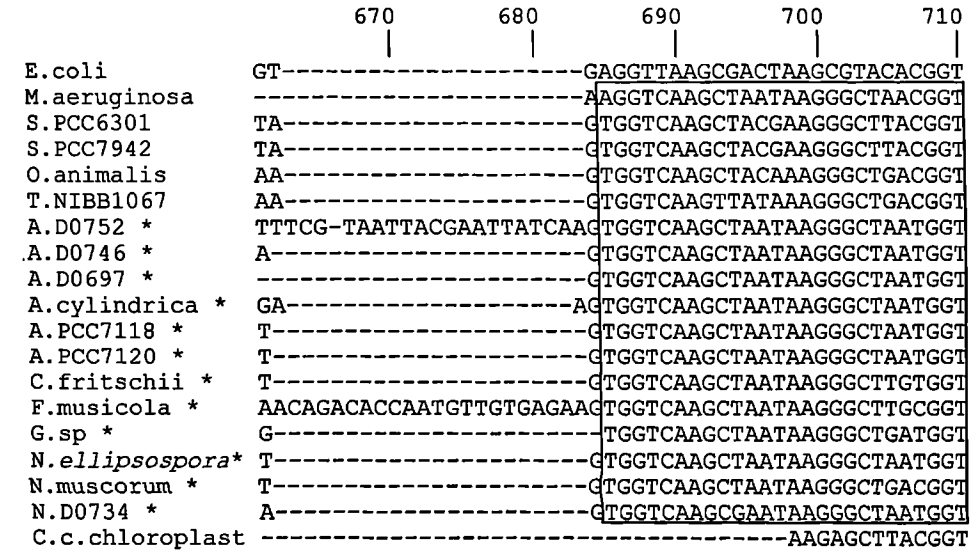
A.PCC7118 * ----------TAGACTGCTGGG--------TAG
A.PCC7120 * ----------TAGACTGCTGGG--------TAG
C.fritschii * ----------TAGACTGCTGGG--------ATG
F.musicola * ----------CAGGCTGCTGGG--------TGA
G.sp * ----------CAAACTGCTGGA--------AAT
N.ellipsospora* ----------TAGACTGCTGGG--------TAG
N.muscorum * ----------CAGACTGCTGGA--------TAG
N.D0734 * ----------TAGACTGCTGAG--------GAA
C.c.chloroplast ----------GTTGTACT--------TCTCAA


490 500 510 520 530 540
E.coli AAATCTGGATCAAGCTGAAAATTGAAACACTGAACAACGAAAGTTGTTC--GTGAGTCTC
M.aeruginosa AGAGTC----C-AG-AGGAACCTTGAAAACTGCATAGAGCTAG--------GTGAAAAAG
S.PCC6301 AGACT-----C-AGCAAGAACCTTGAAAACTGCATAG-AGATGAGAGTG---TAGGTATC
S.PCC7942 AGACT-----C-AGCAAGAACCTTGAAAACTGCATAG-AGATGAGAGTG---TAGGTATC
O.animalis AAGTC-----C-AGCCAGAACCTTGAAAACTGCATAGAAATAAGTCATC---AAAAAGTT
T.NIBB1067 ATA-C-----C-AGTAAGAACCTTGAAAACTGCATAGTCAAAAAAAGTC---AAAGTAAC
A.D0752 * GATTC-----C-AGCCAGAACCTTGAAAACTGCATAGAAACGCGATAG--CA--GGCAGT
A.D0746 * TA--C-----CTAGCCAGAACCTTGAAAACTGCATAGAAACGCGATTA--GAT-AGCAGG
A.D0697 * AAGTC-----C-AGCCAGAACCTTGAAAACTGCATAGTAACGCGA---------GGCAGT
A.cylindrica * AA-AC-----C-AGCCAGAACCTTGAAAACTGCATAGTAACGCGAAT--TAGCAGGCAGA
A.PCC7118 * TA--C-----CTAGCCAGAACCTTGAAAACTGCATAAAAACGCGATTA--GATTAGCAGG
A.PCC7120 * TA--C-----CTAGCCAGAACCTTGAAAACTGCATAAAAACGCGATTA--GATTAGCAGG
C.fritschii * AT-TC-----C-AGCCAGAACCTTGAAAACTGCATAGAGAAGCGAAAAGTAGGTAGTCAG
F.musicola * GAGTC-----C-AGCCAGAACCTTGAAAACTGCATAGAGAAGCGAATG--TAT-GGTAGT
G.sp * A-GTC-----C-AGCCAGAACCTTGAAAACTGCATAGTAACGCGAAAAATAGCAGGCAGA
N.ellipsospora* T--AC-----CTAGCCAGAACCTTGAAAACTGCATAAAAACGCGATTA--GATTAGCAGG
N.muscorum * TTATC-----C-AGCCAGAACCTTGAAAACTGCATAGGAATGCGATTT--AA---GCAGG
N.D0734 * A--CT-----C-AGCCAGAACCTTGAAAACTGCATAGAAACGCGATTA--GA---GCAGG
C.c.chloroplast ATA----------AAATTTTATTATTTGATAATAAAAAAAAGT----AAAGTAAG


550 560 570 580 590 600
E.coli TCAA----------A-----TTTTCG--
M.aeruginosa CCAA----------
S.PCC6301 ACAG----------ACACCATCTTCA--
S.PCC7942 ACAG----------ACACCATCTTCA--
O.animalis TGAG----------AGACATTTACCAGG
T.NIBB1067 TGAA----------CCAGAGGTTAAG--
A.D0752 * CGAAAAA----------GTCAAAAAAAAGACG
A.D0746 * CAGA----------CACAGACAT-GTA
A.D0697 * TCAG----------TAATCAAAA-GCG
A.cylindrica * CGAAACT--GAGTACTGAGGAAAGAGTACCGAGTAATGGGTGAAGAACTCATTGCCGAGT
A.PCC7118 * CAGA----------CACA---------
A.PCC7120 * CAGA----------CACA---------
C.fritschii * TGAACAGT----------GAACAGTAATCAGT
F.musicola * TCA----------GTCAAAAGTGAGCAG
G.sp * CGAAATTTCAAGTAATGAGTGCTGAGTGCTGAGTGCTAAGTGTAAAACACAAAGGCTCCC
N.ellipsospora* CAGA----------CACA---------
N.muscorum * CAGA----------CACA---------
N.D0734 * CAGA----------CACAAGTATTGGA
C.c.chloroplast T----------


610 620 630 640 650 660
E.coli ----CAACTC----------TGAAGTGAAACATCTT----------CGGGTT
M.aeruginosa ----------AAAAGAAGACCGC----------
S.PCC6301 ---TCATCAC----------TTGATTGAGTCAAGTGGGAGAAATGGAAGTCAGAAAGAAG
S.PCC7942 ---TCATCAC----------TTGATTGAGTCAAGTGGGAGAAATGGAAGTCAGAAAGAAG
O.animalis AATTTATTCT----------AGGTGAAAGTCAAATC---AAGCCGACAATCAAACAAAAA
T.NIBB1067 ---TAAAACC----------TTAAAAAAATCTAGTT---CAGGTGTA----GAAAGAAAA
A.D0752 * AAGAGACTGC----AAGTTGGAAAACCAATGAAAA------GCATAATTACGAATTAAGT
A.D0746 * ATAGATGG----------AAGTGTTTGCAGT------GGTGAACACC-AATGTGAT
A.D0697 * AAAGCAGC----------AGATAACTGAAAC------GAGAAAAACC-AATGTAAT
A.cylindrica * AGGAAAGAAA-CAGGAAAGCAGAAAAGTTTGCAA--------GTCGAACAA-AATGTATT
A.PCC7118 * --GACAAA----------CTGTGAATGCAGG------ATA-AAACC--AATGGAAT
A.PCC7120 * --GACAAA----------CTGTGAATGCAGG------ATA-AAACC--AATGGAAT
C.fritschii * AAAAAAACT-------GGTGACTGGTAACTGAAAACT--GAACAGACACCAAAAGTTATT
F.musicola * TGAACAGT-------AATCAAAAGAAAACTGAGAA------CTGAAACAGAAGACTGAGT
G.sp * ATGAAAGTAGTCAGGAAAGAGAAAGAGTTTGCAGA------TGTGAAACCA-AATGTATT
N.ellipsospora* --GACAAA----------CTGTGAATGCAGG------ATA-AAACC--AATGGAAT
N.muscorum * --GACATT----------GGATGTGTATGCA------ATTGAAGCTG-AATTGAAT
N.D0734 * AAGATATA----------AAGTGTTTGCAGG------ATAGAAACC--AATTGTGA
C.c.chloroplast ----------

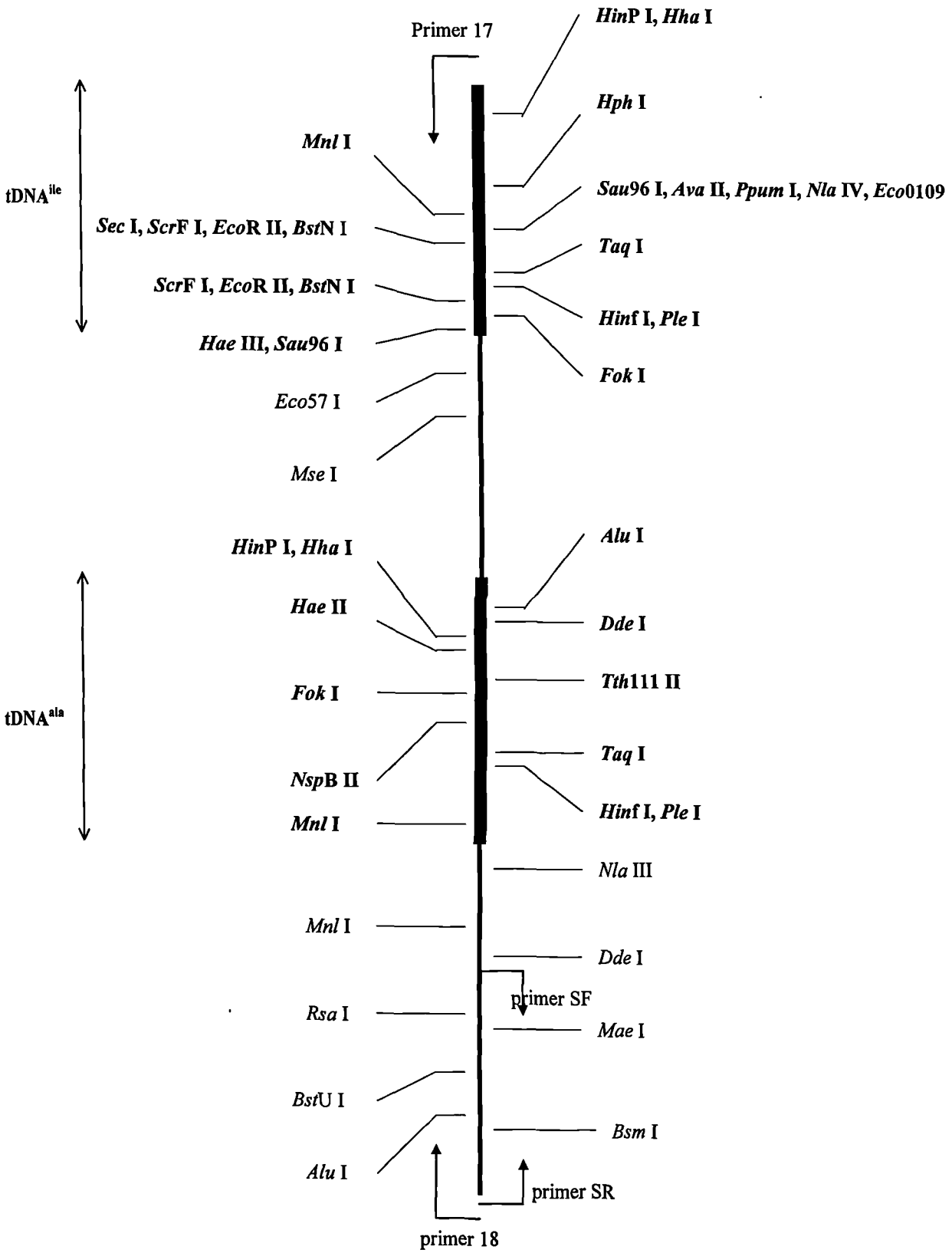

670 680 690 700 710
E.coli GT----------GAGGTTAAGCGACTAAGCGTACACGGT
M.aeruginosa ----------AAGGTCAAGCTAATAAGGGCTAACGGT
S.PCC6301 TA----------GTGGTCAAGCTACGAAGGGCTTACGGT
S.PCC7942 TA----------GTGGTCAAGCTACGAAGGGCTTACGGT
O.animalis AA----------GTGGTCAAGCTACAAAGGGCTGACGGT
T.NIBB1067 AA----------GTGGTCAAGTTATAAAGGGCTGACGGT
A.D0752 * TTTCG-TAATTACGAATTATCAAGTGGTCAAGCTAATAAGGGCTAATGGT
A.D0746 * A----------GTGGTCAAGCTAATAAGGGCTAATGGT
A.D0697 * ----------GTGGTCAAGCTAATAAGGGCTAATGGT
A.cylindrica * GA----------AGTGGTCAAGCTAATAAGGGCTAATGGT
A.PCC7118 * T----------GTGGTCAAGCTAATAAGGGCTAATGGT
A.PCC7120 * T----------GTGGTCAAGCTAATAAGGGCTAATGGT
C.fritschii * T----------GTGGTCAAGCTAATAAGGGCTTGTGGT
F.musicola * AACAGACACCAATGTTGTGAGAAGTGGTCAAGCTAATAAGGGCTTGCGGT
G.sp * G----------TGGTCAAGCTAATAAGGGCTGATGGT
N.ellipsospora* T----------GTGGTCAAGCTAATAAGGGCTAATGGT
N.muscorum * T----------GTGGTCAAGCTAATAAGGGCTGACGGT
N.D0734 * A----------GTGGTCAAGCGAATAAGGGCTAATGGT
C.c.chloroplast ----------AAGAGCTTACGGT

between $tDNA^{ala}$ and the 5' end of 23S rDNA, a very short conserved sequence (5 bp) from position 463 to 467 and a 20 bp sequence from position 498 to 517; the region of high sequence homology is from position 686 to 710, which is at the 5' end of 23S rDNA. High sequence similarities were exhibited in the spacer between $tDNA^{ile}$ and $tDNA^{ala}$, from position 70 to 160 for the heterocystous cyanobacteria, except strain *Chlorogloeopsis fritschii*, which did not contain this spacer. Unicellular strains all possessed a short sequence in this spacer. Sequences of high similarity could also be found for heterocystous strains from position 370 to 376 and 518 to 526. Such similar sequences indicate that heterocystous strains are closely related.

The sequences for *Anabaena* PCC 7118, *Anabaena* PCC 7120 and *Nostoc ellipsospora* were not only similar to each other, but also to other heterocystous filamentous strains, in particular the *Anabaena cylindrica, Nostoc muscorum* and *Nostoc linckia* D0734. High sequence similarites were found for all these, except in those regions from positions 346 to 396 and 527 to 661, where *Anabaena* PCC 7118, *Anabaena* PCC 7120 and *Nostoc ellipsospora* only showed close homology to the *Nostoc* strains. The sequence of the unicellular strains, *Synechococcus* PCC6301 and *Synechococcus* PCC7942, were found to be identical.

Figure 6.14 shows the position of restriction endonuclease sites in the partial cyanobacterial 16S-23S rDNA spacer, deduced from the sequencing data, using the program of full restriction endonuclease sites analysis in GeneJockey II. Those enzymes that cut within the conserved tDNA regions are useful for RFLP analyses, because they cut the DNA from all cyanobacteria tested, and yield polymorphic markers from the variable length spacer. Digested fragments of the same size always exhibited a high

Figure 6.14. Diagrammatic representation of the position of restriction endonuclease sites in the cyanobacterial partial 16S-23S rDNA spacer. Complete restriction maps were obtained by using GeneJockey II package. ▬, tDNA ($tDNA^{ile}$ and $tDNA^{ala}$), —, variable spacer. The restriction endonuclease sites in the tDNAs were normally found for all the cyanobacteria. Sites may be present or absent in the variable spacer depending on the strain. Approximate primer annealing sites are shown for primers 17, 18, SF and SR by arrowed bars. Arrows indicate direction of extension.

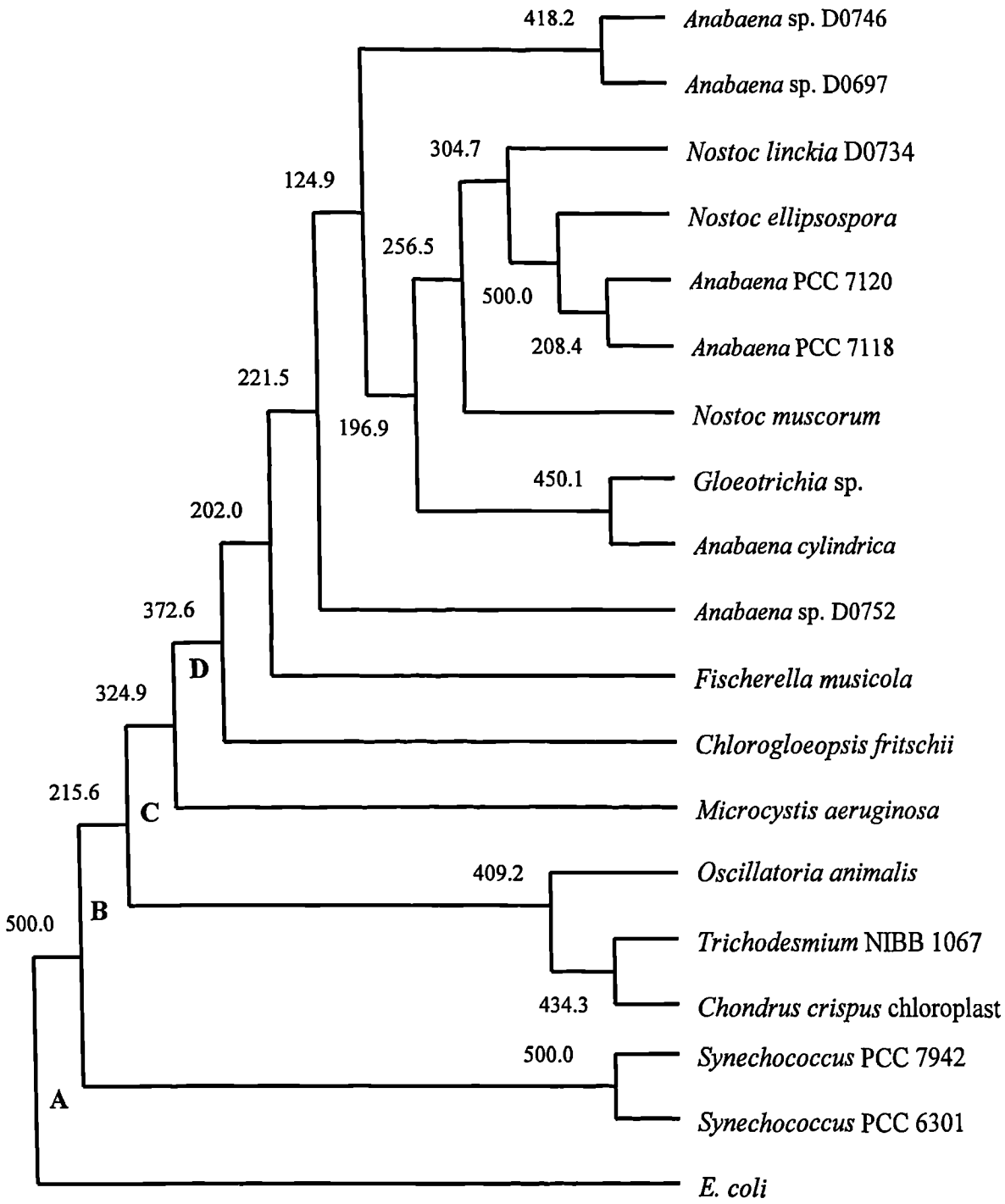

Primer 17
HinP I, Hha I
Hph I
Mnl I
Sau96 I, Ava II, Ppum I, Nla IV, Eco0109
tDNA ile
Sec I, ScrF I, EcoR II, BstN I
Taq I
ScrF I, EcoR II, BstN I
Hinf I, Ple I
Hae III, Sau96 I
Fok I
Eco57 I
Mse I
Alu I
HinP I, Hha I
Hae II
Dde I
Fok I
Tth111 II
tDNA ala
Taq I
NspB II
Hinf I, Ple I
Mnl I
Nla III
Mnl I
Dde I
primer SF
Rsa I
Mae I
BstU I
Bsm I
Alu I
primer SR
primer 18

degree of sequence similarity. However, the enzymes that only cut within the variable spacer region are not useful for RFLPs, because their recognition sequences are not likely to be found in all cyanobacteria. Most of the enzymes that cut within the conserved regions generated similar restriction profiles for all the cyanobacteria, in terms of number of bands, with either one band of the same size, and two of different sizes; or two bands of the same size , and one of different size, with the exception of *Hinf* I, *Taq* I and *Hae* II. From Figure 6.13, sequence differences were found between specific strains at the recognition sites for these three enzymes. At position 54 (in $tDNA^{ile}$), one base pair difference was found between heterocystous strains and non-heterocystous strains, except *Microcystis aeruginosa*, and this position is within the restriction site for *Hinf* I and *Taq* I. This difference proved useful in separating unicellular, non-heterocystous filamentous, and heterocystous filamentous cyanobacteria (see RFLP patterns Fig. 6.5). At position 194-196 ($tDNA^{ala}$), sequence differences were found for *Oscillatoria animalis* and *Trichodesmium* NIBB 1067, whilst all other strains appeared to have the same sequence. This is the position of the only restriction site for *Hae* II. Consequently the partial 16S-23S spacer of *Oscillatoria animalis* and *Trichodesmium* NIBB 1067 are not digested by *Hae* II. Thus *Hae* II would not be as good as *Hinf* I and *Taq* I, which both have one restriction site within each of the conserved regions for $tDNA^{ile}$ and $tDNA^{ala}$, at discriminating between cyanobacteria. Some enzymes in Figure 6.14 only have single or double restriction sites in $tDNA^{ile}$. Such enzymes did not change the regional variation within the spacer.

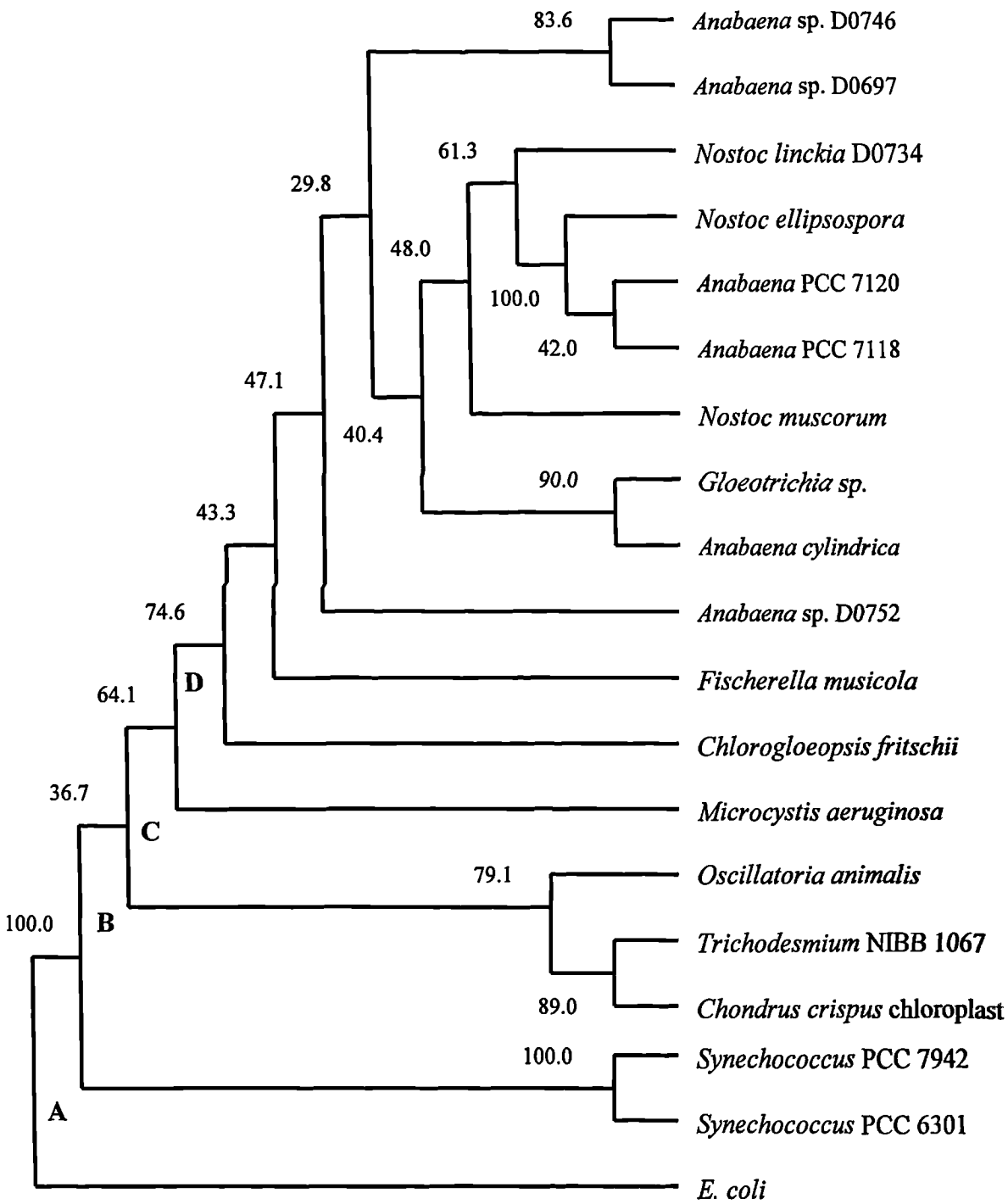
Figure 6.15 shows three consensus trees constructed by using DNA parsimony algorithm with 1, 100, 500 bootstrapping runs, performed on the sequencing data from the partial 16S-23S spacer region of 15 laboratory cyanobacterial strains, together with *Synechococcus* PCC6301 (Accession No. X00346), *Trichodesmium* NIBB1067

Figure 6.15 Consensus trees derived from parsimony method based on partial 16S-23S spacer region sequence analysis. Data from 15 laboratory cyanobacterial strains, two published cyanobacterial sequences of *Synechococcus* PCC 6301 (Tomioka and Sugiura, 1984) and *Trichodesmium* NIBB 1067 (Wilmotte *et al.*, 1994); *E.coli* (Young and Steitz, 1978); and *Chondrus crispus* (red alga) chloroplast (Leblanc *et al.*, 1995) were analysed. The rooted tree was generated by the parsimony method and 500 bootstrapping runs were performed, using the DNAPARS, SEQBOOT and CONSENSE program in the software package PHYLIP. (A): The numbers at the forks indicate the number of times the group consisting of the species which are to the right of that fork occurred among the trees, out of 500 trees. (B): The numbers at the forks indicate the number of times the group consisting of the species which are to the right of that fork occurred among the trees, out of 100 trees. (C): The numbers at the forks indicate the number of times the group consisting of the species which are to the right of that fork occurred among the tree, out of 1 tree. The rDNA spacer was amplified with primer pair 17/18. Clusters A, B, C, D are assigned on each tree.

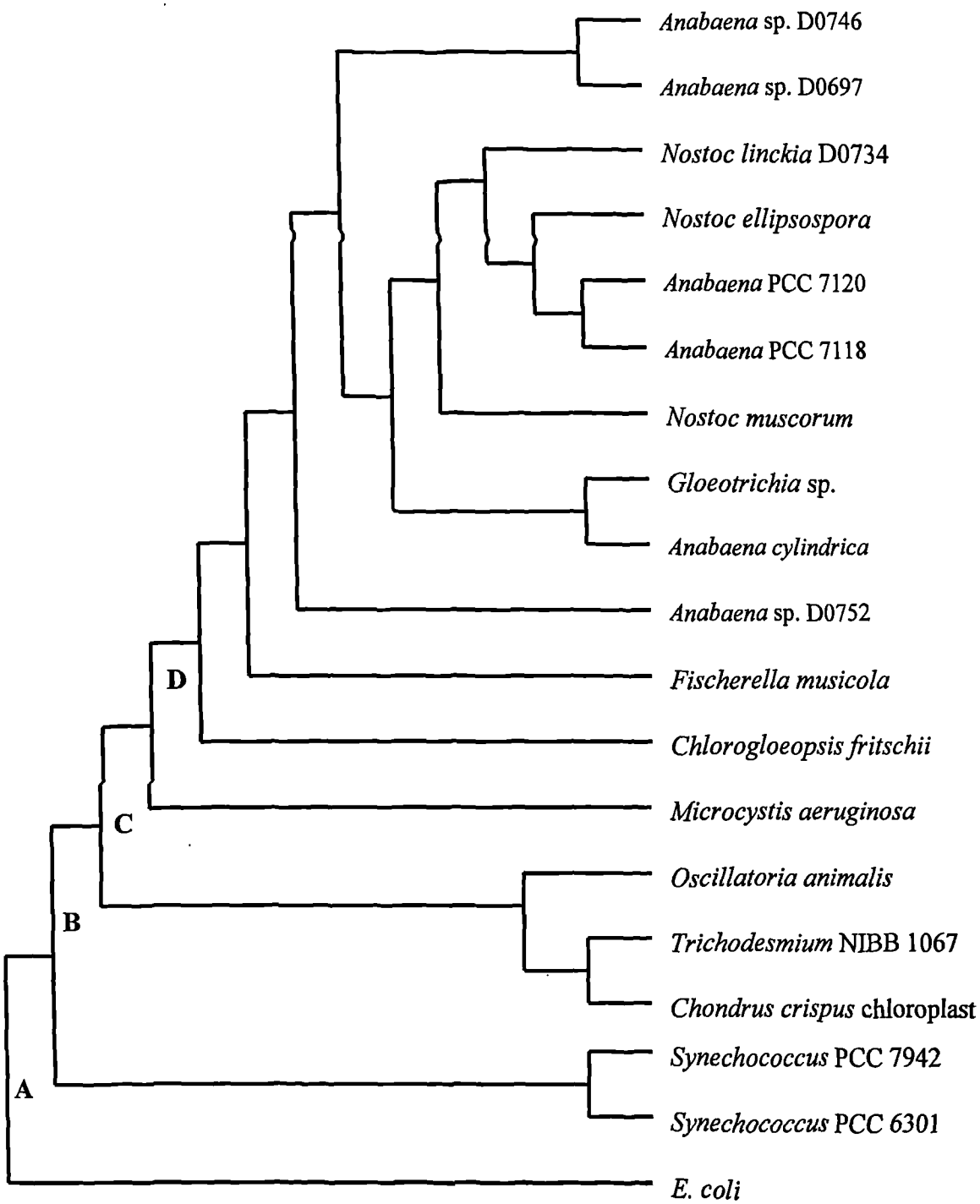
A.


418.2
Anabaena sp. D0746
Anabaena sp. D0697
304.7
Nostoc linckia D0734
124.9
Nostoc ellipsospora
256.5
Anabaena PCC 7120
500.0
208.4
Anabaena PCC 7118
221.5
Nostoc muscorum
196.9
450.1
Gloeotrichia sp.
202.0
Anabaena cylindrica
372.6
Anabaena sp. D0752
D
Fischerella musicola
324.9
Chlorogloeopsis fritschii
215.6
C
Microcystis aeruginosa
409.2
Oscillatoria animalis
500.0
B
Trichodesmium NIBB 1067
434.3
Chondrus crispus chloroplast
500.0
Synechococcus PCC 7942
A
Synechococcus PCC 6301
E. coli

B.


83.6
Anabaena sp. D0746
Anabaena sp. D0697
61.3
Nostoc linckia D0734
29.8
Nostoc ellipsospora
48.0
Anabaena PCC 7120
100.0
42.0
Anabaena PCC 7118
47.1
Nostoc muscorum
40.4
90.0
Gloeotrichia sp.
43.3
Anabaena cylindrica
74.6
Anabaena sp. D0752
Fischerella musicola
64.1
D
Chlorogloeopsis fritschii
36.7
Microcystis aeruginosa
C
79.1
Oscillatoria animalis
100.0
B
Trichodesmium NIBB 1067
89.0
Chondrus crispus chloroplast
100.0
Synechococcus PCC 7942
A
Synechococcus PCC 6301
E. coli

C.


Anabaena sp. D0746
Anabaena sp. D0697
Nostoc linckia D0734
Nostoc ellipsospora
Anabaena PCC 7120
Anabaena PCC 7118
Nostoc muscorum
Gloeotrichia sp.
Anabaena cylindrica
Anabaena sp. D0752
Fischerella musicola
Chlorogloeopsis fritschii
Microcystis aeruginosa
Oscillatoria animalis
Trichodesmium NIBB 1067
Chondrus crispus chloroplast
Synechococcus PCC 7942
Synechococcus PCC 6301
E. coli
A
B
C
D

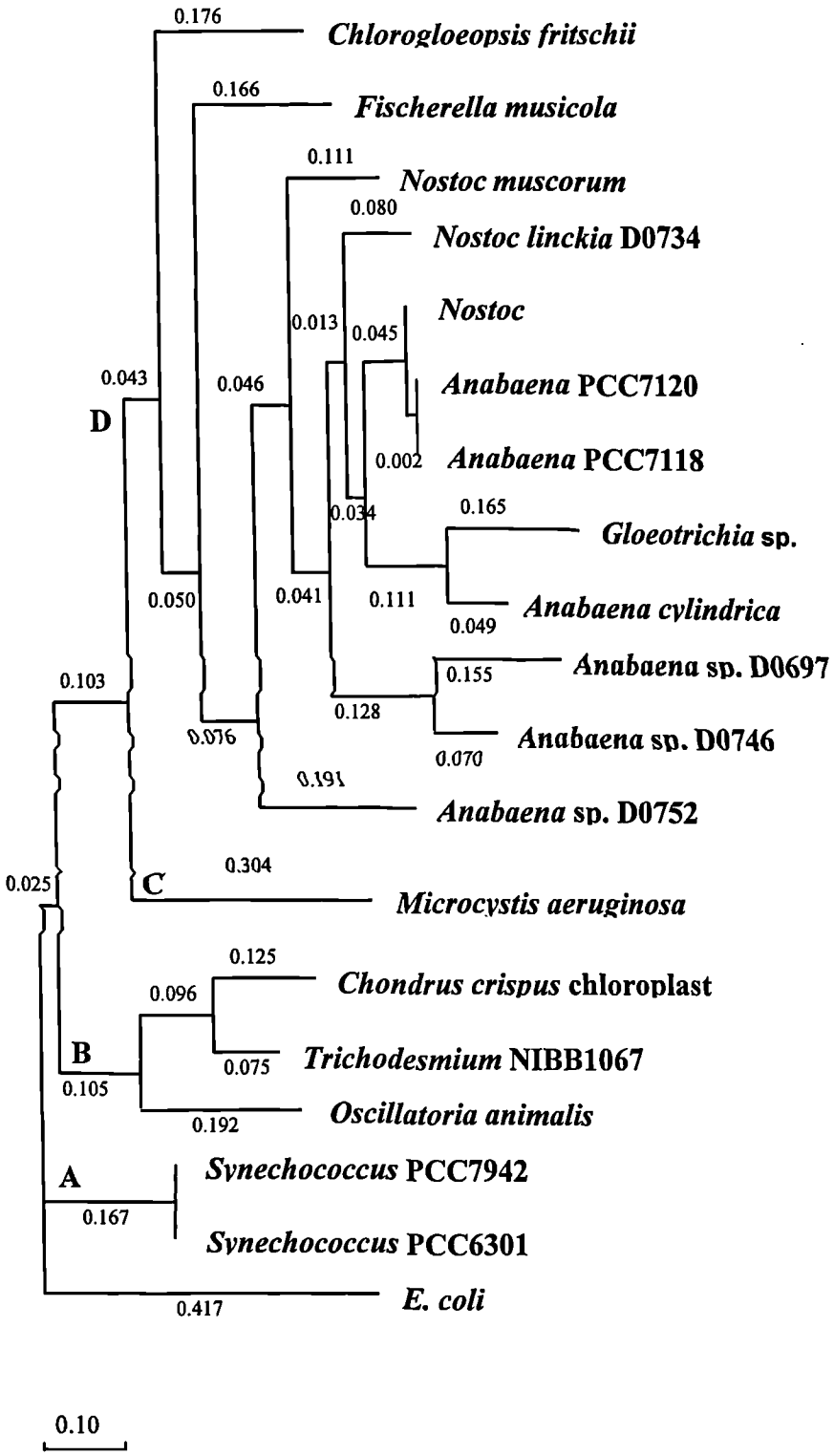
(Accession No. X72871), *E. coli* (Accession No. J01702) and *Chondrus crispus* (red alga) chloroplast (Accession No. Z29521). The three trees show identical results with the majority of branches showing bootstrapping values of 50% or more indicating that the trees are quite robust. *Anabaena* PCC7118 and *Anabaena* 7120 appear close to *Nostoc ellipsospora,* and are closer to other *Nostoc* strains (*Nostoc linckia* D0734 and *Nostoc muscorum*) than to other *Anabaena* strains. The sequence identity of 100% over 446 bases indicates that they are in the same species group (Table 6.4). Furthermore, *Trichodesmium* NIBB 1067 appears close to *Oscillatoria animalis,* but sequence homologies were not found in the aligned sequences, apart from the conserved regions described for all cyanobacteria (Fig. 6.13). The sequence identity of 56.87% indicates that they may not be closely related. Figure 6.16, shows a likelihood tree constructed by using the Maximum Likelihood method for the same strains as those of Figure 6.15. The parsimony tree and likelihood trees are similar, except for the relationship between *Anabaena* and *Nostoc strains. In Figure 6.16, Anabaena* PCC7118, *Anabaena* 7120 and *Nostoc ellipsospora* appear closer to *Anabaena cylindrica* than to *Nostoc muscorum,* and a little more close to *Nostoc linckia* D0734. This suggests that these strains may be located between *Nostoc muscorum* and *Anabaena cylindrica,* which contrasts with the relationship shown in figure 6.15. In both trees (Fig. 6.15 & 6.16), the cyanobacteria were separated into four clusters: (A) *Synechococcus* strains; (B) *Oscillatoria animalis* and *Trichodesmium* NIBB 1067 (together with *Chondrus crispus* chloroplast); (C) *Microcystis aeruginosa*; (D) *Chlorogloeopsis fritschii, Fischerella musicola, Gloeotrichia* sp. and all *Nostoc* and *Anabaena* strains.

Table 6.4 The similarity of partial 16S-23S rDNA spacer sequences from 16 cyanobacterial species.

		Sequence identity (%)*															
length of sequence (bp)	strain	S. PCC 7942	T. NIBB 1067	O. animalis	A. D0697	A. D0746	A. D0752	A. cylindrica	A. PCC7118	A. PCC7120	N. ellipsora	N. muscorum	N. D0734	G. sp	F. musicola	C. fritschii	M. aeruginosa
435	S. PCC 7942	100	53.3	53.83	52.35	54.81	49.14	51.36	51.85	51.85	47.16	50.12	53.09	52.35	50.12	50.37	57.89
394	T. NIBB 1067		100	56.87	52.47	54.12	55.49	51.92	51.92	51.92	48.9	55.49	56.32	48.08	49.73	54.4	52.23
564	*O. animalis*			100	51.71	47.03	49.11	47.94	51.2	51.2	50.84	47.51	50.23	45.69	45.43	45.63	47.73
440	A. D0697				100	61.22	64.88	56.1	60	60	59.76	61.95	62.44	59.02	53.41	47.56	52.23
468	A. D0746					100	59.82	52.97	74.04	74.04	73.73	59.86	67.97	54.57	50.23	51.6	52.23
480	A. D0752						100	58.44	62.41	62.41	62.23	58.67	60.14	56.22	59.11	47.33	50.61
491	A. cylindrica							100	69.15	69.23	67.52	56.29	61.98	68.76	51.3	44.68	53.04
446	A. PCC7118								100	100	100	74.28	78.55	62.89	60.24	51.32	53.44
446	A. PCC7120									100	100	74.28	78.55	62.89	60.27	51.32	53.44
446	N. ellips.										100	74.25	72.6	61.3	59.38	51.2	52.63
451	N. muscorum											100	69.83	57.72	60.81	50.36	50.61
464	N. D0734												100	61.52	55.76	45.85	50.2
619	*G. sp*													100	54.13	54.58	53.04
490	F. musicola														100	50.43	52.23
499	C. fritschii															100	52.63
277	M. aeruginosa																100

* Similarities were based on partial 16S-23S rDNA spacer sequences for 15 cyanobacterial laboratory strains this study and the published sequence for Trichodesmium NIBB 1067. Sequence identity (%) was obtained using GeneJockey II.

Figure 6.16 Maximum likelihood tree based on partial 16S-23S spacer region sequence analysis. Data from 15 various cyanobacterial and two published cyanobacterial sequences of *Synechococcus* PCC 6301 (Tomioka and Sugiura, 1984) and *Trichodesmium* NIBB 1067 (Wilmotte, *et al.*, 1994), *E.coli* (Young and Steitz, 1978) and *Chondrus crispus* (red alga) chloroplast (Leblanc *at al.*, 1995) was used to construct the likelihood tree by the Maximum Likelihood method using DNAML version 3.572c program in the software package PHYLIP. Total 680 trees were examined by program. The rDNA spacer was amplified with primer pair 17/18. Cluster A, B, C, D are assigned.


0.176
Chlorogloeopsis fritschii
0.166
Fischerella musicola
0.111
Nostoc muscorum
0.080
Nostoc linckia D0734
Nostoc
0.013
0.045
0.043
0.046
Anabaena PCC7120
D
0.002
Anabaena PCC7118
0.034
0.165
Gloeotrichia sp.
0.050
0.041
0.111
Anabaena cylindrica
0.049
0.155
Anabaena sp. D0697
0.103
0.128
0.076
Anabaena sp. D0746
0.070
0.191
Anabaena sp. D0752
0.304
0.025
C
Microcystis aeruginosa
0.125
Chondrus crispus chloroplast
0.096
B
0.075
Trichodesmium NIBB1067
0.105
0.192
Oscillatoria animalis
A
Synechococcus PCC7942
0.167
Synechococcus PCC6301
0.417
E. coli
0.10

6.2.3. Design and use of cyano-specific primers from 16S-23S spacer region

From the sequence alignment shown in figure 6.13 using 19 spacer sequences, two short regions of homology of about 20 bp were identified for all the cyanobacteria tested. One within the variable spacer, between $tDNA^{ala}$ and 5' end of 23S rDNA, and one at the 5' end of 23S rDNA. These sequences were not found in the other organisms analysed, and thus showed potential for use in the design of cyano-specific primers. Accordingly two primers SF and SR were synthesised. These primers amplified a region between $tDNA^{ala}$ and 5' end of 23S rDNA (see Fig. 6.1). SF derived from the conserved region from position 498 to 517 (see Fig. 6.13), which was found in all the cyanobacterial strains studied here, and in seven other cyanobacteria (see Wilmotte, 1994); but not in other bacteria as judged by searching the Genbank data base (see Table 6.5). Whilst SR was from the region of high homology found at position 686 to 704, which was also specific for cyanobacteria when compared with other bacteria.

The sequence and physical characteristics of each of these primers are presented in Table 6.6. The $MgCl_2$ concentration and the annealing temperature were tested to optimise the PCR reaction with these primers (Fig. 6.17). A $MgCl_2$ concentration of 2.5mM and annealing temperature of 50 °C generated clear, reproducible bands. Thus these conditions were used in the PCR. No primer-dimer formation occurred when primer pair SR/SF was used for the PCR, as judged by the absence of any band corresponding to amplified primers themselves on gels after amplification. Products could be detected using these primers with about 15pg purified cyanobacterial DNA in a 50µl reaction mixture, with 35 cycles of the PCR (Fig. 6.18). Template DNA concentration dependencies were observed from 15pg/50µl reaction to 150pg/50µl reaction, but they were not observed at 1.5ng/50µl reaction.

Table 6.5 Specificity of cyanobacterial primers

Accession No.	Sequences producing High-scoring Segment Pairs[a]	Identity (%)
(1). primer SF		
emb|X70769|AS16S	Arthrospira sp. gene for 16S rRNA	100
emb|X75044|ASRRNTRN	Arthrospira sp. genes for 16S rRNA, t...	100
emb|X75045|SSRRNTRN	Spirulina sp. genes for 16S rRNA, tRN...	100
emb|X72871|TSITS	Trichodesmium sp. gene for 23S rRNA, ...	100
emb|X00346|ANRN02	Anacystis nidulans 16S-23S spacer region	100
emb|X00512|ANRN03	Anacystis nidulans 23S rRNA gene and ...	100
dbj|D64000|SYCSLRB	Synechocystis sp. PCC6803 complete gene...	91
dbj|D90916|D90916	Synechocystis sp. PCC6803 complete gene...	91
gb|U53123|GCU53123	Geminigera cryophila nucleomorph 18S rRNA...	91
(2). primer SR		
emb|X75044|ASRRNTRN	Arthrospira sp. genes for 16S rRNA, t...	92
emb|X00512|ANRN03	Anacystis nidulans 23S rRNA gene and...	92
emb|X75045|SSRRNTRN	Spirulina sp. genes for 16S rRNA, tRN...	92
emb|X72871|TSITS	Trichodesmium sp. gene for 23S rRNA, ...	92
dbj|D90916|D90916	Synechocystis sp. PCC6803 complete ge...	92
(3). primer SR18		
emb|X00512|ANRN03	Anacystis nidulans 23S rRNA gene and...	100
dbj|D90916|D90916	Synechocystis sp. PCC6803 complete ge...	100
gb|U04806|HSU04806	Human FLT3/FLK2 ligand mRNA, complet...	97
emb|X00012|CHSORN1	Spirodela oligorhiza chloroplast 5' ...	92
gb|L35485|HUMIDS	Homo sapiens iduronate sulphate sulp...	92
gb|U66082|HSU66082	Human contig of seven cosmids from L...	92
(4). primer 18		
emb|X00512|ANRN03	Anacystis nidulans 23S rRNA gene and...	100
gb|U04806|HSU04806	Human FLT3/FLK2 ligand mRNA, complet...	97
emb|X00012|*CHSORN1*	*Spirodela oligorhiza chloroplast 5' ...*	91
gb|L35485|HUMIDS	Homo sapiens iduronate sulphate sulp...	92
gb|U66082|HSU66082	Human contig of seven cosmids from L...	92
gb|M21402|SOYRDNASP	Soybean chloroplast 16S/23S ribosoma...	91
gb|M21453|SPICPRGDG	Spinach chloroplast 16S ribosomal RN...	91
gb|M75722|ALSCP23SA	*Alnus incana chloroplast 23S ribosom...*	91
emb|X59768|CA23SRR	*C.americana 23S rRNA gene*	91
gb|M37149|SOYCPRTG	Soybean chloroplast 16S rRNA (3' end...	91
emb|X01365|CHZM23S	Maize chloroplast DNA for 4.5S and 2...	91
gb|J01446|TOBCPRG23	Tobacco chloroplast 23S and 4.5S rRN...	91
emb|X62099|CHEVIARRN	E.virginiana plastid ribosomal RNA o...	91
emb|X55033|CHPSRRNAO	P.sativum chloroplast DNA of the sin...	91
gb|M37430|PEACPDKAA	Pea Chloroplast 4.5S, 5S, 16S and 23...	91
emb|Z00028|CHZMRRNA	Z.mays chloroplast rRNA-operon	91
gb|M81884|EPFCPCG	Epifagus virginiana chloroplast comp...	91
dbj|D17510|PINCPTRPG	Black pine chloroplast DNA, complete...	91
emb|X04465|CHMPXX	Liverwort Marchantia polymorpha chlo...	91
emb|Z00044|CHNTXX	Tobacco chloroplast genome DNA.	91
emb|X86563|CHZMXX	Z.mays complete chloroplast genome	91
dbj|D90916|D90916	Synechocystis sp. PCC6803 complete g...	91

a, Sequences producing High-scoring Segment Pairs (>90 % identity) were obtained from 312,067 sequences in Genbank data (BLAST e-mail: blast@ncbi.nlm.nih.gov).

Table 6.6 Sequence and physical characteristics of primers SF, SR and SR-18.

	Primer[a]		
Property	SF	SR	SR18
Sequence	5'-GAACCTTGAAAACTGCATAG-3'	5'-AGCCCTTATT AGCTTGACC-3'	5'-TTTGCGGCCGCTCTGTGTGCC-3'
Length (bp)	20	19	21
Dissociation temperature (Tm)	56 °C	56 °C	70 °C
% G+C	40%	47.4%	66.7%

a, primer SF and SR developed in this study; SR18 was part of primer 18.

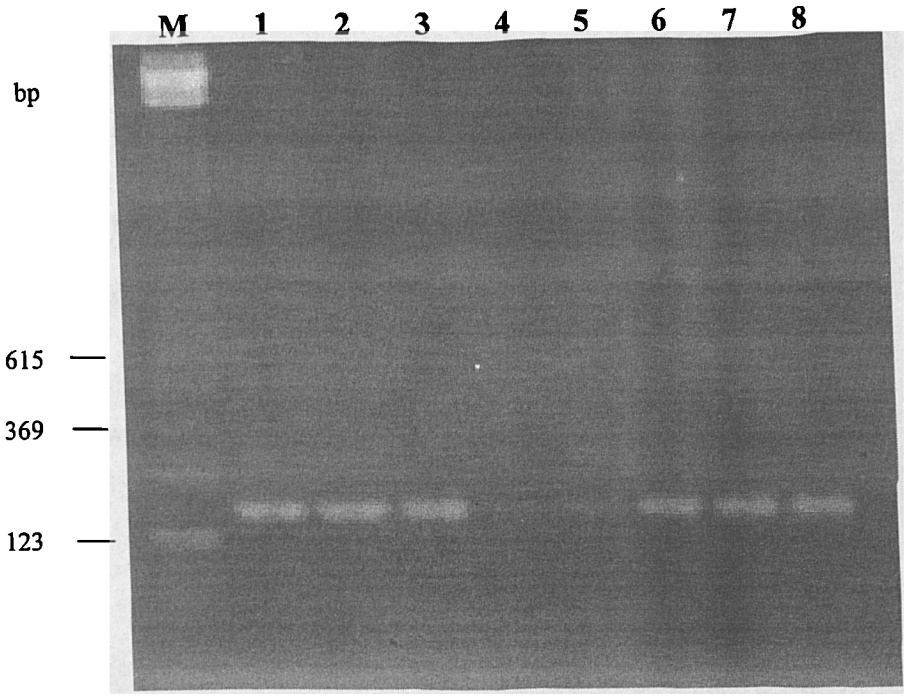


Figure 6.17 Agarose gel elctrophoresis of amplified partial 16S-23S rDNA spacer of *Anabaena cylindrica* at different magnesium chloride concentrations and different annealing temperatures. Lane 1, 5 mM; 2, 2.5mM; 3, 1.5mM; 4, 0.5mM $MgCl_2$. Lane 5, 60 ^{o}C; 6, 55^{o}C; 7, 50^{o}C; 8, 45^{o}C with the magnesium concentration of 2.5mM for the primer SF/SR.

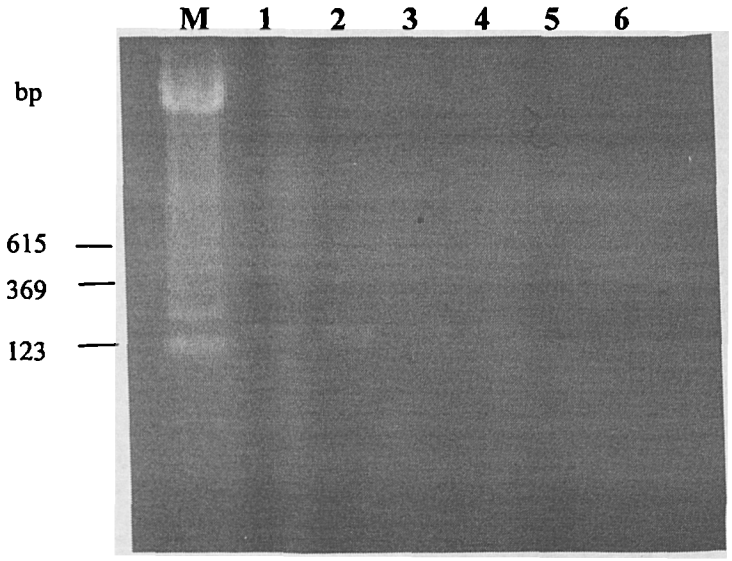


Figure 6.18 Agarose gel elctrophoresis of amplified partial 16S-23S rDNA spacer of *Anabaena cylindrica* at different total genomic DNA concentration. Lane 1, 15ng; 2, 1.5ng; 3, 150pg; 4, 15pg; 5, 1.5pg; 6, 0.15pg in 50µl reaction with the magnesium concentration of 2.5mM for the primer SF/SR.

Specificity of the SF/SR primer pair was checked using DNA from 25 cyanobacteria, from a green algae (isolated from Raby pool) and from *E.coli* K12 (LJMU). A single band of 70-350 bp was generated for each cyanobacterium. No PCR products were obtained from the green algae or *E.coli* DNA (see Fig 6.19). Moreover, this pair of primers showed higher specificity for amplifying cyanobacterial DNA than either primer pair 17/18 or SF/18, which both produced a weak PCR product with green alga DNA and *E.coli* DNA. Whilst SF/SR18 (where SR18 was part (21 bp) of primer 18, see Table 6.5 & 6.6), which did not amplify green alga DNA and *E.coli* DNA, was also more specific than primer pair 17/18 or SF/18. In addition, SF/SR was tested directly on a mixed culture of isolates from a fresh water sample designated sample 1 (see Table 2.3 and section 3.2.4). This sample supposedly contained six strains of two cyanobacterial species, as determined from selective culturing (see Table 3.2) and RAPD data (see section 5.2.3). When the DNA of the sample was amplified using SF/SR, two clear bands were generated (Figure 6.19, lane 30). The larger band (350bp) corresponds to that of *Synechococcus* LJM003 (Fig. 6.19, lane 5) and the smaller band (90 bp) to *Pseudanabaena* LJM001 (Fig. 6.19, lane 9).

The validity of using the primer sequences of SF and SR for identifying cyanobacteria was tested by constructing parsimony consensus (Fig. 6.20) and Likelihood (Fig. 6.21) trees, based on the SF/SR amplified rDNA spacer sequences for cyanobacterial species. The parsimony consensus and likelihood trees are similar, except for the position of *Microcystis aeruginosa*. Furthermore, they are like the trees in figures 6.15 and 6.16 based on the 17/18 amplified spacer sequences, in terms of clusters, showing only one more cluster containing *Oscillatoria animalis*.

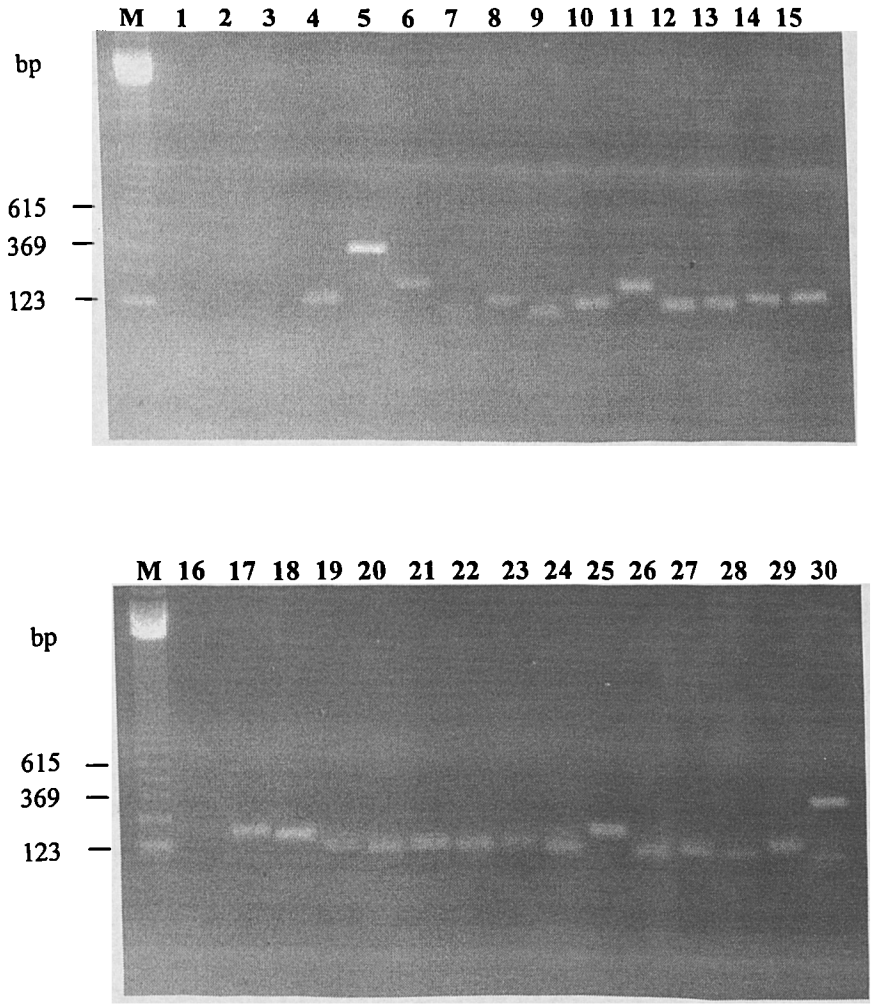
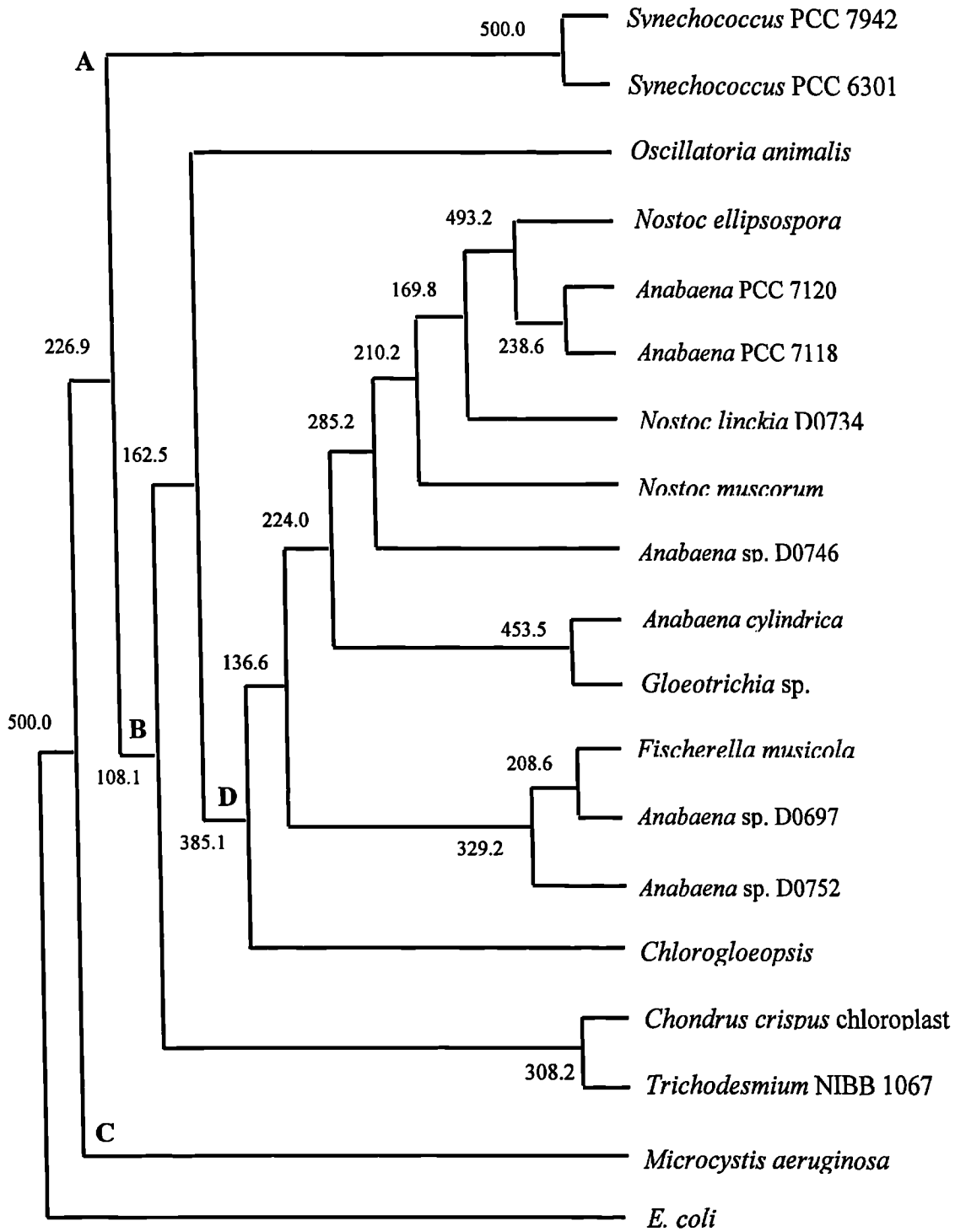


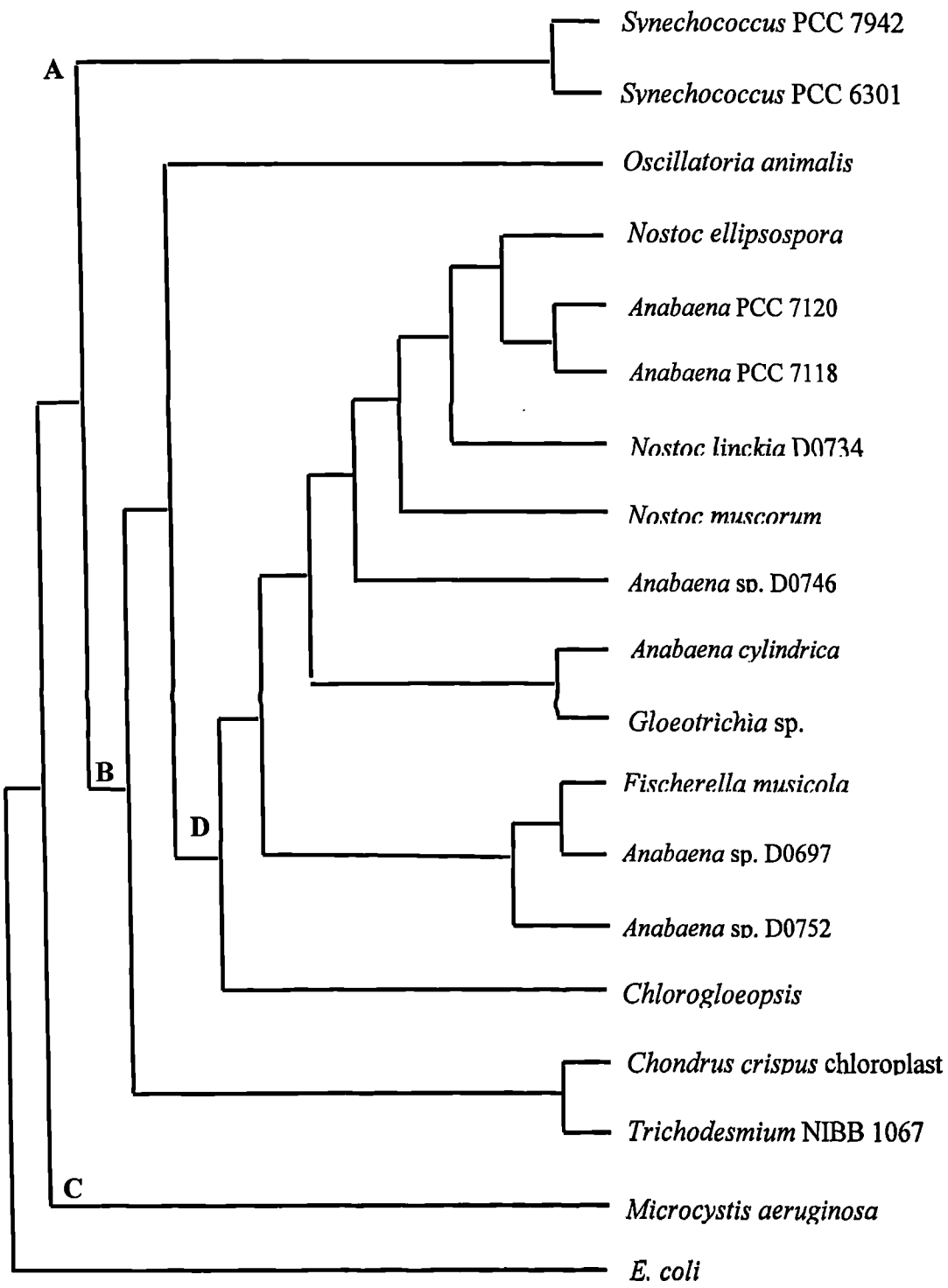
Figure 6.19 Agarose gel elctrophoresis of partial 16S-23S rDNA spacer amplified using primers SF and SR. Lane M, 123bp DNA ladder; 1, DNA negative control; 2, *green alga*; 3, *E. coli*; 4, *Synechococcus* PCC 7942; 5, *Synechococcus* LJM003; 6, *Synechocystis* PCC 6803; 7, *Microsystis aeruginosa*; 8, *Oscillatoria animalis*; *9*, *Pseudanabaena* sp. LJM001; 10, *Nostoc MAC* PCC8009; 11, *Anabaena cylindrica*; 12, *Aphanizomenon gracile*; 13, *Tolypothrix distorta*; 14, *Chlorogloeopsis fritschii*; 15, *Fisherella musicola*; 16, DNA negative control; 17, *Gloeotrichia* sp.; 18, *Anabaena sp.* D0752; 19, *Nostoc* sp. D0766; 20, *Anabaena flos-aquae* D0848; 21, *Anabaena* sp. D0746; 22, *Nostoc* sp. D0740; 23, *Nostoc linckia* D0734; 24, *Nostoc muscorum*; 25, *Nostoc commune;* 26, *Anabaena* PCC 7120; 27, *Anabaena* sp. D0697; 28, *Leptolyngbya* JM014; 29, *Nostoc paleo*; 30, fresh water sample 1.

Figure 6.20. Consensus trees derived from parsimony method based on analysis of the partial 16S-23S spacer region sequence amplified using primers SF and SR. Data from 15 laboratory cyanobacterial strains, two published cyanobacterial sequences of *Synechococcus* PCC 6301 (Tomioka and Sugiura, 1984) and *Trichodesmium* NIBB 1067 (Wilmotte, *et al.*, 1994), *E.coli* (Young and Steitz, 1978) and *Chondrus crispus* (red alga) chloroplast (Leblanc *at al.*, 1995) were analysed. The rooted tree was generated by the parsimony method and 500 bootstrapping runs were performed, using the DNAPARS, SEQBOOT and CONSENSE program in the software package PHYLIP. (A): The numbers at the forks indicate the number of times the group consisting of the species which are to the right of that fork occurred among the trees, out of 500 trees. (B): The numbers at the forks indicate the number of times the group consisting of the species which are to the right of that fork occurred among the trees, out of 1 tree. Clusters A, B, C, D are assigned.

A.


500.0
A
Synechococcus PCC 7942
Synechococcus PCC 6301
Oscillatoria animalis
493.2
Nostoc ellipsospora
169.8
Anabaena PCC 7120
226.9
210.2
238.6
Anabaena PCC 7118
285.2
Nostoc linckia D0734
162.5
Nostoc muscorum
224.0
Anabaena sp. D0746
453.5
Anabaena cylindrica
136.6
Gloeotrichia sp.
500.0
B
108.1
208.6
Fischerella musicola
D
385.1
329.2
Anabaena sp. D0697
Anabaena sp. D0752
Chlorogloeopsis
Chondrus crispus chloroplast
308.2
Trichodesmium NIBB 1067
C
Microcystis aeruginosa
E. coli

B.


A
B
C
D
Synechococcus PCC 7942
Synechococcus PCC 6301
Oscillatoria animalis
Nostoc ellipsospora
Anabaena PCC 7120
Anabaena PCC 7118
Nostoc linckia D0734
Nostoc muscorum
Anabaena sp. D0746
Anabaena cylindrica
Gloeotrichia sp.
Fischerella musicola
Anabaena sp. D0697
Anabaena sp. D0752
Chlorogloeopsis
Chondrus crispus chloroplast
Trichodesmium NIBB 1067
Microcystis aeruginosa
E. coli

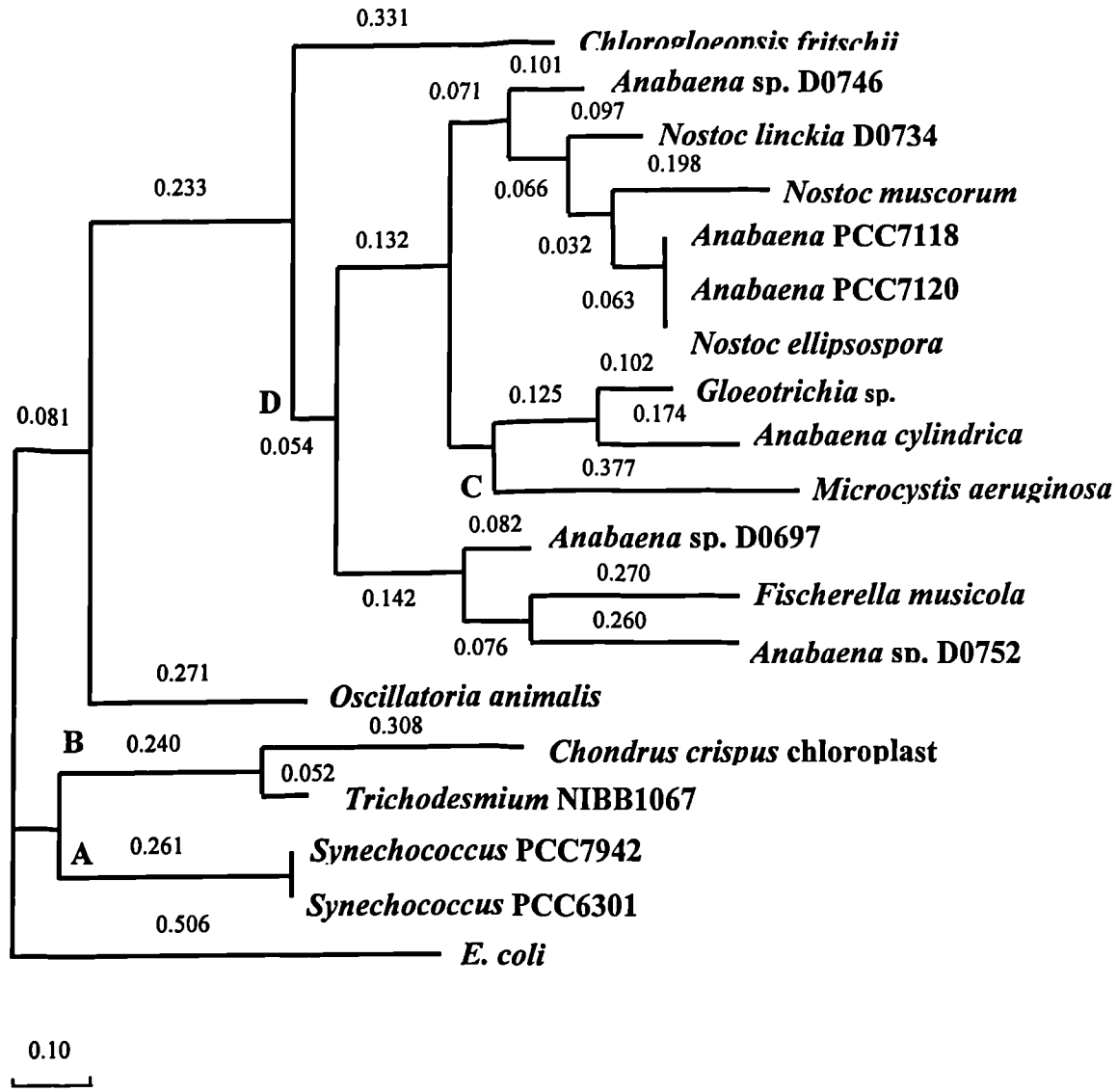


Figure 6.21. Maximum likelihood tree based on SF/SR amplified partial 16S-23S spacer region sequences. Date from 15 various cyanobacterial and two published cyanobacterial sequences of *Synechococcus* PCC 6301 (Tomioka and Sugiura, 1984) and *Trichodesmium* NIBB 1067 (Wilmotte, *et al.*, 1994), *E.coli* (Young and Steitz, 1978) and *Chondrus crispus* (red alga) chloroplast (Leblanc *at al.*, 1995) were used to construct the likelihood tree by Maximum Likelihood method using DNAML version 3.572c program in the software package PHYLIP. Total 751 trees were examined by program.

6.3 Discussion

The results indicate that heterogeneity in both the length and the sequence of the 16S-23S rDNA spacer (or ITS region) is useful for typing cyanobacteria. Clearly PCR analysis of the 16S-23S rDNA spacer region may open the way for a rapid, possibly automatable, general method for cyanobacterial identification and taxonomy. The priming sites for PCR amplification were selected from regions of highly conserved sequences found for example in the 16S rDNA, tDNA and 23S rDNA regions adjacent to the intervening spacer region (see Fig. 6.1). The choice of primers was crucial to the use of spacer variation for species identification. Three primer pairs 1/18, 14/18 and 17/18 (Wilmotte *et al.*, 1993) tested for amplification of the 16S-23S rDNA spacer generated products useful for discriminating between cyanobacterial strains. Multiple DNA bands of different intensities were obtained by using primer pairs 1/18 and 14/18. The intense and reproducible bands have been classified as primary products, the weaker bands as secondary products (see Table 6.1 & Fig. 6.2 a, b). Such classification of product profiles has been reported in the amplification of the 16S-23S rDNA spacer of other bacteria (Jensen *et al.*, 1993). Weak bands were common in the heterocystous strains, which may reflect rDNA heterogeneity for heterocyst differentiation. The pattern of amplified products with primer pair 14/18 enabled clearer distinction between species than that with primer pair 1/18. This suggests that primers closer to the 16S-23S spacer were more discriminatory than those further away. The effect of S1 endonuclease on banding patterns indicated that most secondary products were heteroduplex DNA, containing substantial single-stranded regions and single-stranded DNA (see Table 6.1). These heteroduplexes would migrate more slowly in gels than homoduplexes, because of the single-stranded regions. The heteroduplex structures presumably formed by partial primer sequence matches or cross-hybridisation between similar, but not

identical, spacer amplification products, whilst the formation of single-stranded DNA structures could result from asymmetry in either amplification efficiency or consumption of the primers (Jennies and Stares, 1993). The formation of heteroduplex DNA could be extremely useful in providing additional pattern information by single-strand conformation polymorphisms (SSCP) (Gurtler and Stanisich, 1996), which helps in the differentiation of heterocystous cyanobacteria. Two homoduplex 16S-23S rDNA spacer amplification products were always obtained from heterocystous strains. The variability in products generated for different cyanobacteria with these primer pairs may be due to differential specificity of the primers, variation in copy number and microheterogeneities of the 16S-23S rDNA spacer regions among cyanobacteria.

Only a single band was obtained by using primer pair 17/18, so that species-specific differences were usually detected by digestion of the PCR product with a restriction endonuclease, prior to electrophoresis, and RFLP analysis; or by DNA sequencing. The reasons for the recovery of only a single band with this primer pair may be that: (1) the primers may anneal with only one of the multiple 16S-23S rDNA spacer regions being amplified; (2) only one copy of the 16S-23S rDNA spacer region is actually present per genome of the organism being studied; (3) different PCR products with similar sequence and G+C content generate the same sized band on the gel.

Analysis of length polymorphism and RFLPs of the amplified rDNA spacer using primer pair 17/18 generally permitted discrimination between the cyanobacteria tested to genus and species level. For strains of the same species the same RFLP pattern-type was obtained. Different species and genera had different pattern types, with the exception of *Nostoc ellipsospora, Anabaena* PCC 7120, and *Anabaena* PCC 7118. An identical length and RFLP pattern-type, together with high sequence similarity of the

amplified partial 16S-23S rDNA for these cyanobacteria suggests that they are closely related and may be in the same species group. Caudales and Wells (1992) pointed out that strains of the genus *Nostoc* are closely related to, and often confused with those of the genus *Anabaena*, particularly when morphological characteristics are used for identification. By contrast, on the basis of DNA-DNA hybridisation data and hybridisation patterns with highly repetitive (STRR) DNA sequences the strain *Anabaena* PCC 7120 has been renamed *Nostoc* sp. PCC7120 (Lachance, 1981; Mazel *et al.*, 1990). In addition, the low similarity obtained between the fresh water *Synechococcus* PCC7942 and the marine *Synechococcus* WH7803, on the basis of both length and pattern-type of the spacer, suggests that these strains are not closely related, in line with the hybridisation data (Wood and Townsend, 1990). A comparison of the RFLPs of the 16S rDNA and the 16S-23S rDNA spacer indicates that the 16S-23S rDNA spacer is better for discrimination among the different genera and species of cyanobacteria. This is consistent with the view that the spacer region is generally considered to be under minimal selective pressure (Barry *et al.*, 1991). Its evolutionary rate is ten times greater than that of 16S rDNA (Leblond-Bourger *et al.*, 1996), making it possible to distinguish closely related bacterial species.

Restriction endonuclease sites within the partial cyanobacterial 16S-23S spacer region were identified from the sequencing data (Fig. 6.14). Some of the enzymes cut in the conserved region, so proving useful for RFLP analysis, whilst others cut only in the variable spacer and so were not as useful, because the recognition sequence was not found in all cyanobacteria. The useful enzymes could be placed into three groups: (1) Enzymes that have only a single restriction site in $tDNA^{ala}$: *Dde* I, *Alu* I, *Nsp*B II, *Tth*111 II and *Hae* II. (2) Enzymes that have a single restriction site in $tDNA^{ala}$, and a single restriction site in $tDNA^{ile}$, generating one band of the same size (within $tDNA^{ile}$), and

two or more of different sizes, from all cyanobacteria. They are *Mnl* I, *Fok* I, *Hin*P I or *Hha* I. The restriction profiles using an enzyme from group 1 and group 2 should be similar, except for individual differences in the spacer. (3) Enzymes that have a single restriction site in $tDNA^{ala}$, and in heterocystous strains and *Microcystis aeruginosa* a single restriction site in $tDNA^{ile}$, generating further polymorphisms between non-heterocystous strains (excepting *Microcystis aeruginosa*) and heterocystous strains. They are *Taq* I, *Hinf* I and *Ple* I. Thus for analysis of length polymorphism and RFLPs of the amplified rDNA spacer using primer pair 17/18, the sequencing data indicates that restriction enzymes should be chosen from these groups, preferably two or three enzymes from group 1 and/or 2, and one or two from group 3. Such choices are in line with those that were made for RFLP-PCR (section 6.2.1), on the basis of digesting PCR-amplified products. Four restriction enzymes were chosen from twenty tested: two (*Dde* I and *Alu* I) are in group 1, and two (*Hinf* I and *Taq* I) are in group 3. In this study, the restriction map of the 16S-23S rDNA spacer deduced from the sequencing data thus confirms that the restriction enzymes used for analysis of RFLPs of the amplified spacer using primer pair 17/18 were very suitable.

The trees based on RFLPs (Fig. 6.6) and sequencing data (Fig. 6.15 and 6.16), all show clustering of the unicellular, rod-shaped cyanobacterial strains (genera *Synechococcus*); the non-heterocystous, filamentous strains (genera *Oscillatoria*, *Phormidium* and *Trichodesmium*); the unicellular, spherical-shaped strains (genera *Microcystis*, *Chroococcus*, *Gloeocapsa* and *Synechocystis*) and the heterocystous strains (genera *Anabaena*, *Nostoc*, *Gloeotrichia*, *Fischerella* and *Chlorogloeopsis*), but the trees are not identical. This is largely due to the limitation of the RFLP analysis. To generate RFLPs only a small portion of the rDNA spacer is scanned. Small sequence variations affecting restriction enzyme sites can lead to changes in RFLP profiles for sequences

that are otherwise very similar. This may lead to re-grouping of closely related species. For example, *Anabaena cylindrica*, *Anabaena* PCC 7120 and *Nostoc muscorum* contain very similar sequences between the two tDNAs, but a four base deletion appears at position 119-122 in *Anabaena cylindrica*, which is not found in these other strains (Fig. 6.13). This four base difference produces one band of a different size in RFLP profiles among these strains. As a consequence *Anabaena cylindrica* is positioned further away from *Anabaena* PCC 7120 and *Nostoc muscorum* in the distance tree based on RFLP data than in that based on sequencing data. Thus RFLP pattern type does not necessarily correlate fully with the extent of sequence similarity. RFLP analysis of the amplified rDNA spacer thus provides a useful method for rapid identification and typing of cyanobacteria, but there are drawbacks to its use in establishing taxonomic relationships.The clustering of cyanobacterial strains shown from the trees is consistent with that of the 16S rRNA sequence trees (Wilmotte, 1994).

From sequence alignment of the cyanobacterial 16S-23S spacer regions, the sequences of the unicellular strains, *Synechococcus* PCC6301 and *Synechococcus* PCC7942, were identical. This is consistent with the results of RFLP analysis of genomic DNA from strains PCC6301 and PCC7942, which indicated that the two genomes were identical (Golden *et al.*, 1989; Wood and Townsend, 1990) and suggests that these two strains are possibly of the same species or sub-species. The sequence identity of 100%, over 446 bases, for *Nostoc ellipsospora, Anabaena* PCC 7120, and *Anabaena* PCC 7118 (Table 6.4) indicates that they may be in same species group. This supports the findings of Lachance (1981) that *Anabaena* PCC 7120 and *Anabaena* PCC 7118 were more closely related to *Nostoc* strains than to other *Anabaena* strains, on the basis of DNA-DNA hybridisation data. *Trichodesmium* NIBB 1067 appeared close to *Oscillatoria animalis* on the basis of the sequence trees (Fig. 6.15 and 6.16), but

sequence homologies were not found from the aligned sequences, apart from the conserved regions, described for all the cyanobacteria (Fig. 6.13). The sequence identity of 56.87% indicated that they may not be closely related. From sequences of the 16S rDNA, Wilmotte and colleagues (1994) found that *Trichodesmium* NIBB 1067 and *Oscillatoria* PCC7515 showed 94.9% sequence similarity. Nevertheless, the genotypic heterogeneity exhibited by a number of strains assigned to the genus *Oscillatoria* hinders a taxonomic decision on the separate existence of the genera *Trichodesmium* and *Oscillatoria.*

The high sequence similarities found for heterocystous strains suggest that these strains are closely related. In the `spacer between $tDNA^{ile}$ and $tDNA^{ala}$, high sequence similarity was found for all heterocystous cyanobacteria, excepting *Chlorogloeopsis* (*Chlorogloea*) *fritschii* which lacks this spacer. This species produces '*Gloeocapsa*' like aggregates (Rippka, 1988), which is a characteristic of unicellular spiracle-shaped strains. Furthermore, the unicellular spiracle-shaped strains of *Microcystis* also lacked this spacer, and other unicellular strains exhibited a shorter sequence in this spacer. In the other spacer, gaps are found in the sequences of the unicellular strains when aligned with those of the heterocystous strains. These gaps presumably reflect the genetic distance between the different strains: the more gaps there are in the sequence of one strain relative to another, the greater distance between the strains. As a consequence sequence alignment becomes increasingly more difficult and less meaningful as sequence diversity between strains increases. However, this can be compensated for by provision of sequence information for strains that are intermediate to the unrelated strains. Wilmotte (1994) concluded that no meaningful alignment of the rDNA spacer sequences of their tested strains was possible. However, in this study, useful information on the extent of sequence similarity between selected cyanobacterial strains has been

obtained based on the alignment of the partial 16S-23S rDNA spacer sequences. Furthermore, since the spacer sequence is short, and of variable length (300-750 bp), it is easy to sequence and analyse, and the variation shown for different species can be exploited in the identification and taxonomy of cyanobacteria.

Apart from the two tRNA gene sequences that were conserved in all the cyanobacterial strains, except *Microcystis aeruginosa* (which did not have $tDNA^{ala}$), three other conserved regions were present in all the cyanobacteria tested. Two conserved regions were found in the middle of the variable length spacer between $tDNA^{ala}$ and 23S rDNA. The third conserved region was at the 5' end of the 23S rDNA. By searching the Genbank data base the two conserved regions of about 20bp appeared more specific for cyanobacteria than other bacteria, making them candidates for cyano-specific primers. The pair of primers, SF and SR, developed from these regions, successfully amplified cyanobacterial DNA generating a product of between 70bp and 350bp, but did not amplify DNA from the green alga or *E.coli*. In order to identify isolates, the SF/SR amplified product could be sequenced and data correlated with parsimony consensus and maximum likelihood trees (Fig. 6.20 and 6.21); such trees were found to be similar to those based on the 17/18 amplified sequences (Fig. 6.15 and 6.16). Given the specificity and other properties of the SF/SR primers, the relatively short size of the SF/SR amplified product and the reliability of automated sequencing (as demonstrated here, see Table 6.3), a rapid PCR-based approach to identifying cyanobacteria directly from DNA recovered from environmental samples could be developed (see section 8.2). It should be noted that for direct automated sequencing of PCR products a high degree of specificity of DNA polymerase must be used for amplification of template DNA to reduce errors caused by normal *Taq* polymerase. An example of such a polymerase is DyNAZyme II, which was used in this study.

Thus PCR amplification and RFLP analysis of the 16S-23S rDNA spacer provide a potentially useful approach to the identification of cyanobacteria. With more data it should be possible to generate a database of RFLP-patterns, in order to identify unknown isolates by the matching of pattern-type and determining similarity to laboratory strains from phenetic analysis. Furthermore, it is possible to detect more than one cyanobacterial strain in a mixed culture from the variability in length and RFLP pattern-type of the rDNA spacer. In addition, sequence variation within the spacer shows promise for use in the identification and phylogeny of cyanobacteria. Moreover, from sequence alignment cyano-specific primer sequences have been designed, opening up possibilities for their use in the rapid identification of cyanobacteria directly from water samples. Exploitation of spacer-region variation should thus provide a rapid and reliable method of identifying cyanobacteria.

Chapter 7

Morphological and genetic methods in identification and taxonomy of cyanobacteria

7.1 Introduction

Cyanobacteria taxonomy has been approached in different ways, all with one major goal: defining those phenotypic properties which are taxonomically useful. Because the phenotype is the product of genetic and environmental factors, the stability of certain characteristics is a function of the properties of the environment. Cyanobacteria exhibit a wide range of specific environmental requirements. Conversely, their growth and metabolic activities leave specific impacts on the environment they occupy. The diversity in form and function of cyanobacteria appears to be the result of a long evolutionary process. Fossils similar to cyanobacteria have been reported from about 3.5 billion years (Ga) old sedimentary rocks (Schopf and Walter, 1983), while diversified assemblages of taxa that are morphologically comparable to modern cyanobacterial genera and species existed in rocks 1.0 to 2.0 Ga of age (Knoll and Golubic, 1991). The proper ages of some cyanobacterial genera from fossil evidence are listed in Figure 7.1 (Schopf, *et al.* 1987; Knoll and Golubic, 1991; Woese, 1994).

Diversity of cyanobacteria is expressed by their morphological, biochemical, and physiological properties, which enable them to settle and persist in a wide range of habitats. As in all other organism groups, various cyanobacterial properties are phenotypic expressions of particular genotypes. Traditionally, the task of systematic practice was to analyse, evaluate, and order phenotypes, and to provide a practical frame of reference for purposes of comparing, identifying and quantifying organisms. Implicit in this effort was the ambition to re-construct the phylogenetic history of the observed diversity and, in the process, to assess the relatedness between organisms. This indirect approach met several objective limitations. One of them is, for example, the difficulty to

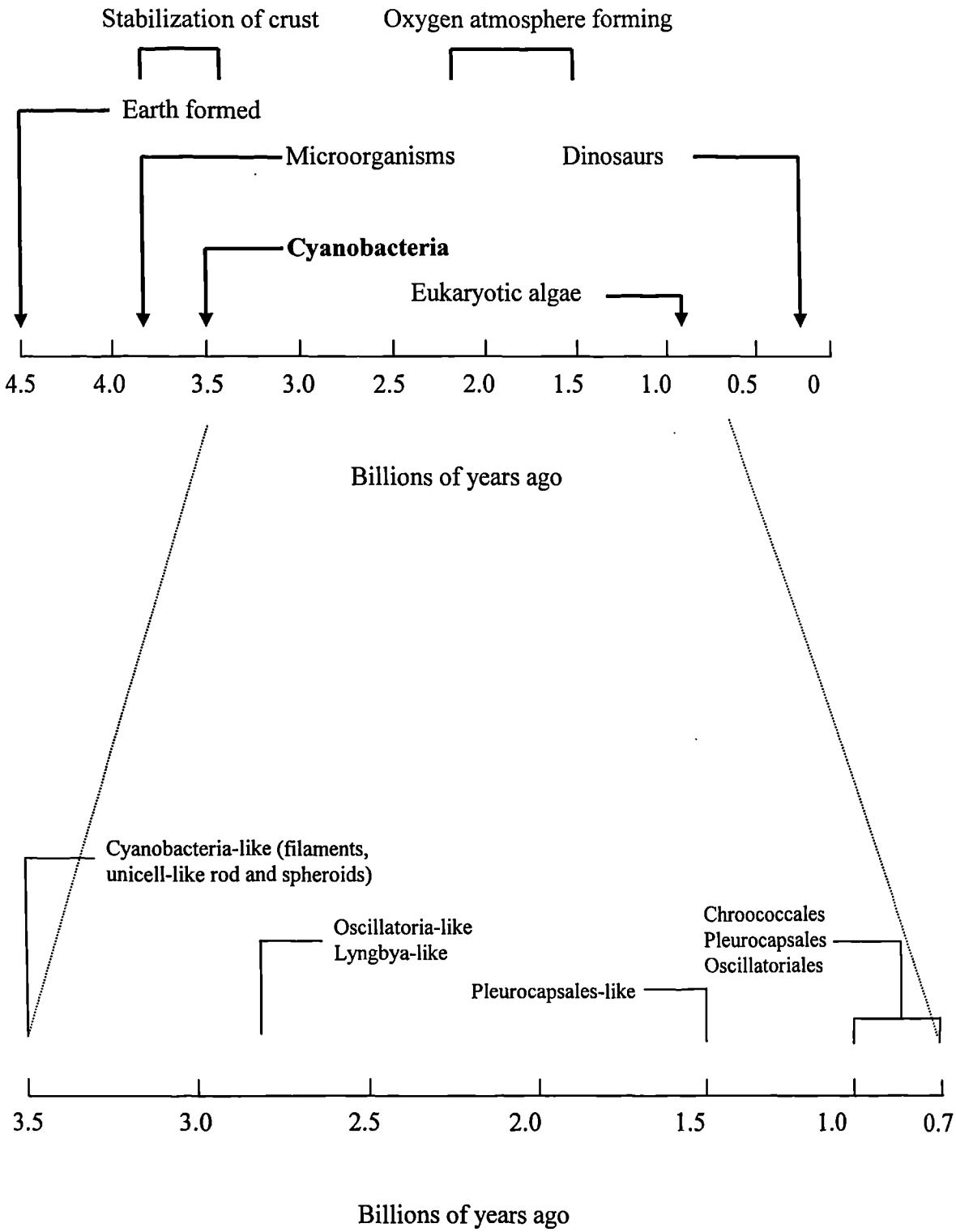


Figure. 7.1 Time line for the planet Earth and the age of cyanobacteria from fossil evidence (Schopf, *et al.* 1987; Knoll and Golubic, 1991; Woese, 1994).

recognise and separate inherited traits from the environmentally induced ones. Another one is the difficulty to decide whether similar phenotypes reflect a close taxonomic relation (homology), or are the result of convergent evolution (analogy) (Wilmotte and Golubic, 1991).

As a result of the morphological simplicity of most prokaryotes, their classification was based largely on physiological properties, as expressed in pure cultures. Only when applied with relatively large and morphologically complex prokaryotes, such as cyanobacteria could a systematic practice rely on morphological criteria. Similar ecological functions of cyanobacteria and eukaryotic algae have traditionally prompted a similar systematic treatment of their natural populations (Geitler, 1925, 1932). While field studies relied mostly on morphological analyses of natural populations, laboratory studies concentrated on culture characterisations. Since the early 1970's the latter approach was applied to those cyanobacteria that were transferred and maintained in culture collections (Rippka et al., 1979; Rippka, 1988), although the differences between the two taxonomic treatments have yet to be reconciled. Both treatments, however, rely largely on characterisation of phenotypes. The limitations discussed above apply to all studies of phenotypic expression, irrespective of whether natural populations or pure cultures are studied, and whether morphological or physiological criteria are applied. However, morphology may change depending on environmental conditions (Evans *et al.*, 1976) and the diversity of strains within a culture may be suppressed by selective culturing conditions (Doers and Parker, 1988). Photosynthetic pigment content, lipid composition, differentiated cell structures and other characters may also be subject to change because of the variable expression of cyanobacterial gene products in culture (Kenyon *et al.*, 1972; Klein *et al.*, 1973). The

limitations of morphological and physiological approaches have prompted the development of molecular biological techniques for identification of cyanobacteria.

Edelman, Stanier, Leach and Herdman (1973) suggested that increasing knowledge concerning genotypic relationships would lead to a more satisfactory classification of the cyanobacteria. The idea that evolutionary changes were recorded within the molecular architecture of organisms, and that they could be studied by comparing the sequences of marcromolecular elements, such as nucleic acid bases and amino acids, was proposed 30 years ago by Zuckerkandl and Pauling (1965). In the years that followed, molecular biology and genetics achieved significant methodological advancements and break-throughs. These methods opened the way towards a more direct evaluation of genetic information. However it soon became evident that none of them proved to be the panacea to modern taxonomy, and that each methodological improvement carried a price in terms of newly introduced biases and artefacts. In the use of rRNA nucleotide sequences to study animal phylogeny, Nielsen (1989) warned: "It is important to point out that the molecular data do not provide unequivocal phylogenetic trees and must be treated with just as much criticism, care, and tact as the traditional morphological characters". A certain practical experience with the use of rRNA sequences (whether partial or complete) for the taxonomy of different kinds of organisms has meanwhile been accumulated, and is reflected in the current literature. Thus, it becomes possible to evaluate the advantages and shortcomings of these new methods and to define more realistically what can be expected from them for the cyanobacteria in the future.

In this study, several rapid molecular methods (developed in chapters 5 and 6) were compared with the morphological method, and comment on conflicts and

consensus between morphological data and the positions of the cyanobacterial strains in the genetic data of this study. Comparisons were also made between the molecular data in this study and the 16S rRNA sequencing phylogenetic tree (Wilmotte, 1994).

7.2 Results

An analysis of the 16S-23S rDNA spacer region of cyanobacterial isolates was carried out by using both RFLP-PCR and DNA sequencing. RFLP profiles of the amplified 16S-23S rDNA spacer were generated by using restriction enzyme *Hin*fI, *Dde*I, *Alu*I and *Taq*I which were used in section 6.2.1. The RFLP pattern and pattern-type of isolates are shown in Figure 7.2 and Table 7.1. They showed potentially useful discrimination among the different genera and species for isolates. A dendrogram (Fig.7.3) was constructed on the basis of pair-wise genetic distances, using the combined RFLP data from the four restriction enzymes for all the laboratory strains and isolates used. In the tree (Fig. 7.3), the cyanobacteria were separated into seven clusters: (A) *Synechococcus* strains; (B) *Pseudanabaena* strains; (C) *Leptolyngbya* strains; (D) *Nostoc palaeo*; (E) *Oscillatoria* and *Phormidium* strains; (F) *Chroococcidiopsis* LJM013, *Chroococcus prescottii*, *Gloeocapsa* sp., *Microcystis aeruginosa,* and *Synechocystis* PCC6803; (G) *Aphanizomenon gracile*, *Chlorogloeopsis fritschii*, *Fischerella musicola*, *Gloeotrichia* sp., *Tolypothrix distorta* and all *Nostoc* and *Anabaena* strains.

Figure 7.4 shows the consensus tree constructed by using DNA parsimony algorithm with 500 bootstrapping runs performed on the sequencing data from the partial 16S-23S spacer region (between primer 17 and 18) of 20 laboratory cyanobacterial strain and 13 isolates, together with *Arthrospira* sp. PCC7345 (Accession

Figure 7.2. Diagramatic representation of RFLP patterns of amplified rDNA products (using primers 17 and 18) **from selected cyanobacterial isolates and laboratory strains with the restriction enzymes *Hin*fI, *Dde*I, *Alu*I and *Taq*I.** The numbers below the tracks correspond to the RFLP patterns in Table 6.2 and 7.1.

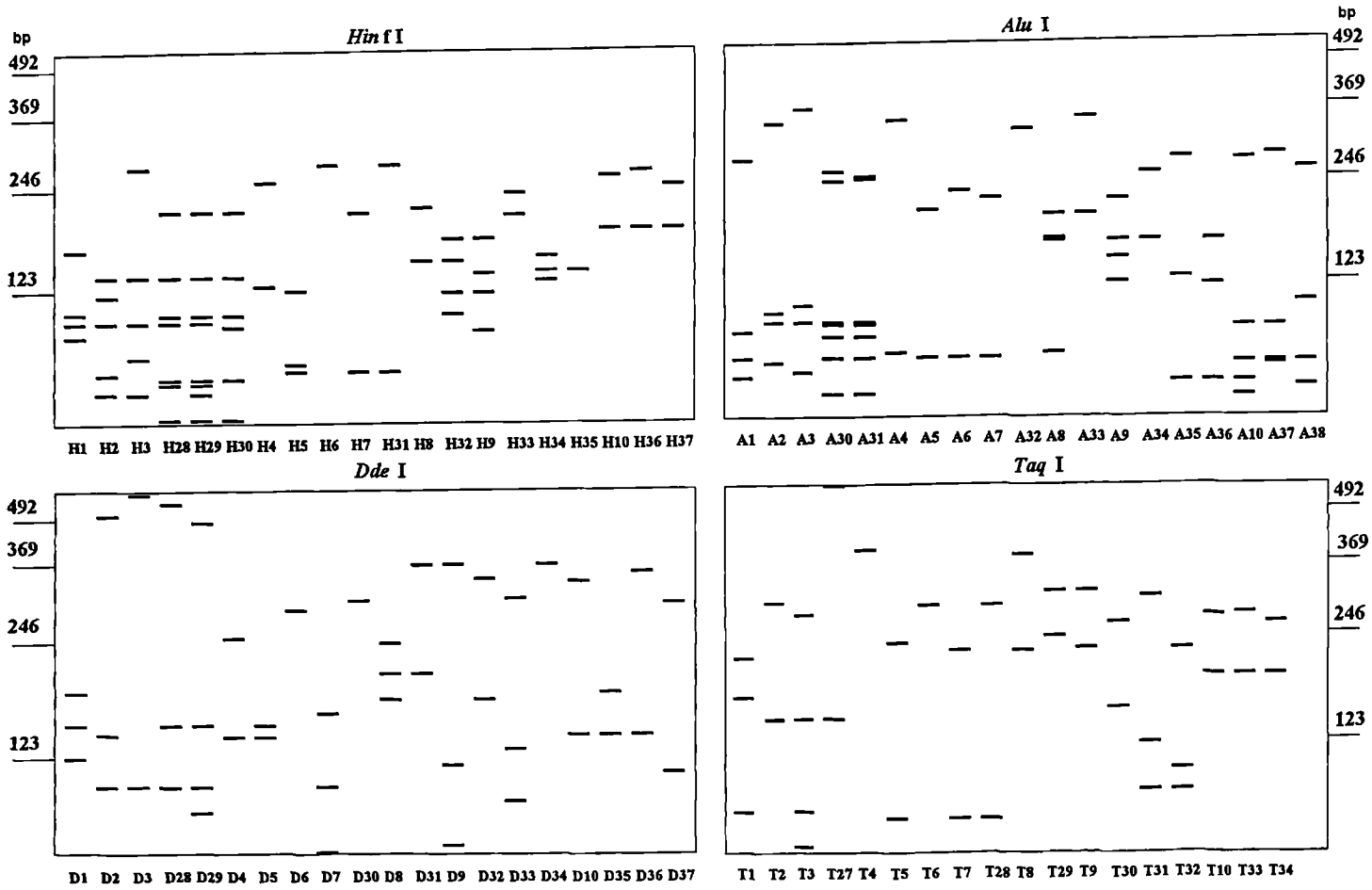

bp
492
369
246
123
Hin f I
H1 H2 H3 H28 H29 H30 H4 H5 H6 H7 H31 H8 H32 H9 H33 H34 H35 H10 H36 H37
Alu I
bp
492
369
246
123
A1 A2 A3 A30 A31 A4 A5 A6 A7 A32 A8 A33 A9 A34 A35 A36 A10 A37 A38
Dde I
492
369
246
123
D1 D2 D3 D28 D29 D4 D5 D6 D7 D30 D8 D31 D9 D32 D33 D34 D10 D35 D36 D37
Taq I
492
369
246
123
T1 T2 T3 T27 T4 T5 T6 T7 T28 T8 T29 T9 T30 T31 T32 T10 T33 T34

Table 7.1. RFLP patterns and pattern-types obtained for cyanobacterial isolates.

Isolates[a]	Pattern-type[b]	*Hinf* I	*Dde* I	*Alu* I	*Taq* I
Unicellular					
Chroococcidiopsis LJM013	AD	H31	D30	A32	T28
Synechococcus LJM002	AE	H30	D29	A31	T27
Synechococcus LJM003	AF	H29	D28	A30	T27
Synechococcus LJM004	AG	H28	D28	A30	T27
Filamentous non-heterocystous					
Leptolyngbya LJM 012	AH	H35	D34	A36	T32
Leptolyngbya LJM 014	AI	H34	D33	A35	T31
Phormidium LJM 017	AK	H32	D31	A33	T29
Pseudanabaena JM 001	J	H10	D10	A10	T10
Pseudanabaena JM 005	J	H10	D10	A10	T10
Pseudanabaena JM 006	J	H10	D10	A10	T10
Pseudanabaena JM 007	J	H10	D10	A10	T10
Pseudanabaena JM 009	AL	H10	D35	A10	T10
Pseudanabaena JM 011	AM	H36	D36	A37	T33
Pseudanabaena JM 016	AL	H10	D35	A10	T10
Pseudanabaena JM 019	AN	H37	D37	A38	T34

a, Isolates identified to genus level on basis of morphological criteria (see section 3.2.4 and Table 3.2).

b, Details of the patterns (H28-H37, D28-D37, A30-A38 and T27-T34) for amplified (primer pair 17/18) rDNA digests are given in Fig. 7.2. Pattern-type derives from the pattern combination with the four test restriction enzymes.

Figure 7.3. Dendrogram indicating genetic distance among cyanobacterial laboratory strains and isolates. The genetic distance from the node, based on the RFLP profiles of 48 strains, is indicated above each branch. The clusters A, B, C, D, E, and F are designated according to branch forming and genetic distance. The less closely related the strains are the greater the genetic distance.

Cyanobacterial isolates are shown in bold.

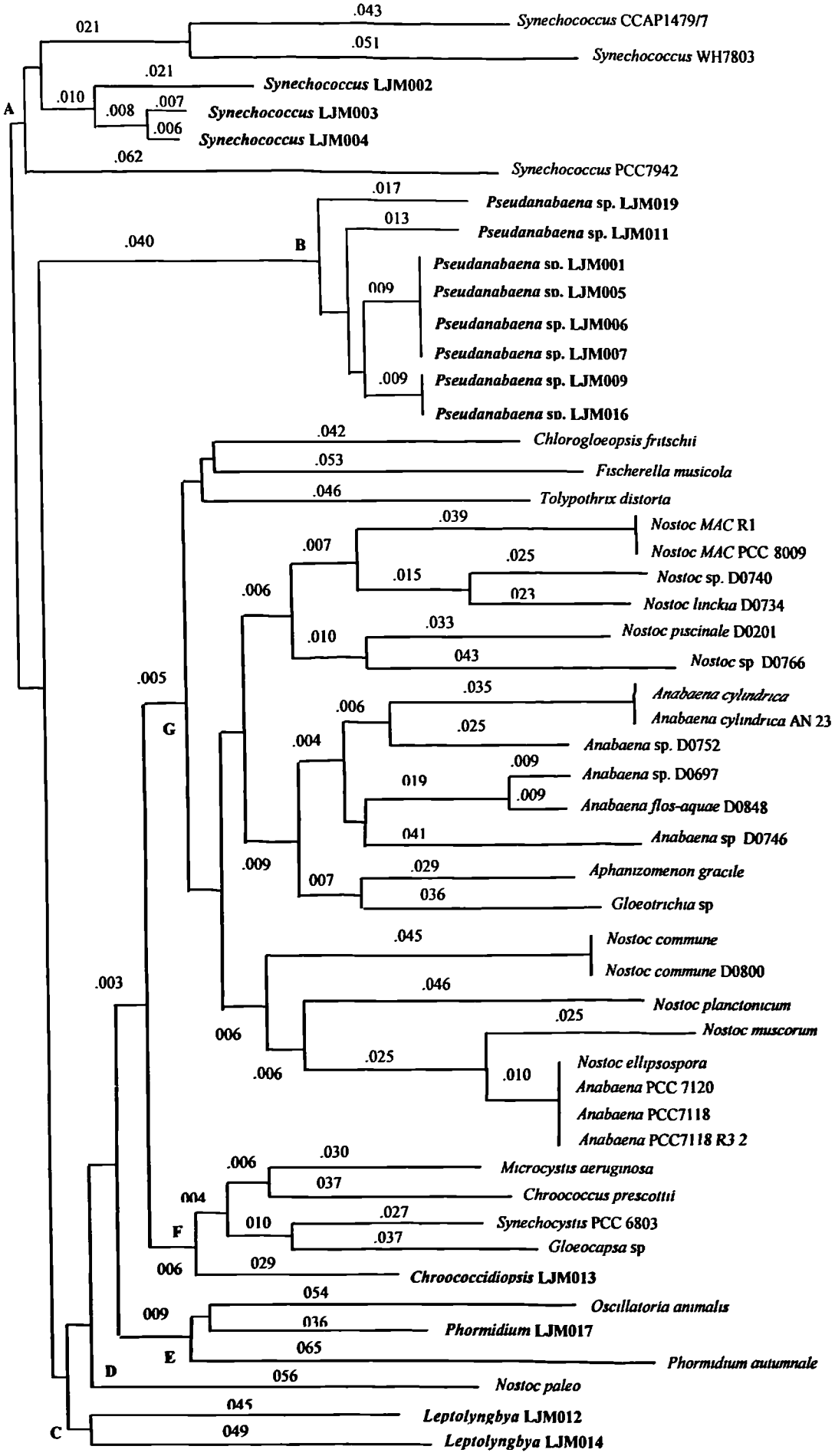
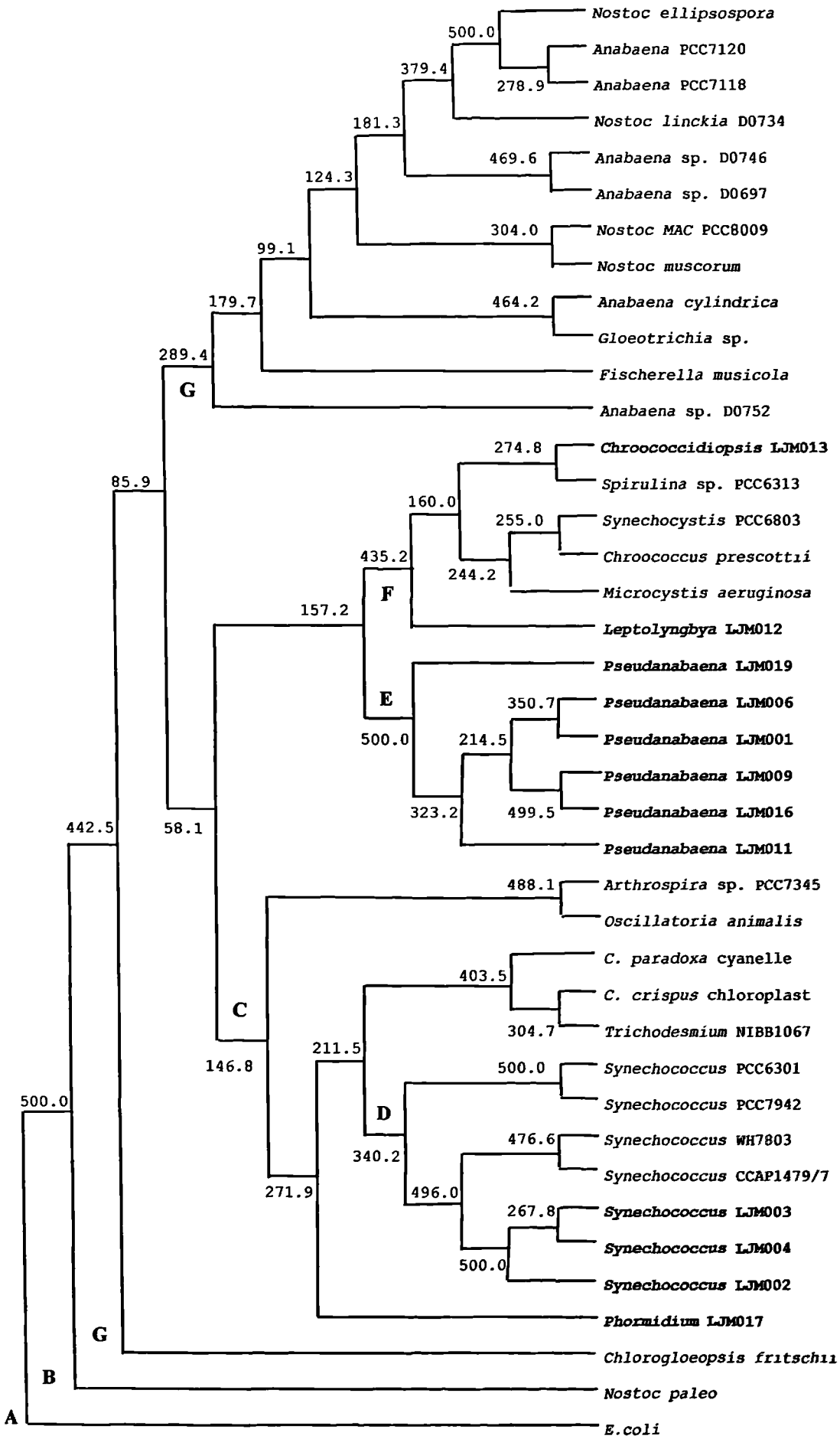

.043
Synechococcus CCAP1479/7
021
.051
Synechococcus WH7803
.021
Synechococcus LJM002
.010
.008
.007
Synechococcus LJM003
A
.006
Synechococcus LJM004
.062
Synechococcus PCC7942
.017
Pseudanabaena sp. LJM019
013
Pseudanabaena sp. LJM011
.040
B
Pseudanabaena sp. LJM001
009
Pseudanabaena sp. LJM005
Pseudanabaena sp. LJM006
Pseudanabaena sp. LJM007
.009
Pseudanabaena sp. LJM009
Pseudanabaena sp. LJM016
.042
Chlorogloeopsis fritschii
.053
Fischerella musicola
.046
Tolypothrix distorta
.039
Nostoc MAC R1
.007
Nostoc MAC PCC 8009
.025
.015
Nostoc sp. D0740
.006
023
Nostoc linckia D0734
.033
.010
Nostoc piscinale D0201
043
Nostoc sp D0766
.005
.035
Anabaena cylindrica
.006
Anabaena cylindrica AN 23
G
.025
.004
Anabaena sp. D0752
.009
Anabaena sp. D0697
019
.009
Anabaena flos-aquae D0848
041
Anabaena sp D0746
.009
.029
Aphanizomenon gracile
007
036
Gloeotrichia sp
.045
Nostoc commune
Nostoc commune D0800
.003
.046
Nostoc planctonicum
.025
Nostoc muscorum
006
.025
Nostoc ellipsospora
.006
.010
Anabaena PCC 7120
Anabaena PCC7118
Anabaena PCC7118 R3 2
.006
.030
Microcystis aeruginosa
037
Chroococcus prescottii
004
.027
010
Synechocystis PCC 6803
F
.037
Gloeocapsa sp
006
029
Chroococcidiopsis LJM013
054
Oscillatoria animalis
009
036
Phormidium LJM017
E
065
Phormidium autumnale
D
056
Nostoc paleo
045
Leptolyngbya LJM012
C
049
Leptolyngbya LJM014

Figure 7.4. Consensus trees derived from parsimony method based on partial 16S-23S spacer region sequence analysis. Sequencing data from 21 laboratory strains and 12 isolates (this study), together with *Arthrospira* sp. PCC7345 (Nelissen *et al.*, 1994), Spirulina sp. PCC6313 (Nelissen *et al.*, 1994), *Synechococcus* PCC6301 (Tomioka and Sugiura, 1984), *Trichodesmium* NIBB1067 (Wilmotte, 1994), *E. coli* (Young and Steitz, 1978), *Cyanophora paradoxa* cyanelle (Janssen *et al.*, 1987) and *Chondrus crispus* (red alga) chloroplast (Leblanc *et al.*, 1995) from the Genbank were used to constract the tree. The rooted tree was generated by the parsimony method and 500 bootstrapping runs were performed, using the DNAPARS, SEQBOOT and CONSENSE program in the software package PHYLIP. The numbers at the forks indicate the number of times the group consisting of the species which are to the right of that fork occurred among the trees, out of 500 trees. Clusters A, B, C, D, E, F, G are indicated. The cyanobacterial isolates are shown in bold.


Nostoc ellipsospora
Anabaena PCC7120
Anabaena PCC7118
Nostoc linckia D0734
Anabaena sp. D0746
Anabaena sp. D0697
Nostoc MAC PCC8009
Nostoc muscorum
Anabaena cylindrica
Gloeotrichia sp.
Fischerella musicola
Anabaena sp. D0752
Chroococcidiopsis LJM013
Spirulina sp. PCC6313
Synechocystis PCC6803
Chroococcus prescottii
Microcystis aeruginosa
Leptolyngbya LJM012
Pseudanabaena LJM019
Pseudanabaena LJM006
Pseudanabaena LJM001
Pseudanabaena LJM009
Pseudanabaena LJM016
Pseudanabaena LJM011
Arthrospira sp. PCC7345
Oscillatoria animalis
C. paradoxa cyanelle
C. crispus chloroplast
Trichodesmium NIBB1067
Synechococcus PCC6301
Synechococcus PCC7942
Synechococcus WH7803
Synechococcus CCAP1479/7
Synechococcus LJM003
Synechococcus LJM004
Synechococcus LJM002
Phormidium LJM017
Chlorogloeopsis fritschii
Nostoc paleo
E.coli
500.0
379.4
278.9
181.3
469.6
124.3
304.0
99.1
179.7
464.2
289.4
G
274.8
85.9
160.0
255.0
435.2
244.2
F
157.2
E
350.7
500.0
214.5
323.2
499.5
58.1
442.5
488.1
403.5
C
304.7
211.5
146.8
500.0
D
476.6
340.2
271.9
496.0
267.8
500.0
G
B
A

No. X75044), *Spirulina* sp. PCC6313 (Accession No. X75045), *Synechococcus* PCC6301 (Accession No. X00346), *Trichodesmium* NIBB1067 (Accession No. X72871), *E. coli* (Accession No. J01702), *Cyanophora paradoxa* cyanelle (Accession No. M19493) and *Chondrus crispus* (red alga) chloroplast (Accession No. Z29521). The majority of branches show bootstrapping values of 50% or more indicating that the trees are quite robust. Figure 7.5 and Figure 7.6 shows the DNA distance trees constructed by using the Maximum Likelihood method and the Neighbour joining method based on the same sequences as in Figure 7.4. The DNA parsimony tree (Fig. 7.4) and the DNA distance tree (Fig. 7.5) are identical. The DNA distance tree with the Neighbour joining method (Fig. 7.6) is largely congruent with other two trees (Fig. 7.4 and 7.5), except the group of *Synechococcus* and group of *Pseudanabaena* exchanged position, and *Chlorogloeopsis fritschii* returned into the heterocystous strain cluster. In the three trees (Fig. 7.4, 7.5 and 7.6), the strains used were separated into seven clusters: (A) *E. coli*; (B) *Nostoc palaeo*; (C) *Arthrospira* sp. PCC7345, *Oscillatoria animalis*, *Phormidium* LJM017 and *Trichodesmium* NIBB 1067 (together with *Chondrus crispus* chloroplast and *Cyanophora paradoxa* cyanelle); (D) *Synechococcus* strains; (E) *Pseudanabaena* strains; (F) *Chroococcidiopsis* LJM013, *Chroococcus prescottii*, *Synechocystis* PCC6803, *Microcystis aeruginosa, Spirulina* sp. PCC6313 and *Leptolyngbya* LJM012; (G) *Chlorogloeopsis fritschii*, *Fischerella musicola*, *Gloeoetrichia* sp. and all *Nostoc* and *Anabaena* strains. All the clusters of cyanobacteria shows great distance to cluster A--*E.coli*. Observations of the branches, presented below, gives interesting information on the genotypic relationships among the cyanobacteria within each cluster and morphological features are presented below:

1. Branches A from RFLP dendrogram and branches D from sequences trees (*Synechococcus* cluster)

Figure 7.5. Maximum likelihood tree based on partial 16S-23S spacer region sequence analysis. Sequencing data from 21 laboratory strains and 12 isolates (this study) together with *Arthrospira* sp. PCC7345 (Nelissen *et al.*, 1994), *Spirulina* sp. PCC6313 (Nelissen *et al.*, 1994), *Synechococcus* PCC6301 (Tomioka and Sugiura, 1984), *Trichodesmium* NIBB1067 (Wilmotte, 1994), *E. coli* (Young and Steitz, 1978), *Cyanophora paradoxa* cyanelle (Janssen *et al.*, 1987) and *Chondrus crispus* (red alga) chloroplast (Leblanc *et al.*, 1995) from the Genbank, were used to construct the tree by the Maximum Likelihood method using DNAML version 3.572c program in the software package PHYLIP. Total 4168 trees were examined by program. Clusters A, B, C, D, E, F, G are indicated. The cyanobacterial isolates are shown in bold.

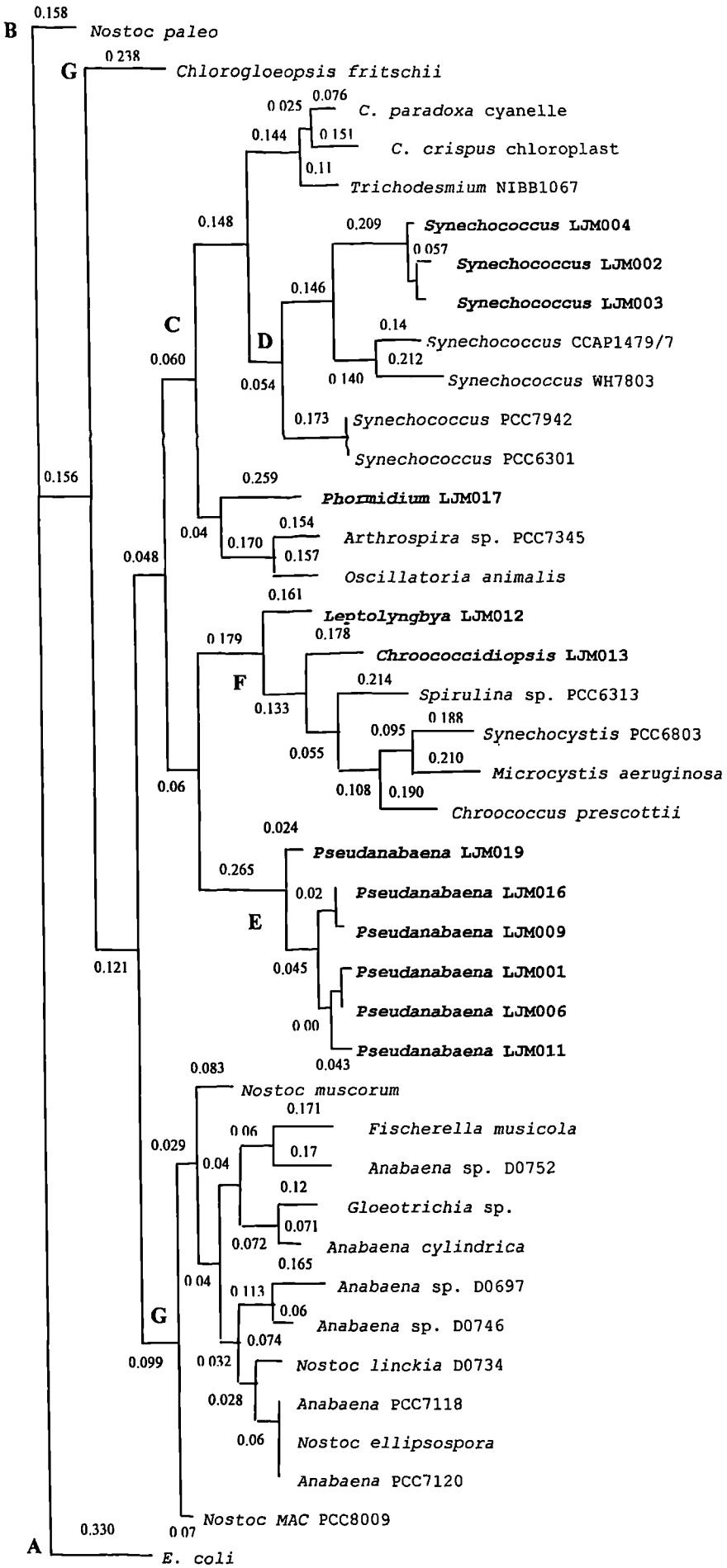
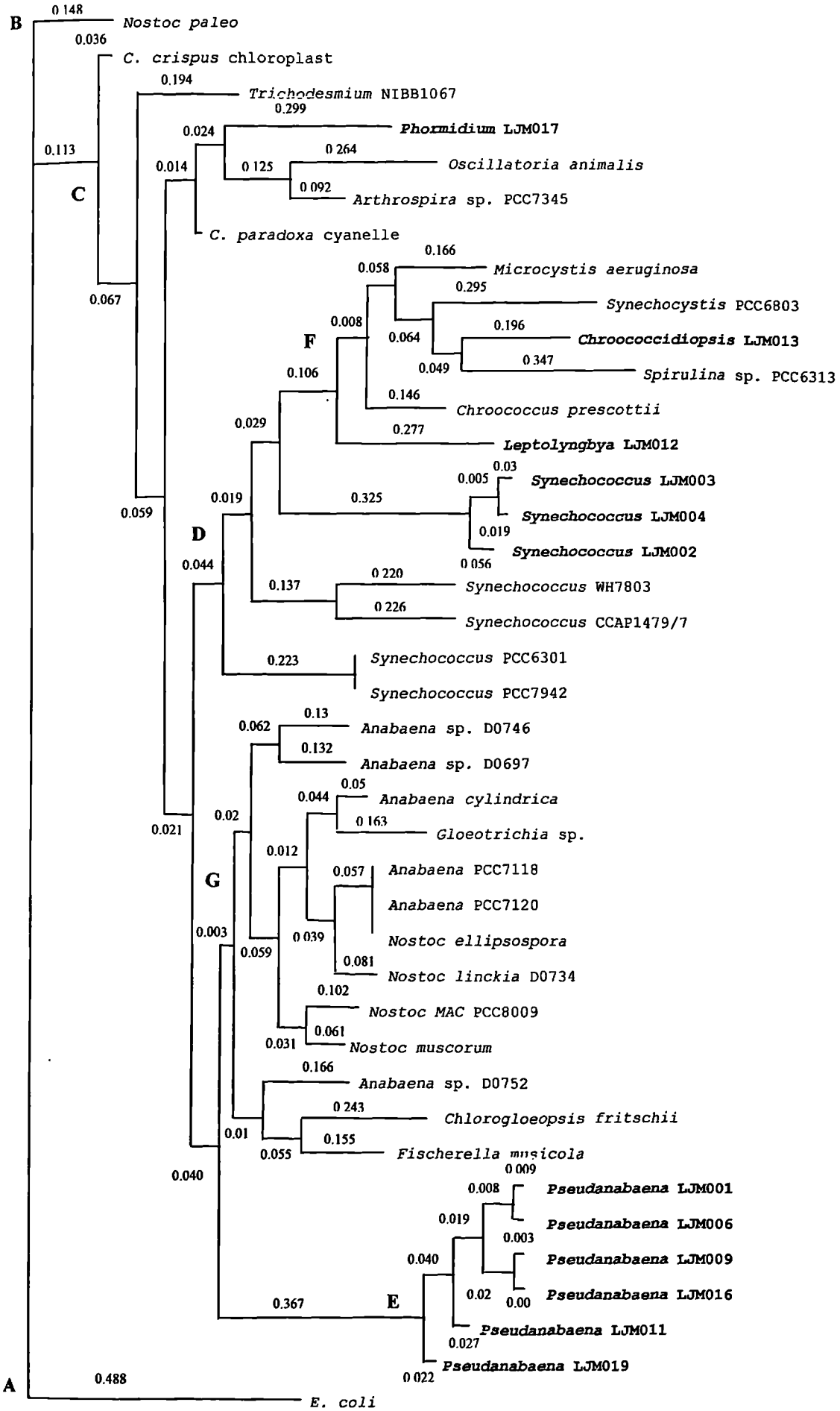

B
0.158
Nostoc paleo
G
0.238
Chlorogloeopsis fritschii
0.025
0.076
C. paradoxa cyanelle
0.144
0.151
C. crispus chloroplast
0.11
Trichodesmium NIBB1067
0.148
0.209
Synechococcus LJM004
0.057
Synechococcus LJM002
0.146
Synechococcus LJM003
C
D
0.14
Synechococcus CCAP1479/7
0.060
0.212
0.140
Synechococcus WH7803
0.054
0.173
Synechococcus PCC7942
Synechococcus PCC6301
0.156
0.259
Phormidium LJM017
0.04
0.154
0.170
Arthrospira sp. PCC7345
0.048
0.157
Oscillatoria animalis
0.161
Leptolyngbya LJM012
0.179
0.178
Chroococcidiopsis LJM013
F
0.214
Spirulina sp. PCC6313
0.133
0.188
0.095
Synechocystis PCC6803
0.055
0.210
Microcystis aeruginosa
0.06
0.108
0.190
Chroococcus prescottii
0.024
Pseudanabaena LJM019
0.265
0.02
Pseudanabaena LJM016
E
Pseudanabaena LJM009
0.121
0.045
Pseudanabaena LJM001
Pseudanabaena LJM006
0.00
Pseudanabaena LJM011
0.043
0.083
Nostoc muscorum
0.171
0.06
Fischerella musicola
0.029
0.17
Anabaena sp. D0752
0.04
0.12
Gloeotrichia sp.
0.071
0.072
Anabaena cylindrica
0.165
0.04
0.113
Anabaena sp. D0697
0.06
G
Anabaena sp. D0746
0.074
0.099
0.032
Nostoc linckia D0734
0.028
Anabaena PCC7118
0.06
Nostoc ellipsospora
Anabaena PCC7120
Nostoc MAC PCC8009
0.330
0.07
A
E. coli

Figure 7.6. Distance tree derived from neighbour-joining method based on partial 16S-23S spacer region sequence analysis. Sequencing data from 21 laboratory strains and 12 isolates (this study) together with *Arthrospira* sp. PCC7345 (Nelissen *et al.*, 1994), *Spirulina* sp. PCC6313 (Nelissen *et al.*, 1994), *Synechococcus* PCC6301 (Tomioka and Sugiura, 1984), *Trichodesmium* NIBB1067 (Wilmotte, 1994), *E. coli* (Young and Steitz, 1978), *Cyanophora paradoxa* cyanelle (Janssen *et al.*, 1987) and *Chondrus crispus* (red alga) chloroplast (Leblanc *et al.*, 1995) from the Genbank, were used to construct the tree by Neighbour-joining method using Neighbour-joining/UPGMA version 3.572c program in the software package PHYLIP. Clusters A, B, C, D, E, F, G are indicated. The cyanobacterial isolates are shown in bold.

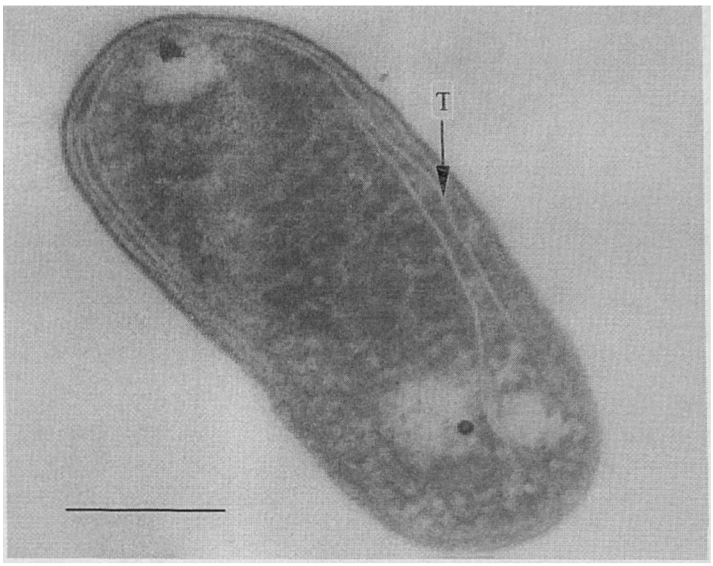

B
0.148
Nostoc paleo
0.036
C. crispus chloroplast
0.194
Trichodesmium NIBB1067
0.299
Phormidium LJM017
0.024
0.113
0 264
0.014
0 125
Oscillatoria animalis
C
0 092
Arthrospira sp. PCC7345
C. paradoxa cyanelle
0.166
0.058
Microcystis aeruginosa
0.295
0.067
Synechocystis PCC6803
0.008
0.196
F
0.064
Chroococcidiopsis LJM013
0.049
0 347
Spirulina sp. PCC6313
0.106
0.146
Chroococcus prescottii
0.029
0.277
Leptolyngbya LJM012
0.03
0.005
Synechococcus LJM003
0.019
0.325
0.059
Synechococcus LJM004
D
0.019
Synechococcus LJM002
0.044
0 056
0 220
0.137
Synechococcus WH7803
0 226
Synechococcus CCAP1479/7
0.223
Synechococcus PCC6301
Synechococcus PCC7942
0.13
0.062
Anabaena sp. D0746
0.132
Anabaena sp. D0697
0.05
0.044
Anabaena cylindrica
0.02
0 163
0.021
Gloeotrichia sp.
0.012
0.057
Anabaena PCC7118
G
Anabaena PCC7120
0.003
0 039
Nostoc ellipsospora
0.059
0.081
Nostoc linckia D0734
0.102
Nostoc MAC PCC8009
0.061
0.031
Nostoc muscorum
0.166
Anabaena sp. D0752
0 243
Chlorogloeopsis fritschii
0.01
0.155
0.055
Fischerella musicola
0.040
0 009
0.008
Pseudanabaena LJM001
0.019
Pseudanabaena LJM006
0.003
0.040
Pseudanabaena LJM009
0.02
0.00
Pseudanabaena LJM016
0.367
E
Pseudanabaena LJM011
0.027
Pseudanabaena LJM019
0 022
A
0.488
E. coli

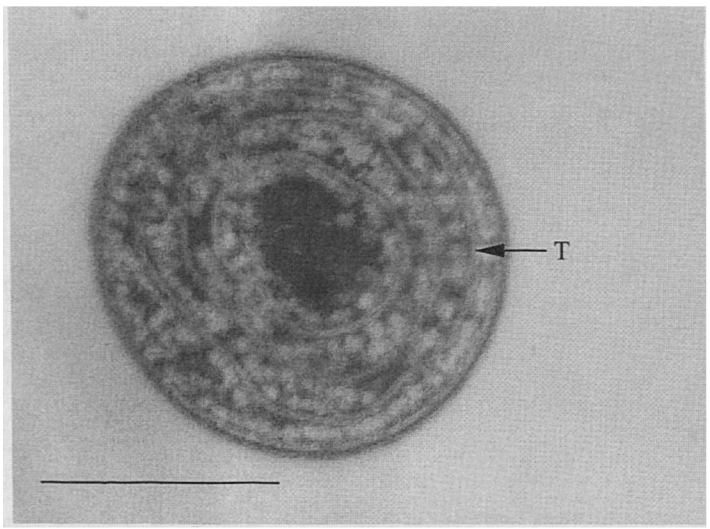
The trees of Figures. 7.3, 7.4, 7.5 and 7.6 show that all six *Synechococcus* strains form three sub-clusters, with a distance between each sub-cluster (Figs. 7.3, 7.5 and 7.6). Fresh water *Synechococcus* strains PCC6301 and PCC7942, which contain identical sequences of the rDNA spacer, were indicated at same position in one sub-cluster. The other three fresh water *Synechococcus* strains LJM002, 003 and 004 form a tight sub-cluster, the DNA distance between strain LJM003 and LJM004 is less than from any one of them to LJM002. The strain *Synechococcus* CCAP1479/7 and WH7803 form another sub-cluster, but the distance between the two strains is large.

All these strains are simple, rod-shaped, unicellular forms, with distinguished by the division of elongated to cylindrical cells by binary fission in one plane, the presence of thylakoids, and the absence of a thick sheath. The differences between these strains relate to cell size, pigment content and habitat. *Synechococcus* strains PCC7942, LJM002, LJM003, LJM004 and LJM020 were isolated from fresh water, and can only grow in low salt tolerance media. *Synechococcus* CCAP1479/7 isolated from brackish water, can grow in both freshwater and sea water media. *Synechococcus* WH7803 is a marine strain, and can only grown in sea water media. *Synechococcus* WH7803 is different from other strains used, only containing PE (the culture colour is pink); whilst the other strains do not contain PE. Figure 7.7 shows TEM microphotographs of strains *Synechococcus* CCAP1479/7 and WH7803. Morphological change depending on selective culturing conditions was found with *Synechococcus* PC7942. Figure 7.8 C shows the microscopy photographs of strain *Synechococcus* PCC7942 forming long 'snake' shaped cells under high light intensity or lower CO_2 supplement than normal conditions. Figure 7.8 B shows that the 'snake' is a single unhealthy cell, compared with a normal cell (Figure 7.8 A). The other *Synechococcus* strains only showed slight changes when culturing conditions were changed.

Figure 7.7. Transmission electron micrograph of a thin section of A, *Synechococcus* CCAP1479/7 and B, *Synechococcus* WH 7803. bar, 0.5 μm. T, thylakoid.

A.





B.

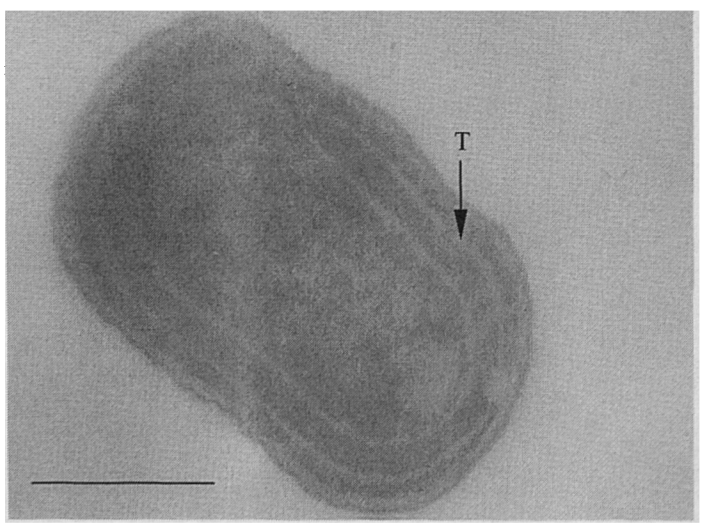
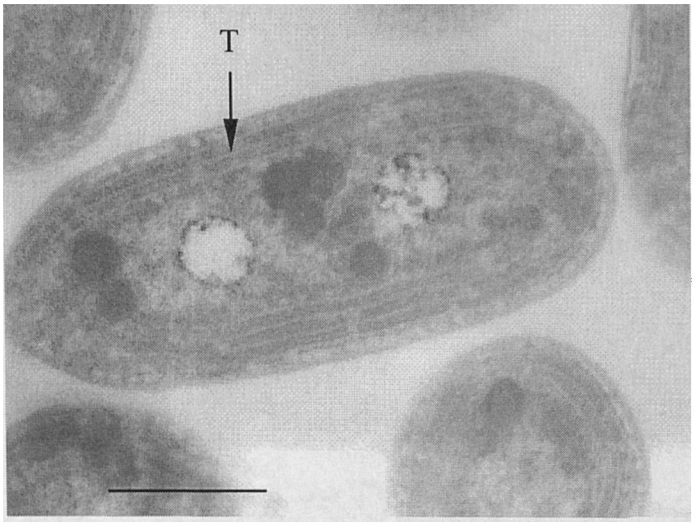
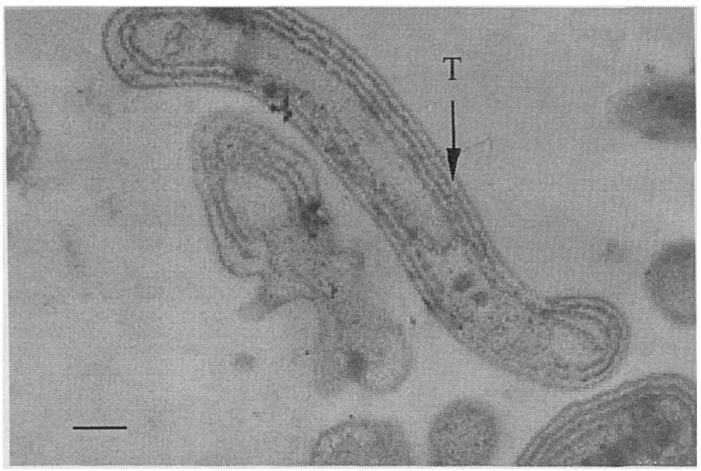


Figure 7.8. Micrograph of *Synechococcus* PCC7942. A) transmission electron micrograph of a thin section of healthy cell of strain PCC7942, bar, 0.5 μm; B) transmission electron micrograph of a thin section of unhealthy cell of strain PCC7942, bar, 0.5 μm; C) micrograph of unhealthy cells of strain PCC7942, bar, 10 μm. T, thylakoid.

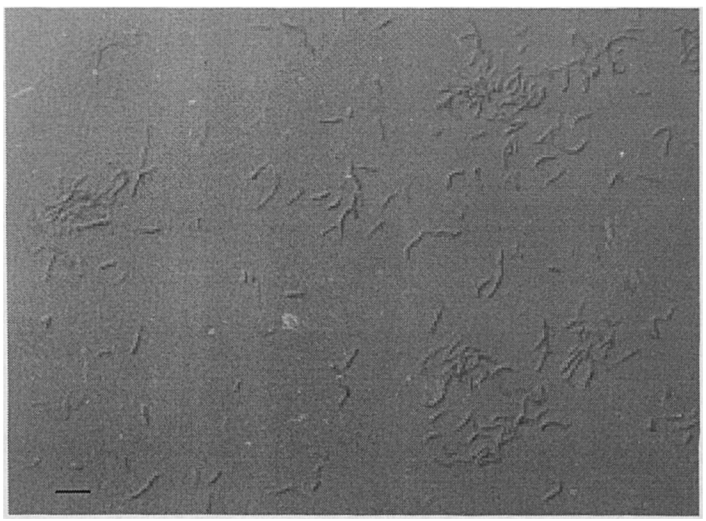
A.



B.



C.



2 Branches B from RFLP dendrogram and branches E from sequences trees (*Pseudanabaena* cluster)

The tree of Figure 7.3 includes eight *Pseudanabaena* strains, the tree of Figures 7.4, 7.5 and 7.6 only include six of them. All the *Pseudanabaena* strains in each tree form a tight cluster. The four trees show the same four sub-clusters; *Pseudanabaena* sp. LJM019 in one sub-cluster with a large distance to the other three sub-clusters; *Pseudanabaena* sp. LJM011 in another sub-cluster with a slight smaller sub-cluster distance to the other sub-clusters than strain LJM019; *Pseudanabaena* LJM009 and LJM016 in another sub-cluster; *Pseudanabaena* LJM001, 005, 006 and 007 in the fourth sub-cluster. The sub-cluster distances between the *Pseudanabaena* clusters are about ten times lower than the sub-cluster distances in the *Synechococcus* cluster.

3 Branches E from RFLP dendrogram and branches C from sequences trees (Other non-heterocystous filamentous strains)

This cluster contain different strains from the RFLP and sequence trees. In the RFLP tree (Fig. 7.3), this cluster contain three strains, *Oscillatoria animalis*, *Phormidium autumnale* and *Phormidium* LJM017, each strain being separated by a great distance. In sequence trees (Fig. 7.4, 7.5 and 7.6), this complex branch contains exclusively simple filamentous strains, the cyanelle of *Cyanophora paradoxa*, and the chloroplast of *Chondrus crispus*. *Arthrospira* sp. PCC7345 and *Oscillatoria animalis* form one sub-cluster. *Phormidium* LJM017 forms a sub-cluster, and *Trichodesmium* NIBB1067 together with *Chondrus crispus* chloroplast and *Cyanophora paradoxa* cyanelle form a sub-cluster. The great distance was also found between each sub-cluster.

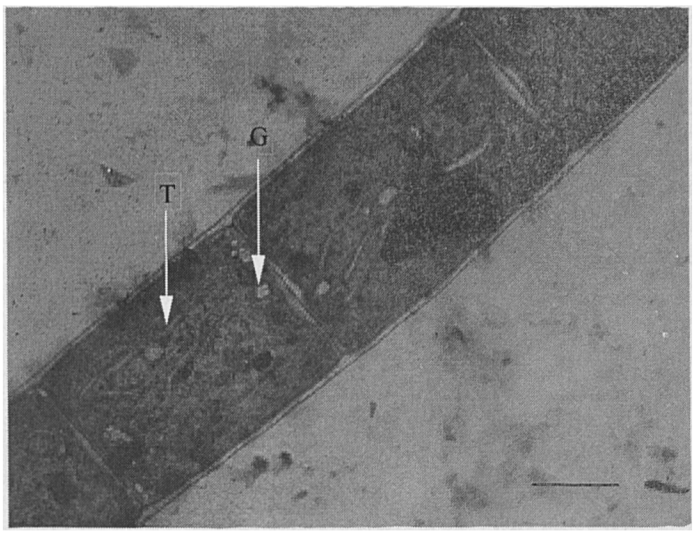
4 Branches D from RFLP dendrogram and branches B from sequences trees

This cluster only contains one strain *Nostoc palaeo.* The RFLP dendrogram shows that this strain is located at a non-heterocystous filamentous strains cluster. The sequence trees show that this strain locates at the early branch far away from other cyanobacterial clusters. This strain which was not listed in Table 2.1 was a gift with no reference from University of Central Lancashire. The morphology of the strain was re-examined as follows: filamentous cyanobacteria with no heterocysts and akinetes, producing cylindrical trichome waved, unconstricted or little constricted at the cross walls; ability to form thin sheaths around the trichome dependence on the culture conditions, lacking false branching, cells ± isodiametric or longer than wide, polar gas vacuoles, thylakoids present located peripherally and perpendicular to the walls. The diameter of trichome ranges from about 2.0 to 2.5 μm, cell length:width is 1.0--1.5, Trichomes do not contain PE, but synthesise nitrogenase, and are photoheterotrophic positive for sucrose. Colonies are pea green in colour and mats type. Figure 7.9 shows the TEM and microscopy photographs of *Nostoc palaeo.*

5 Branches F from RFLP dendrogram and sequences trees

Branches F from RFLP dendrogram contain five strains grouping into three sub-clusters, *Chroococcidiopsis* LJM013 in one sub-cluster, *Chroococcus prescottii* and *Microcystis aeruginosa* in one sub-cluster, *Gloeocapsa* sp. and *Synechocystis* PCC6803 in one sub-cluster. The great distances are not only shown between sub-clusters, but also between strains in same sub-cluster. Branches F from sequences trees are heterogeneous branches containing unicellular and filamentous strains. *Leptolyngbya* LJM012 locates at the early branch cluster F in the sequence tree, but is in cluster C in the RFLP dendrogram close to other simple filamentous strains. The morphological result was shown in chapter 3.

A.



B.

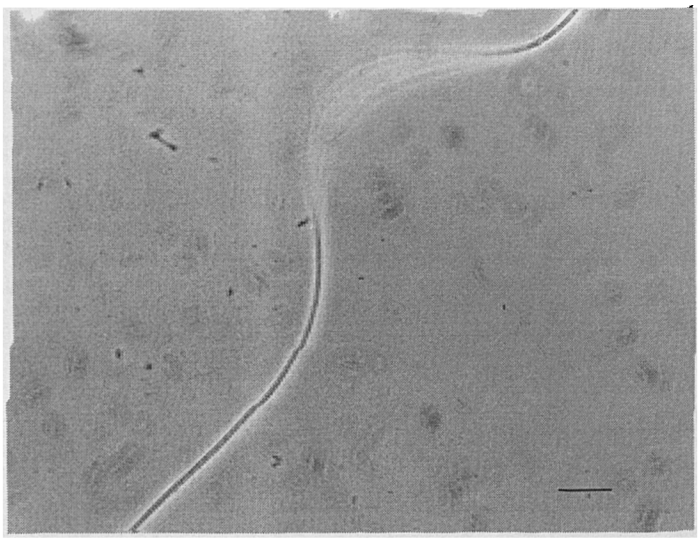


Figure 7.9. Micrograph of *Nostoc palaeo*. A) transmission electron micrograph of a thin section of strain *Nostoc palaeo*, bar, 1.0 μm; B) micrograph of trichome of strain *Nostoc palaeo*, bar, 20 μm. T, thylakoid. G, gas vesicle.

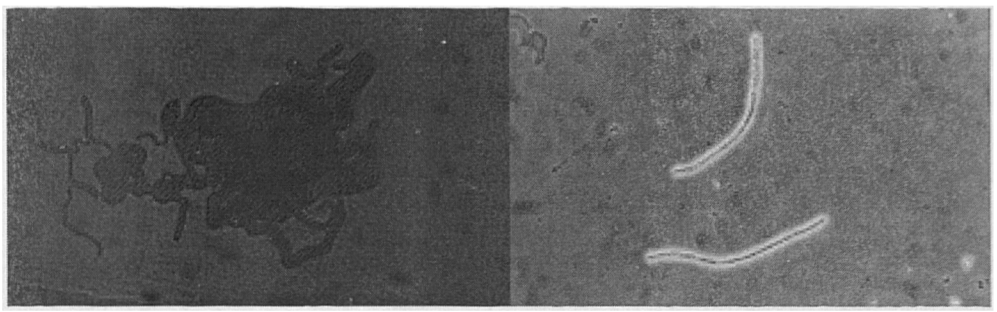
6 Branches G from RFLP dendrogram sequences trees

(Heterocystous filamentous strains)

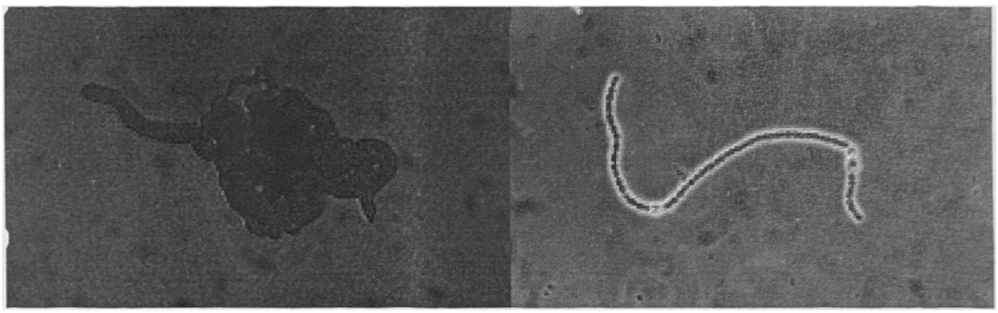
This cluster contain all the heterocystous filamentous strains. In sequences trees, this cluster divided into six sub-clusters: *Chlorogloeopsis fritschii* and *Fisherella musicola* in one sub-cluster; *Anabaena* sp. D0752 in one sub-cluster; *Anabaena* sp. D0697 and D0746 in one sub-cluster; *Gloeoetrichia* sp. and *Anabaena cylindrica* in one sub-cluster; *Nostoc* muscorum and MAC PCC8009 in one sub-cluster; *Nostoc linckia* D0734, *Nostoc ellipsospora*, *Anabaena* PCC7118 and *Anabaena* PCC7120 in one sub-cluster. In the RFLP dendrogram, this cluster divided into four sub-clusters: *Chlorogloeopsis fritschii*, *Fisherella musicola* and *Tolypothrix distorta* in one sub-cluster; *Nostoc MAC* R1, *MAC* PCC8009, *Nostoc* sp. Do740, *Nostoc linckia* D0734, *Nostoc piscinale* D0201 and *Nostoc* sp. D0766 in one sub-cluster; *Nostoc commune*, *Nostoc commune* D0800, *Nostoc planctonicum*, *Nostoc muscorum*, *Nostoc ellipsospora*, *Anabaena* PCC7118 and *Anabaena* PCC7120 in one sub-cluster; *Aphanizomenon gracile*, *Gloeoetrichia* sp., *Anabaena cylindrica*, *Anabaena* sp. D0752, D0697 and D0746 in one sub-cluster. Figure 7.10 shows a micrograph of *N.ellipsospora*, *Anabaena* PCC7118 and *Anabaena* PCC7120. *N.ellipsospora* and *Anabaena* PCC7120 produce heterocysts, Anabaena PCC7118 do not produce heterocysts, and trichome shorter than *Anabaena* PCC7120 and *N.ellipsospora*. All three strains perform small life cycle (step 3-14-15-16-17 of Komarek and Anagnostidis 1989).

The parsimony consensus (Fig. 7.11), Likelihood distance (Fig. 7.12) and Neighbour joining distance (Fig. 7.13) trees were also constructed based on the SF/SR amplified rDNA spacer sequence. The parsimony consensus tree and likelihood tree are similar, except for the position of *Synechococcus* PCC7942 and PCC6301, *Phormidium* LJM017 and *Chondrus crispus* chloroplast. Furthermore they are similar in terms of

A



B



C

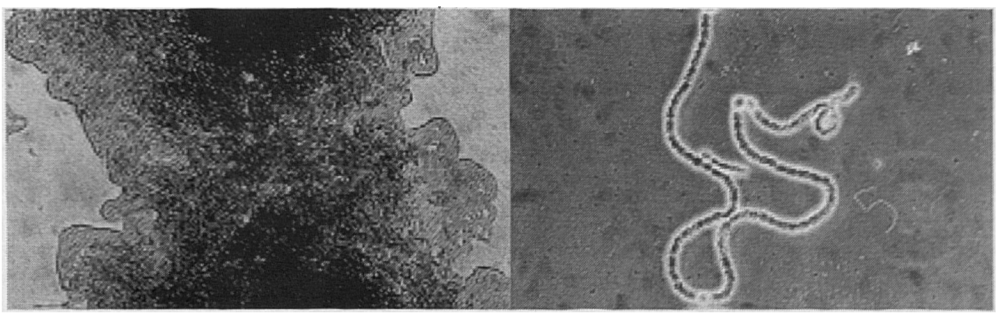


Figure 7.10. Micrographs of trichome or clony of some heterocystous strain. A) *Anabaena* PCC7118; B) *Anabaena* PCC7120; C) *Nostoc* ellipsospora.

Figure 7.11. Consensus trees derived from parsimony method based on partial 16S 23S spacer region (between two specific primers) sequence analysis. Sequencing data from 21 laboratory strains and 12 isolates (this study) together with *Arthrospira* sp. PCC7345 (Nelissen *et al.*, 1994), *Spirulina* sp. PCC6313 (Nelissen *et al.*, 1994), *Synechococcus* PCC6301 (Tomioka and Sugiura, 1984), *Trichodesmium* NIBB1067 (Wilmotte, 1994), *E. coli* (Young and Steitz, 1978), *Cyanophora paradoxa* cyanelle (Janssen *et al.*, 1987) and *Chondrus crispus* (red alga) chloroplast (Leblanc *et al.*, 1995) from the Genbank were used to constract the tree. The rooted tree was generated by the parsimony method and 500 bootstrapping runs were performed, using the DNAPARS, SEQBOOT and CONSENSE program in the software package PHYLIP. The numbers at the forks indicate the number of times the group consisting of the species which are to the right of that fork occurred among the trees, out of 500 trees. Clusters A, B, C, D, E, F, G are indicated. The cyanobacterial isolates are shown in bold.

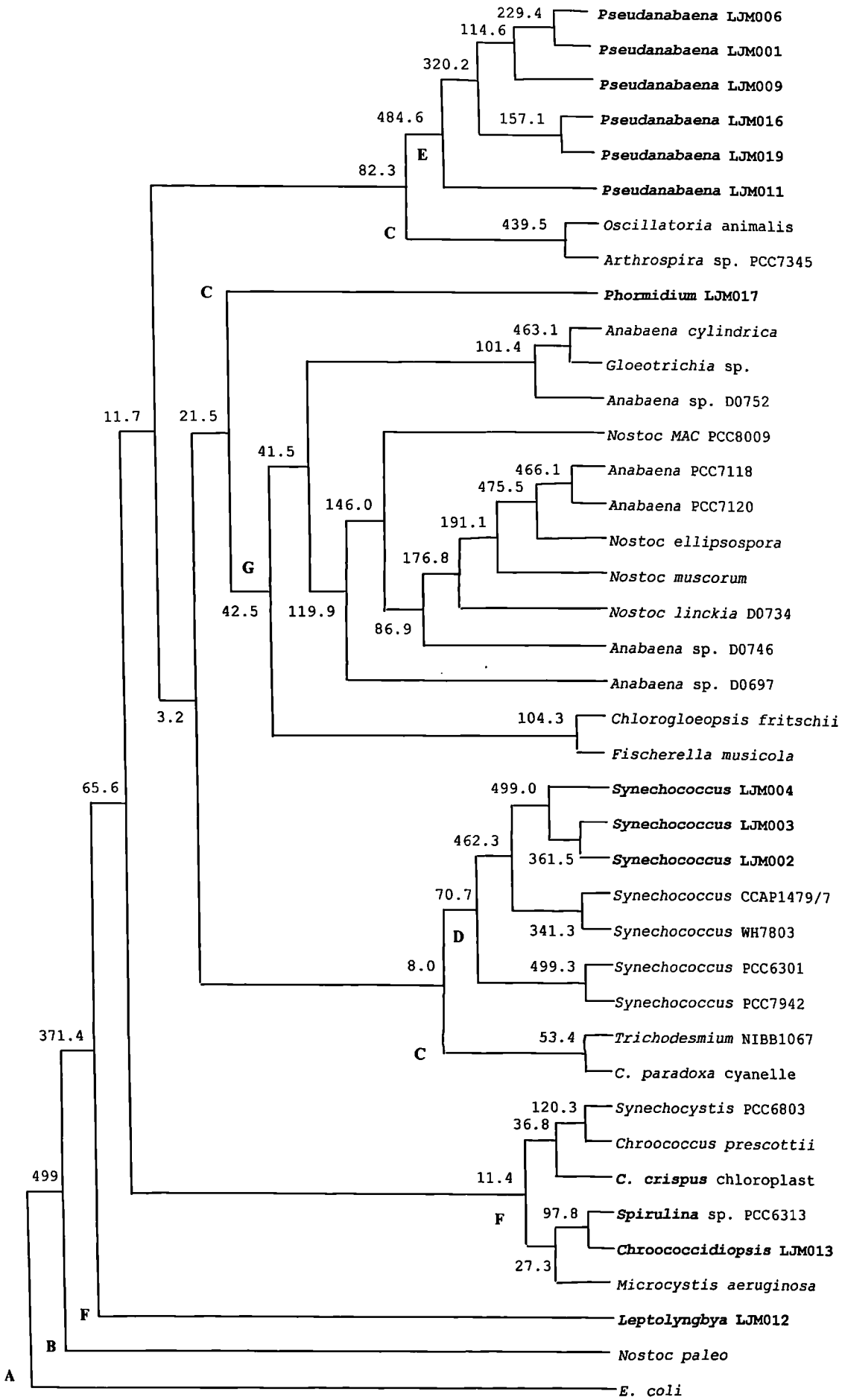

229.4
Pseudanabaena LJM006
114.6
Pseudanabaena LJM001
320.2
Pseudanabaena LJM009
484.6
157.1
Pseudanabaena LJM016
E
Pseudanabaena LJM019
82.3
Pseudanabaena LJM011
C
439.5
Oscillatoria animalis
Arthrospira sp. PCC7345
C
Phormidium LJM017
463.1
Anabaena cylindrica
101.4
Gloeotrichia sp.
Anabaena sp. D0752
11.7
21.5
Nostoc MAC PCC8009
41.5
466.1
Anabaena PCC7118
475.5
146.0
Anabaena PCC7120
191.1
Nostoc ellipsospora
176.8
G
Nostoc muscorum
42.5
119.9
Nostoc linckia D0734
86.9
Anabaena sp. D0746
Anabaena sp. D0697
3.2
104.3
Chlorogloeopsis fritschii
Fischerella musicola
65.6
499.0
Synechococcus LJM004
Synechococcus LJM003
462.3
361.5
Synechococcus LJM002
70.7
Synechococcus CCAP1479/7
D
341.3
Synechococcus WH7803
8.0
499.3
Synechococcus PCC6301
Synechococcus PCC7942
371.4
53.4
Trichodesmium NIBB1067
C
C. paradoxa cyanelle
120.3
Synechocystis PCC6803
36.8
Chroococcus prescottii
499
11.4
C. crispus chloroplast
F
97.8
Spirulina sp. PCC6313
Chroococcidiopsis LJM013
27.3
Microcystis aeruginosa
F
Leptolyngbya LJM012
B
Nostoc paleo
A
E. coli

Figure 7.12 Maximum likelihood tree based on partial 16S 23S spacer region (between two specific primers) sequence analysis. Sequencing data from 21 laboratory strains and 12 isolates (this study) together with *Arthrospira* sp. PCC7345 (Nelissen *et al.*, 1994), *Spirulina* sp. PCC6313 (Nelissen *et al.*, 1994), *Synechococcus* PCC6301 (Tomioka and Sugiura, 1984), *Trichodesmium* NIBB1067 (Wilmotte, 1994), *E. coli* (Young and Steitz, 1978), Cyanophora paradoxa cyanelle (Janssen *et al.*, 1987) and *Chondrus crispus* (red alga) chloroplast (Leblanc *et al.*, 1995) from theGenbank, were used to constract the tree by Maximum Likelihood method using DNAML version 3.572c program in the software package PHYLIP. Total 4882 trees were examined by program. Clusters A, B, C, D, E, F, G are indicated. The cyanobacterial isolates are shown in bold.

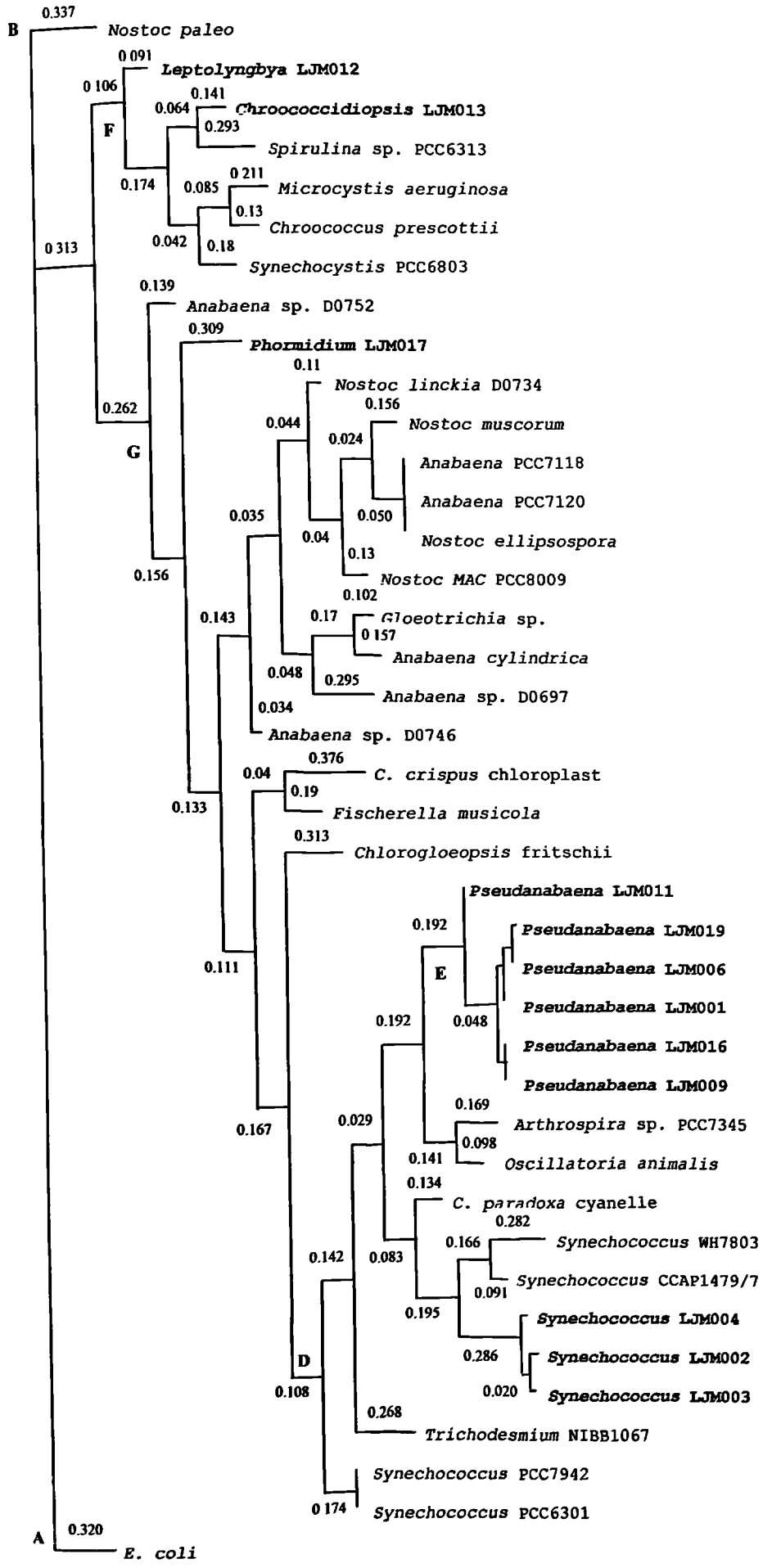
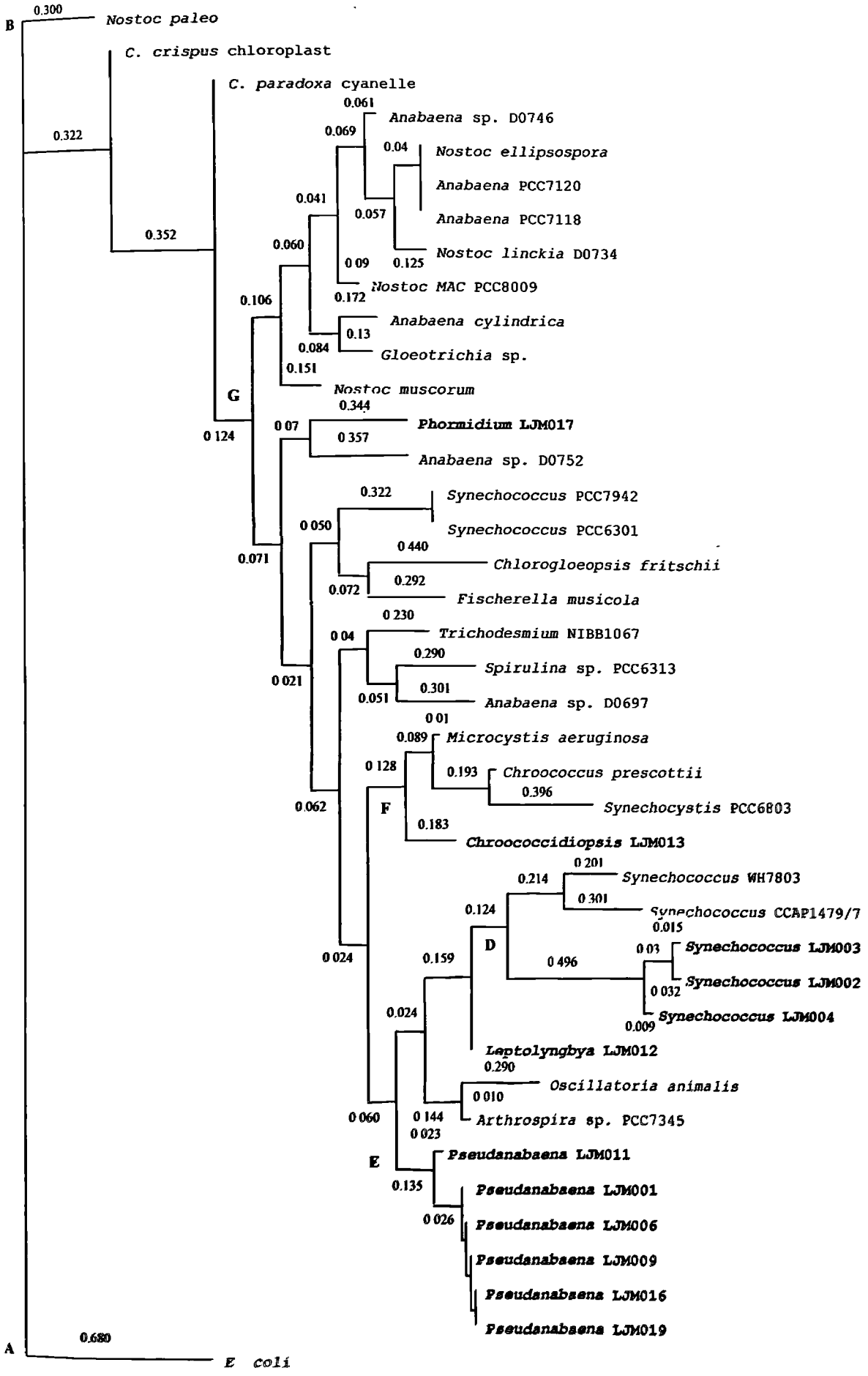

B
0.337
Nostoc paleo
0.091
Leptolyngbya LJM012
0.106
0.141
0.064
Chroococcidiopsis LJM013
F
0.293
Spirulina sp. PCC6313
0.174
0.211
0.085
Microcystis aeruginosa
0.13
Chroococcus prescottii
0.042
0.18
Synechocystis PCC6803
0.313
0.139
Anabaena sp. D0752
0.309
Phormidium LJM017
0.11
Nostoc linckia D0734
0.262
0.156
0.044
Nostoc muscorum
0.024
G
Anabaena PCC7118
Anabaena PCC7120
0.035
0.050
0.04
Nostoc ellipsospora
0.13
Nostoc MAC PCC8009
0.156
0.102
0.143
0.17
Gloeotrichia sp.
0.157
Anabaena cylindrica
0.048
0.295
Anabaena sp. D0697
0.034
Anabaena sp. D0746
0.376
0.04
C. crispus chloroplast
0.19
0.133
Fischerella musicola
0.313
Chlorogloeopsis fritschii
Pseudanabaena LJM011
0.192
Pseudanabaena LJM019
E
Pseudanabaena LJM006
0.111
Pseudanabaena LJM001
0.192
0.048
Pseudanabaena LJM016
Pseudanabaena LJM009
0.169
0.029
Arthrospira sp. PCC7345
0.098
0.167
0.141
Oscillatoria animalis
0.134
C. paradoxa cyanelle
0.282
0.166
Synechococcus WH7803
0.142
0.083
Synechococcus CCAP1479/7
0.091
0.195
Synechococcus LJM004
D
0.286
Synechococcus LJM002
0.108
0.020
Synechococcus LJM003
0.268
Trichodesmium NIBB1067
Synechococcus PCC7942
0.174
Synechococcus PCC6301
A
0.320
E. coli

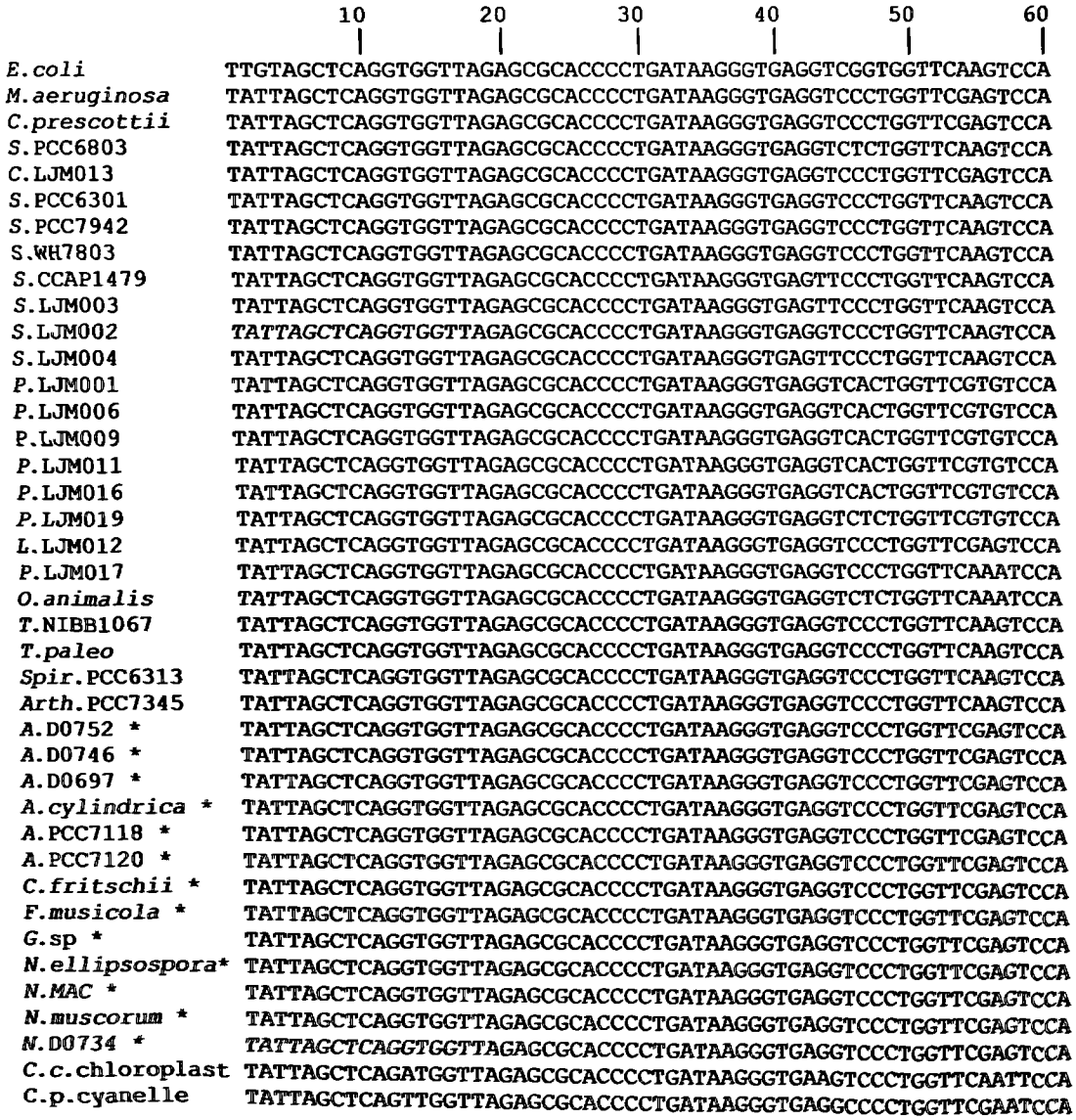
Figure 7.13. Distance tree derived from neighbour-joining method based on partial 16S-23S spacer region (between two specific primers) sequence analysis. Sequencing data from 21 laboratory strains and 12 isolates (this study) together with Arthrospira sp. PCC7345 (Nelissen *et al.*, 1994), Spirulina sp. PCC6313 (Nelissen *et al.*, 1994), *Synechococcus* PCC6301 (Tomioka and Sugiura, 1984), *Trichodesmium* NIBB1067 (Wilmotte, 1994), *E. coli* (Young and Steitz, 1978), Cyanophora paradoxa cyanelle (Janssen *et al.*, 1987) and *Chondrus crispus* (red alga) chloroplast (Leblanc *et al.*, 1995) from the Genbank were used to constract the tree by Neighbor-joining method using DNADIST and Neighbor-joining/UPGMA version 3.572c program in the software package PHYLIP. Clusters A, B, C, D, E, F, G are indicated. The cyanobacterial isolates are shown in bold.


0.300
B
Nostoc paleo
C. crispus chloroplast
C. paradoxa cyanelle
0.061
Anabaena sp. D0746
0.069
0.04
Nostoc ellipsospora
0.322
Anabaena PCC7120
0.041
0.057
Anabaena PCC7118
0.352
0.060
Nostoc linckia D0734
0 09
0.125
Nostoc MAC PCC8009
0.172
0.106
Anabaena cylindrica
0.13
0.084
Gloeotrichia sp.
0.151
Nostoc muscorum
G
0.344
Phormidium LJM017
0 07
0 124
0 357
Anabaena sp. D0752
0.322
Synechococcus PCC7942
0 050
Synechococcus PCC6301
0 440
0.071
Chlorogloeopsis fritschii
0.292
0.072
Fischerella musicola
0 230
Trichodesmium NIBB1067
0 04
0.290
Spirulina sp. PCC6313
0 021
0.301
0.051
Anabaena sp. D0697
0 01
0.089
Microcystis aeruginosa
0 128
0.193
Chroococcus prescottii
0.396
Synechocystis PCC6803
0.062
F
0.183
Chroococcidiopsis LJM013
0 201
0.214
Synechococcus WH7803
0 301
0.124
Synechococcus CCAP1479/7
0.015
D
0 03
Synechococcus LJM003
0 024
0.159
0 496
Synechococcus LJM002
0 032
0.024
Synechococcus LJM004
0.009
Leptolyngbya LJM012
0.290
Oscillatoria animalis
0 010
0 060
0 144
Arthrospira sp. PCC7345
0 023
E
Pseudanabaena LJM011
0.135
Pseudanabaena LJM001
0 026
Pseudanabaena LJM006
Pseudanabaena LJM009
Pseudanabaena LJM016
Pseudanabaena LJM019
A
0.680
E coli

clusters to those in Figure 7.4 and 7.5 based on the 17/18 amplified spacer sequence except cluster C arrangment. In Figure 7.11, the majority of branches of clusters D and E showing bootstrapping values of 50% or more indicating that the branches are quite robust. The majority of branches of other high diverse strains show bootstrapping values of below 50%, except the strain *Arthrospira* sp. PCC7345 with *Oscillatoria animalis*, *Anabaena cylindrica* with *Gloeotrichia* sp., *Anabaena* PCC7118 and PCC7120 with *Nostoc ellipsospora*, *Nostoc palaeo*, and *E. coli*. In the Neighbour joining tree (Fig. 7.13), it is confusing that the positions of *Synechococcus* PCC7942 and PCC6301, *Trichodesmium* NIBB 1067 and *Spirulina* sp. PCC6313 are moved into heterocystous strain cluster.

Partial cyanobacterial 16S-23S rDNA spacer sequences from amplified PCR products using primers 17/18 (which amplify $tDNA^{ile}$, spacer, $tDNA^{ala}$, spacer and 5' end of 23S rDNA) were recovered, except for an estimated 5 nucleotides at the 5' end of $tDNA^{ile}$ for cyanobacteria by automated sequencing. Conserved nucleotide positions were aligned using the CLUSTAL W program for 20 laboratory cyanobacterial strain and 13 isolates, together with Arthrospira sp. *PCC7345* (Nelissen *et al.*, 1994), Spirulina sp. *PCC6313* (Nelissen *et al.*, 1994), *Synechococcus* PCC6301 (Accession No. X00346), *Trichodesmium* NIBB1067 (Accession No. X72871), *E. coli* (Accession No. J01702), Cyanophora paradoxa cyanelle (Janssen *et al.*, 1987) and *Chondrus crispus* (red alga) chloroplast (Accession No. Z29521), to determine the extent of sequence homlogy (Figure 7.14). It was noticed that *Chroococcidiopsis* LJM013, *Chroococcus prescottii*, *Synechocystis* PCC6803, *Microcystis aeruginosa*, *Spirulina* sp. PCC6313 and *Leptolyngbya* LJM012 do not contain conserved region $tDNA^{ala}$. Except the conserved regions described in chapter 6, more high homology regions were found present in the cyanobacterial strains within same species or same genera: Three conserved regions are

Figure 7.14. Alignment of 16S-23S rDNA spacer sequences from various cyanobacterial species. Sequences start from the 5' end of $tDNA^{ile}$ to the beginning of 23S rDNA. Alignment was obtained for sequences from 21 laboratory strains and 12 isolates (this study) together with *Arthrospira* sp. PCC7345 (Accession No. X75044), *Spirulina* sp. PCC6313 (Accession No. X75045), *Synechococcus* PCC6301 (Accession No. X00346), *Trichodesmium* NIBB1067 (Accession No. X72871), *E. coli* (Accession No. J01702) *Cyanophora paradoxa* cyanelle (Accession No. M19493) and *Chondrus crispus* (red alga) chloroplast (Accession No. Z29521) from the Genbank, using the CLUSTAL W program version 1.6 (http://www.ibcp.fr/clustalw.html). Alignment across the $tDNA^{ile}$ (position 1-70) and $tDNA^{ala}$ (163-238) sequence was very good, but gaps were present within the spacer region. Some of these gaps were manually removed. A-Adenine, T-Thymine, G-Guanine, C-Cytosine. - , absence of nucleotide. Numbers above the sequences represent base positions. *, heterocystous strains. The regions of high homology were boxed.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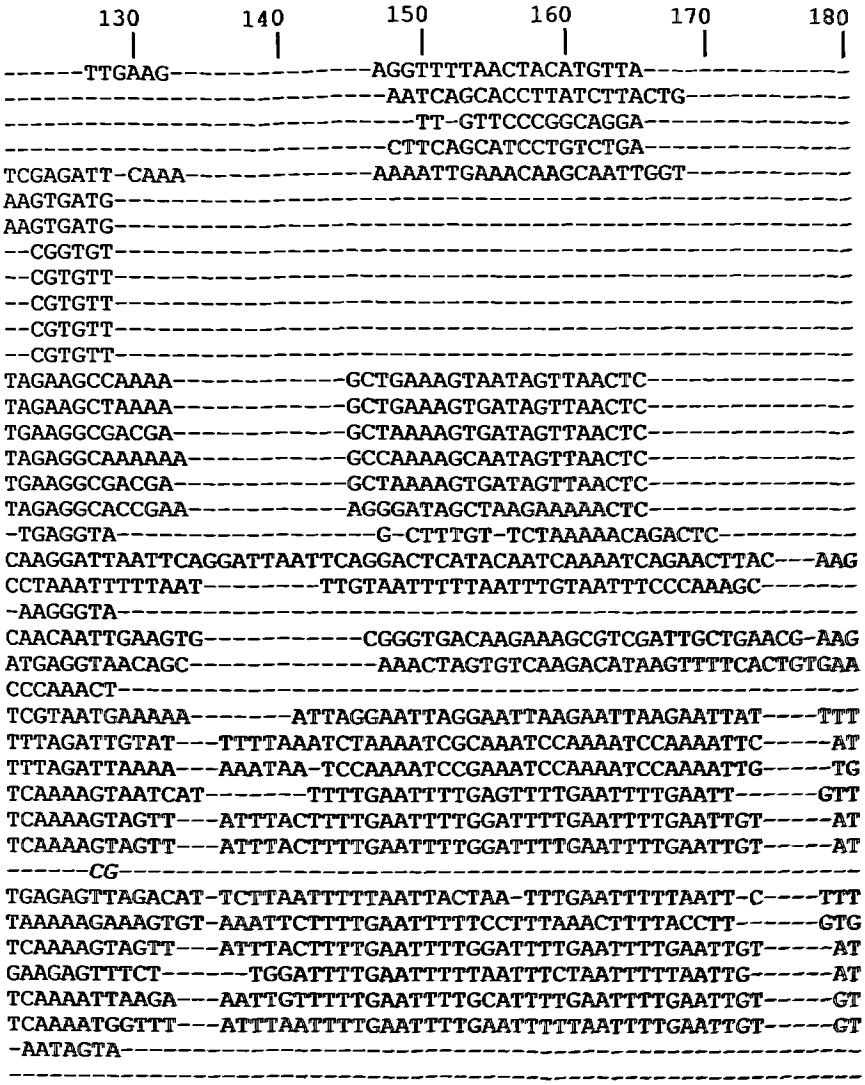
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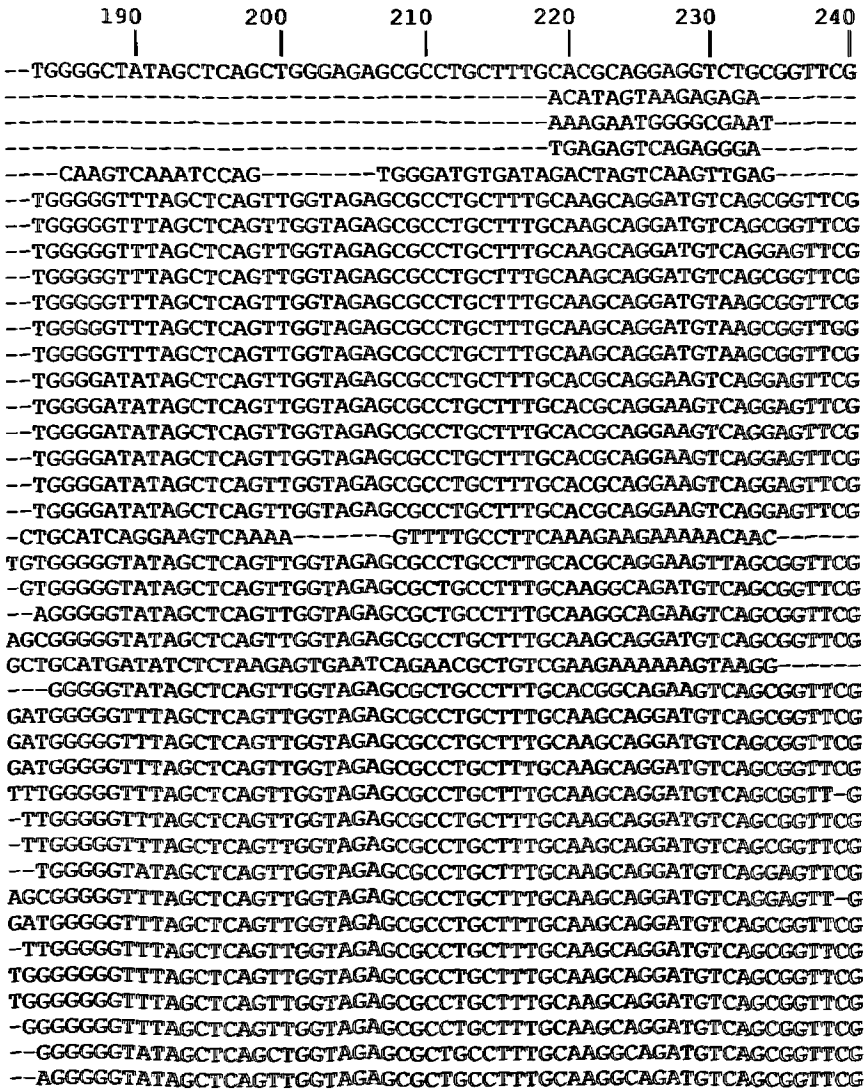
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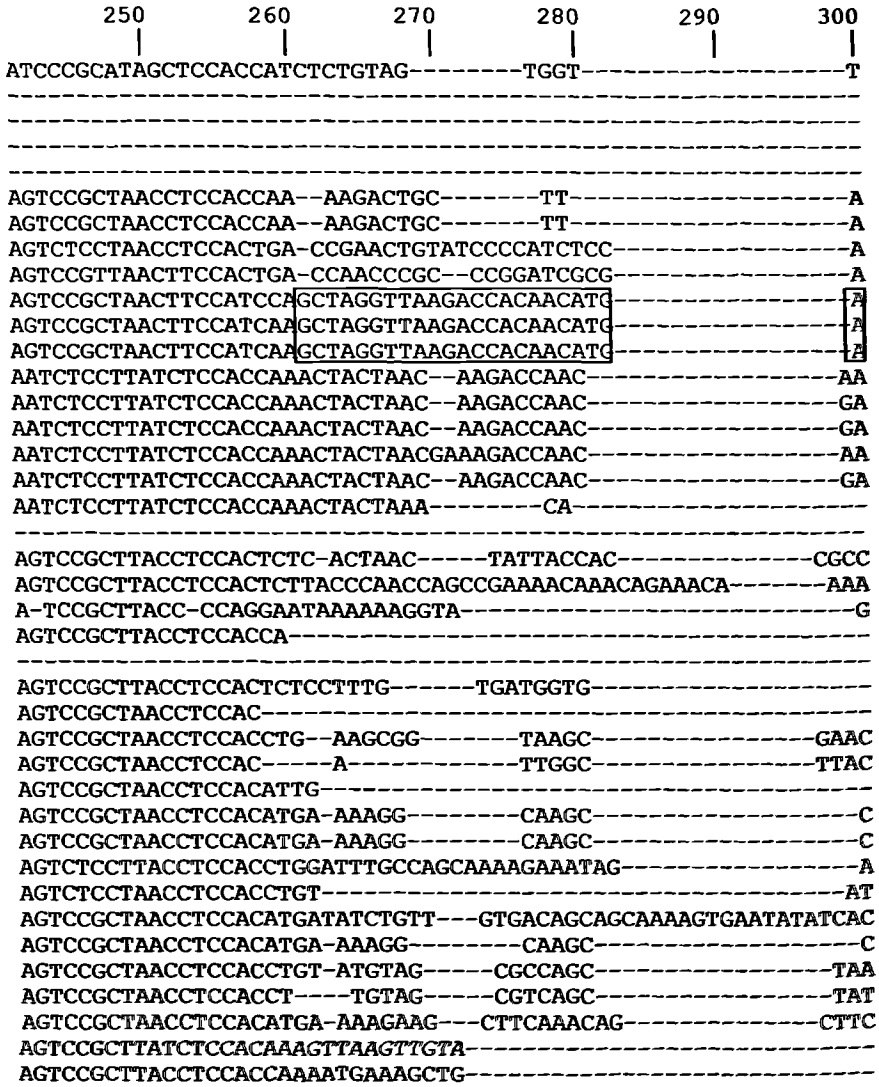
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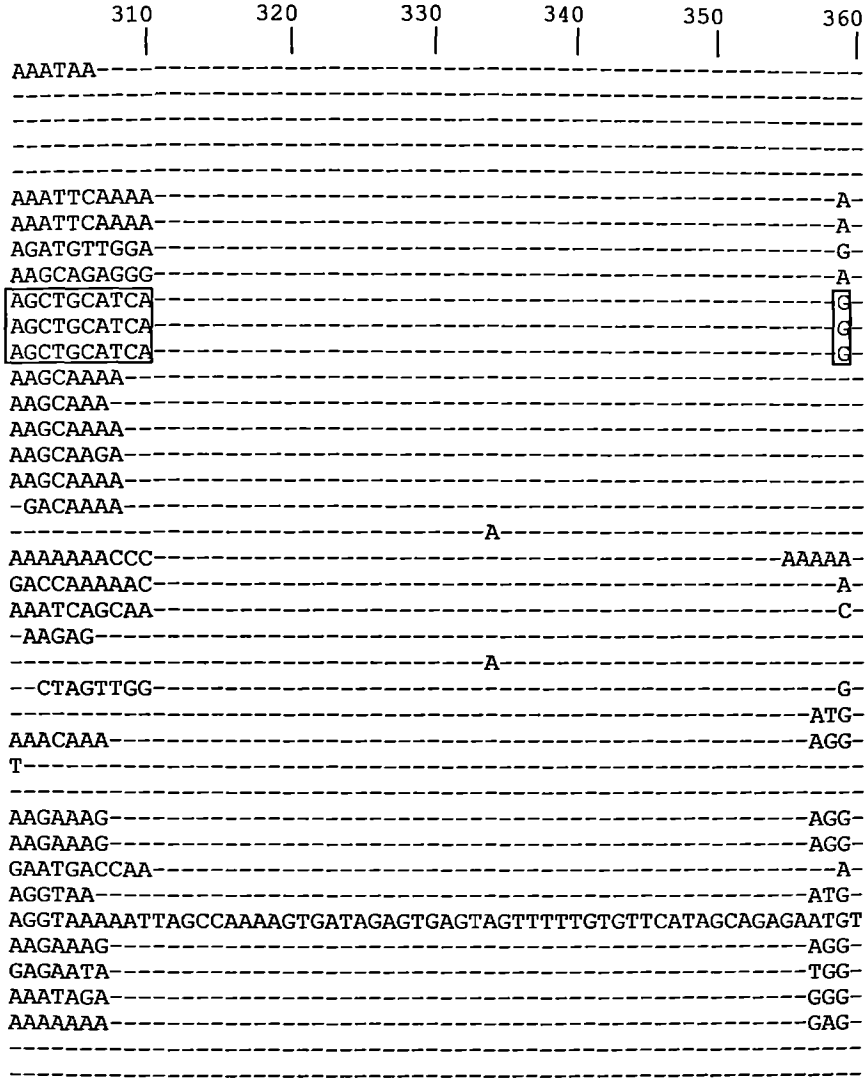
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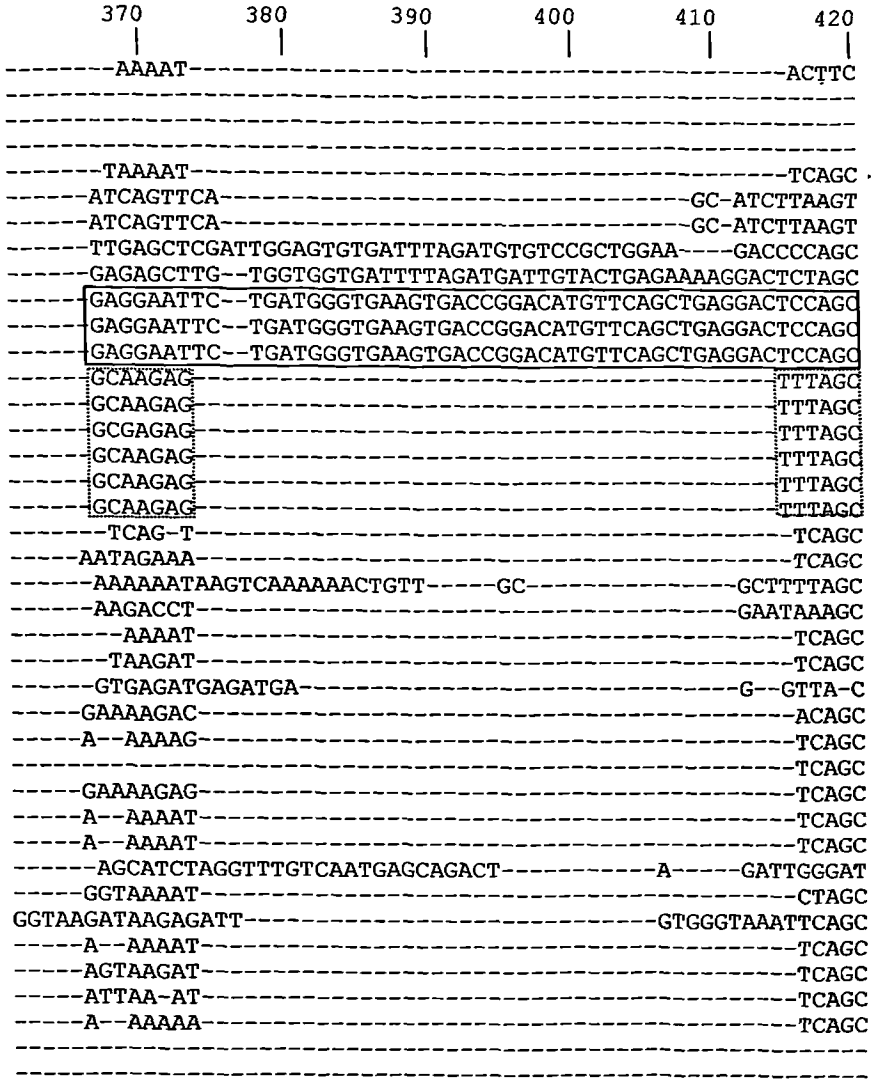
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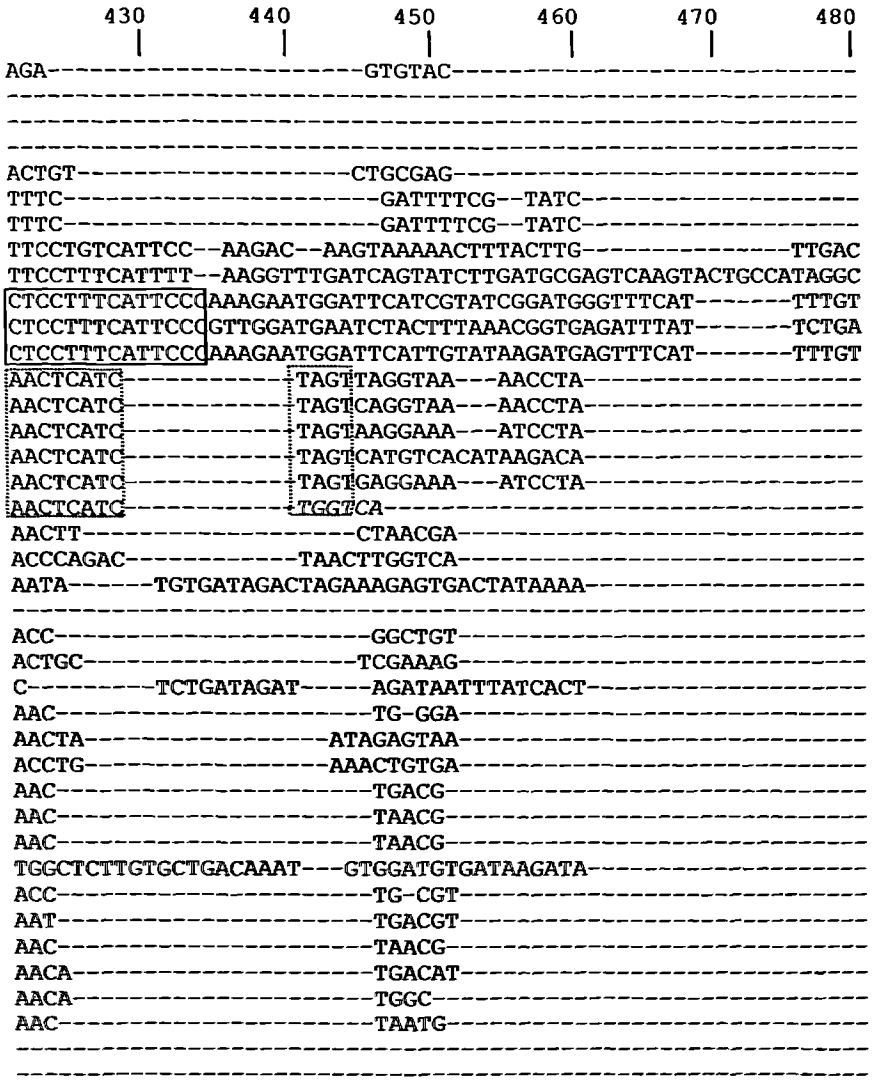
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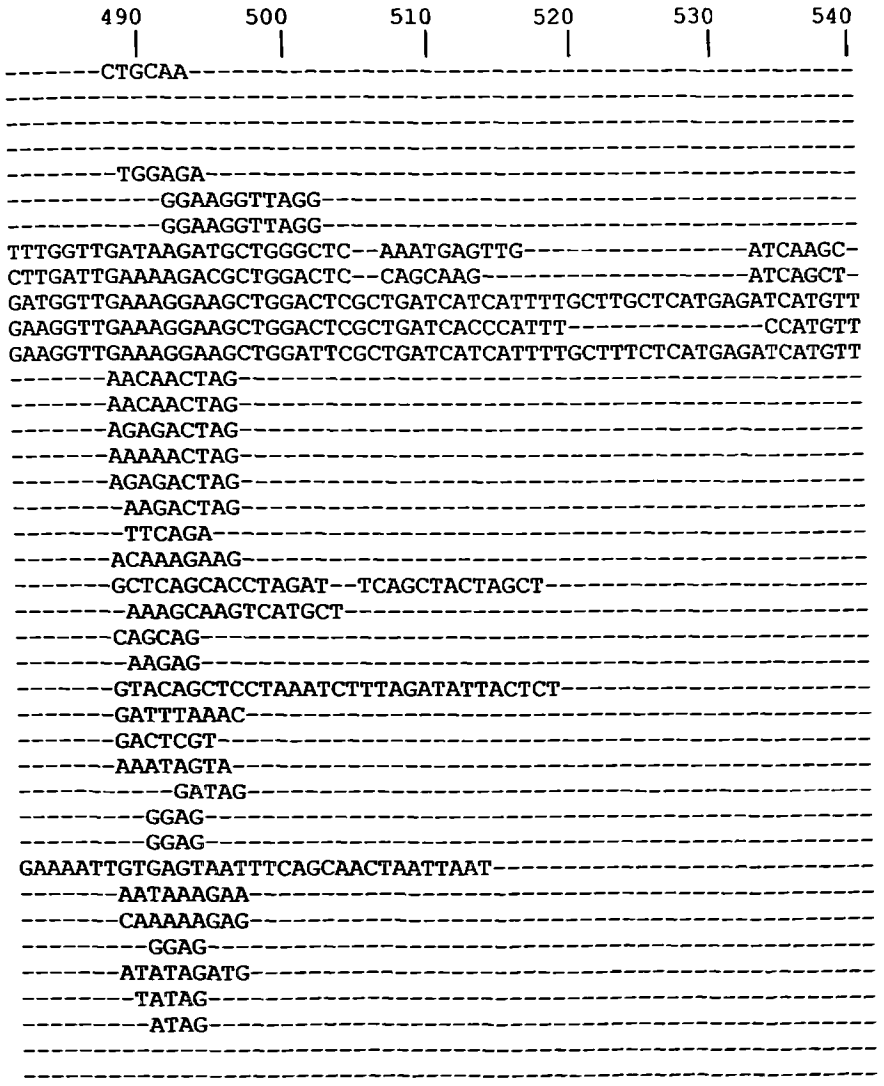
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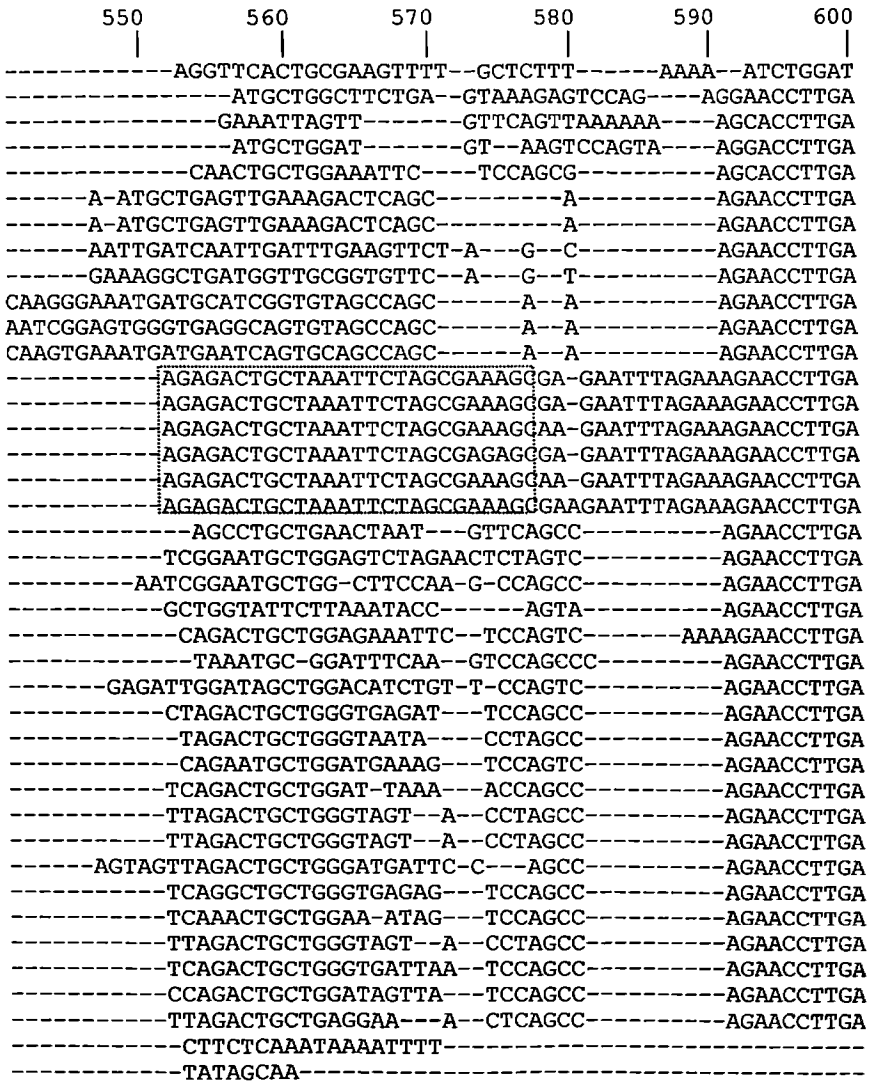
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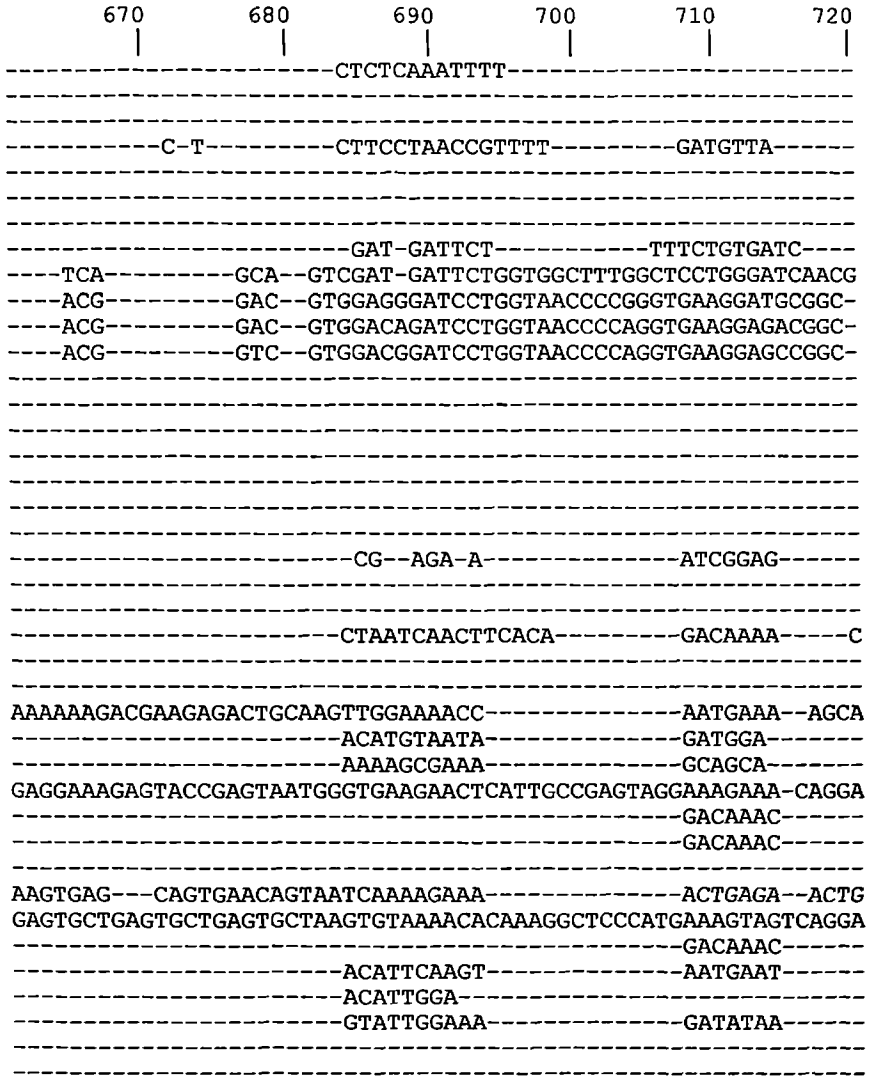
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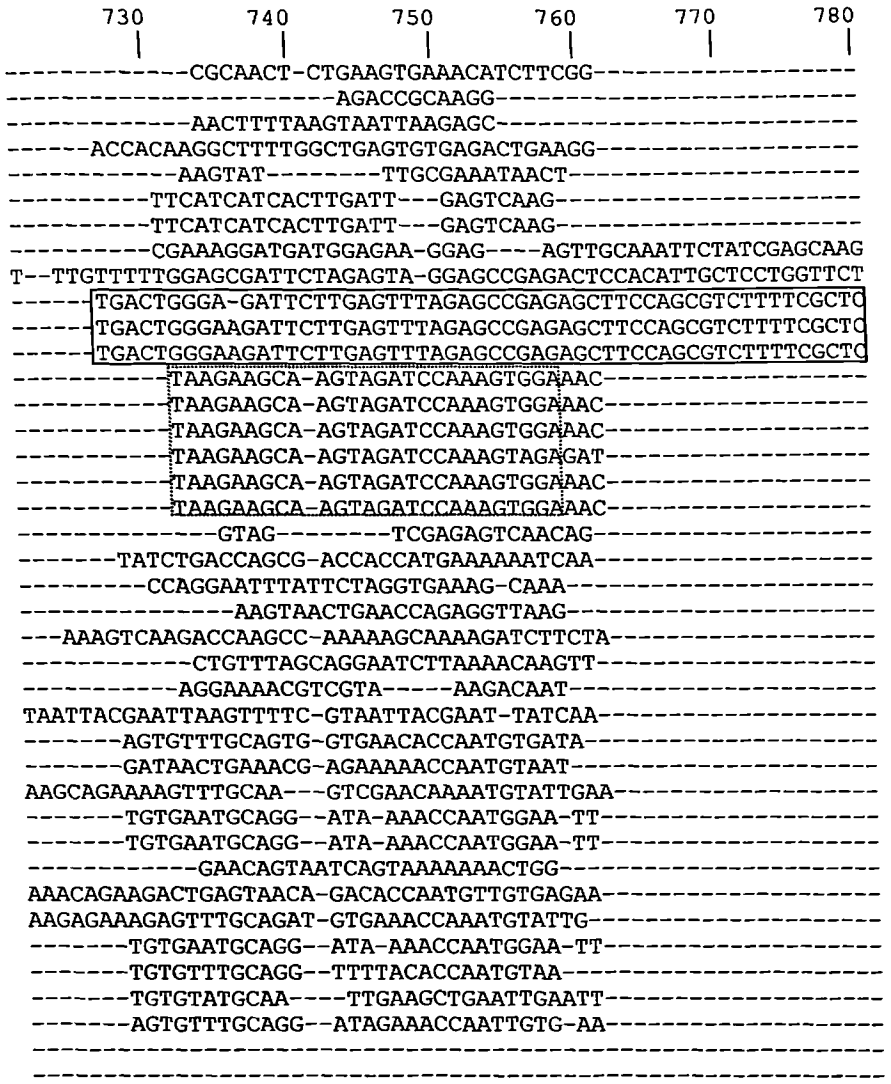
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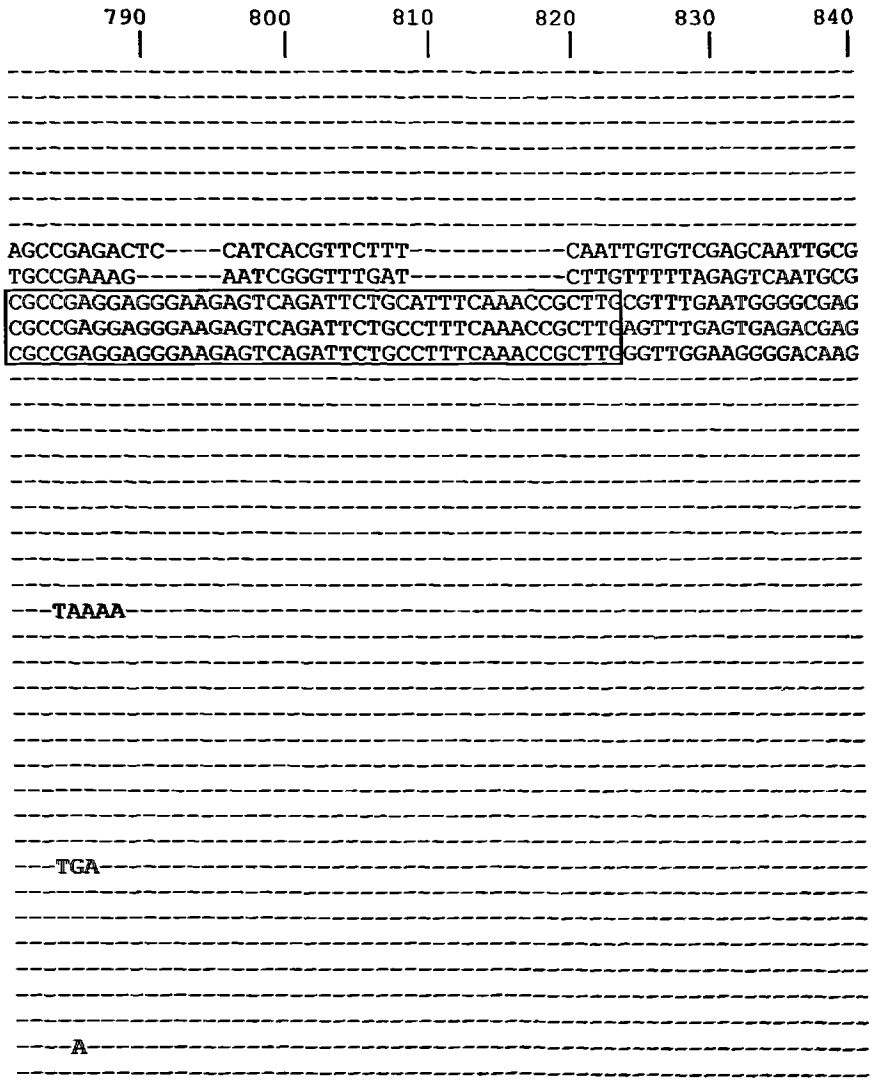
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AGGGCTTGCGGTGGATACCTAGGCACACAGAGCGGCCGCAAA
AGGGCTGATGGTGGATACCTAGGCACACAGAGCGGCCGCAAA
AGGGCTAATGGTGGATACCTAGGCACACAGAGCGGCCGCAAA
AGGGCTCATGGTGGATACCTAGGCACACAGAGCGGCCGCAAA
AGGGCTGACGGTGGATACCTAGGCACACAGAGCGGCCGCAAA
AGAGCTAATGGTGGATACCTAGGCACACAGAGCGGCCGCAAA
AGAGCTTACGGTGGATACCTTGGCATTTAGAAGCGATG-AAG
AGGGCTTACGGTGGATACCTAGGCATTCAGAAGCGATG-AAG
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within the variable spacer between $tDNA^{ala}$ and the 5' end of 23S rDNA among *Synechococcus* LJM002, 003 and 004, two very long conserved sequences (about 90 bp) from position 261 to 434 and from position 727 to 823, and a 42 bp sequence from position 892 to 936. Three conserved regions are within the variable spacer between $tDNA^{ala}$ and the 5' end of 23S rDNA among *Pseudanabaena* LJM001, 005, 006, 007, 009, 011, 016 and 019, a 25 bp sequence from position 367 to 444, a 21 bp sequence from position 552 to 580 and another 25 bp sequence from position 731 to 757.

7.3 Discussion

By aligning the sequence, more conserved regions in the variable length spacer were found between the strains assessed in same species or genus. These are the best candidates for which to design cyanobacterial genera or species-specific primers or probes. This will be very useful to detect presence of particular cyanobacterial species or genus in environmental samples more rapidly, such as toxigenic species. Thus the 16S-23S rDNA spacer sequence does provide a potentially useful molecule for the identification of cyanobacteria.

The phylogenetic significance of trees using each genetic method were compared with morphological phenotypes. The morphological taxonomic groups (see Table 1.1) appear largely consistent with the phylogenetic information obtained to date. In Neighbour-joining (sequence) trees, all the cyanobacterial strains were separated into three large group, (1) Simple filamentous strains (*Oscillatoriales* or section III); 2 Unicellular strains (*Chroococcales* & *Pleurocapsales* or section I & II); (3 Heterocystous strains (*Nostocales* & *Stigonematales* or section IV & V). In Parsimony and Maximum Likelihood sequence trees and the RFLP dendrogram, the unicellular

group was separated by a simple filamentous group into two clusters; unicellular rod-shaped strains with division in one plane and unicellular spherical shaped strains with division in more than one plane.

These four isolates and three laboratory strains, are simple rod-shaped unicellular forms, traditionally classified within the *Synechococcus*. In phylogenetic studies, all these strains grouped into one large cluster with three far related sub-clusters (Fig. 7.3, 7.4, 7.5 and 7.6). This clearly indicates further generic division in this morphologically simplest genera. In contrast with current the taxonomic system, Rippka and Herdman (1992) have changed genera *Synechococcus* to *Synechococcus* group which contain five clusters, and Komarek and Anagnostidis (1986) put them into two families containing thirteen genera. The isolates, *Synechococcus* strains LJM002, LJM003, LJM004 and LJM020 are morphological very similar; *Synechococcus* strains LJM003 and LJM004 are a little more close than others (Fig. 3.22). This were confirmed by phylogenetic study (Fig. 5.16, 7.3, 7.4, 7.5 and 7.6). Marine strain *Synechococcus* WH7803 shows a far distance to both groups of freshwater strains, even if it is morphologically very similar to the isolates (Fig. 3.6A, 3.7, 3.8, 3.9 and 7.7). This marine strain was proved unrelated to other freshwater strains, in line with DNA-DNA hybridisation data (Wood and Townsend, 1990). *Synechococcus* CCAP1479/7 and WH7803 were grouped in one sub-cluster, but distant, in line with morphological data, a little similar in habitat and a little different in cell structure and pigment content. It suggests that they may be in two closer groups, than with other two. In the tree topologies, the *Synechococcus* group did not separate and form clusters with other simple filamentous strains. This provide a better result than the 16S rRNA-sequence data reviewed by Wilmotte (1994).

The *Pseudanabaena* cluster contain eight freshwater isolates. They are far away from other simple filamentous genera. This supports the finding of Geitler (1982) and Guglielmi and Cohen-Bazire (1984) that the genera *Pseudanabaena* is rather different from other Oscillatoriace. All eight strains form tight clusters (Fig. 3.16B, 7.3, 7.4, 7.5 and 7.6). This suggested that these strains should belong to the same genus. Four sub-clusters were drawn based on the distance: *Pseudanabaena* sp. LJM019; *Pseudanabaena* sp. LJM011; *Pseudanabaena* LJM009 and LJM016; *Pseudanabaena* LJM001, 005, 006 and 007. This suggests that these four sub-clusters probably are four species. This appear largely consistent with morphological data (chapter 3), except for strains LJM001, LJM005, LJM006 and LJM007. They were separated into two groups because strains LJM001 and LJM005 do not contain PE, and because of slight morphological differences.

The heterogeneous branch containing unicellular spherical-shaped and filamentous strains is only found in sequences trees. This branch unifies the remaining four of the set of eleven unicellular strains studied. Four strains are from four different genera based on morphological classification. It is in agreement with the tree topologies of Figures 7.5 and 7.6. The isolate, *Chroococcidiopsis* LJM013 shows in one sub-cluster without other strains, but the distance is not far. In morphological classification, *Chroococcidiopsis* is placed in order *Pleurocapsales* (section II), different from *Chroococcus prescottii*, *Microcystis aeruginosa* and *Synechocystis* PCC6803 in order *Chroococcales* (section I). This strain also shows high morphological similarity with the other three strains, for example, repeated binary fission in three planes to produce a more or less regular cubical cell in an aggregate. In line with cluster G of the 16S rRNA tree (Wilmotte, 1994), unicellular strains with binary fission in more than one plane (in section I) and baeocyte-forming strains (section II) form one cluster. This

suggests that these groups are probably in one Section or Order. The presence of *Spirulina* in this lineage is surprising, because the morphology of this truly multicellular form is rather complex: it consists of an helical trichome with cross-walls visible only by electron microscopy. However, the reason that *Spirulina* PCC6313 and *Leptolyngbya* LJM012 are placed in this cluster may be that they both lack $tDNA^{ala}$ as do other unicellular strains in this cluster. In the RFLP dendrogram, *Leptolyngbya* LJM012 located at the early branch of simple filamentous strains with *Leptolyngbya* LJM014 is at a great distance. This suggests that these strains probably belong to different genera.

In sequence trees, other simple filamentous strains form a complex branch with the cyanelle of *Cyanophora paradoxa* and the chloroplast of *Chondrus crispus* (red alga), in line with branch F of the 16S rRNA sequence tree(Wilmotte, 1994). Four cyanobacterial filamentous strains form a loose group. This indicates that four strains may be from four different genera. *Arthrospira* sp. PCC7345 is close to *Oscillatoria animalis* and not to *Spirulina* sp. PCC6313, although it has helical trichomes like the latter strain (see Fig. 17, Rippka et al., 1979). The genus *Arthrospira* and *Spirulina* were fused in the same genera, *Spirulina*, by Geitler (1932) and Rippka et al., (1979). Later, Rippka and Herdman (1992) recognised the separation of the two genera, already proposed by Anagnostidis and Komarek (1988) and Castenholz (1989). Ultrastructural studies (Guglielmi and Cohn-Bazire, 1982) supported this separation and indicated the presence of one circle of pores in the peptidoglycan layer near the cross-walls in *Arthrospira* sp. PCC7345 and several *Oscillatoria* sp. strains sensu Rippka et al. (1979). The presence of one circle of pores was not tested on *Oscillatoria animalis.*

A early branch for cyanobacteria in all sequence trees is *Nostoc palaeo.* This suggests that this strain probably is very ancient (viz. the name palaeo). There is no

evidence that this strain could belong to any group of cyanobacteria, except in the Neighbour-joining tree. It was shown to have a little close relatedness to simple filamentous strains, but not closely related. In the RFLP Dendrogram, *Nostoc palaeo* located in the simple filamentous group (cluster D) with a large distance from other strains. No evidence from morphology results indicated that *Nostoc palaeo* is a heterocystous strain, as no heterocysts and akinetes production was found in this study. Other morphological characters (Fig. 7.9), such as cylindrical trichome waved, unconstricted or little constricted at the cross walls; ability to form thin sheaths around the trichome, false branching lacking, cells ± isodiametric or longer than wide; polar gas vacuoles, shows that it may be belong to a gas vacuole-producing genus of the *Phormidiaceae* familia (Anagnostidis and Komarek, 1988). To rename this strain will need more phenotypic and phylogenetic information.

All heterocystous strains are situated in the same cluster, in line with branch H of the 16S rRNA sequence tree(Wilmotte, 1994). The genotypic unity of the two strains from section V, or the Order Stigonematales, are located at the early branches of this cluster, and form a loose cluster away from other heterocystous strains. The distance between two strains clearly shows that two strains belong to different genera, and *Fisherella musicola* appears a little close to other heterocystous strain than *Chlorogloeopsis fritschii*, in contrast with the morphology of two strains. *Chlorogloeopsis fritschii* (same strain with PCC 6718) which contains both unicellular and heterocystous filamentous morphology, forms cell aggregates, short filaments and heterocysts (see Fig. 79-84 in Rippka *et al.*, 1979). *Fisherella musicola* in which cell division occurs in more than one plane, forms filaments with true branches and heterocysts. *Anabaena* and *Nostoc* strains in this cluster appear mixed together. By contrast, Caudales and Wells (1992) pointed out that strains of the genus *Nostoc* are

closely related to, and often confused with those of the genus *Anabaena*, particularly when morphological characteristics are used for identification. *Nostoc ellipsospora, Anabaena* PCC 7120, and *Anabaena* PCC 7118 have been discussed in chapter 6, and placed in the same species group of *Nostoc*. From this study, morphologies of *N.ellipsospora* and *Anabaena* PCC7120 were very similar (Fig. 7.10), they both produce heterocysts, but Anabaena PCC7118 does not produce heterocysts. All three strains perform a small life cycle (step 3-14-15-16-17 of Komarek and Anagnostidis 1989). Rippka and Herdman (1992) have renamed *Anabaena* PCC 7120 and *Anabaena* PCC 7118 as *Nostoc* PCC7120 and *Anabaena* PCC 7118, and placed them in the Nostoc cluster 3. Komarek and Anagnostidis (1989) moved *Nostoc linckia, Nostoc ellipsospora* and *Anabaena variabilis* to a new genus *Trichormus*. By contrast, the few strains Nostoc linckia D0734, *Nostoc ellipsospora*, Anabaena PCC7118 and *Anabaena* PCC7120 four strains form a sub-cluster in the sequence tree.

The sequence trees were also successfully constructed based on the primers SF/SR amplified rDNA spacer sequence. The trees based on the primers SF/SR amplified rDNA spacer sequence and the trees based on the primers 17/18 amplified rDNA spacer sequence are similar, except the Neighbour joining tree. It is a little confusing that the positions of *Synechococcus* PCC7942 and PCC6301, *Trichodesmium* NIBB 1067 and *Spirulina* sp. PCC6313 are moved into the heterocystous strain cluster in the Neighbour-joining tree based on the SF/SR amplified sequence. The majority of branches of highly diverse strains which show great distance in the distance tree, show very low bootstrapping values in parsimony tree, except the groups with clear relationships. For example, the Synechococcus group. It is not surprising that the distance and parsimony methods give different branch topologies for the basic nodes that are not supported by the bootstrap analysis. Otherwise, using the single spacer

sequence (SF/SR) without tDNA will increase the variation (gaps) of alignment of sequences. This will affect on the particular packages used which are not designed for too many gaps per sequence. In the heterocystous cluster, the *Nostoc* strains and Anabaena strains are clearly separated into two sub-clusters. This proved again the SF/SR primers potential usefulness for identification of cyanobacteria, and suggests that it is not a very suitable analysis for highly diverse strains.

To involve more strains from the same morphological species or genera may be useful in confirming or contradicting the present scheme. The assessment may be useful in pointing out positive as well as negative correlations that are emerging when different methods are applied to the same organisms. This approach may help in formulating questions for further research, and determining the best candidate for future phylogenetic analyses.

The evolution information (Fig. 7.1) based on fossil record available to date, which is by necessity limited to morphological assessment, is largely consistent with the results of molecular phylogeny discussed in here: (a) Both, coccoid and filamentous forms are found very early, preserved in sedimentary rocks of Archean age (3500 Ma). (b) Endolithic forms that reproduce with baeocytes were found preserved in mid-proterozoic rocks (1500 Ma). (c) helically wound filaments were found in the strata (700 Ma), while heterocystous forms, and forms with true branching have not been convincingly documented in the Proterozoic fossil record of cyanobacteria.

Hillis (1987) pointed out that morphological and molecular systematic techniques each have distinct advantages for phylogenetic reconstruction ... studies that combine thc two approaches can thereby maximise both information content and

usefulness. Interestingly, the traditional system of cyanobacterial classification based on morphology proved to be more consistent with the emerging phylogenetic information, than the traditional systems of other phototrophic bacteria, which were based on functional rather than structural phenotypes. In this study, some morphological criteria proved very useful, such as cell formation or style (unicellular, filamentous or heterocystous), cell division patterns, colony formation, cell structure; some morphological criteria needed to be modify for particular strains, such as cell length and shape change depending on culture condition, thylakoids located irregularly or peripherally in same strain. The pigment composition can only be used to separate strains in same species.

In theory it would be desirable that taxonomy be based on phylogeny, because the evolutionary processes are the ones responsible for the diversification of a group of organisms. However, the possibility cannot be excluded that convergent evolution has taken place too often at the morphological level, making the use of morphological characters difficult. There is already an indication that this may be the case with morphologically simple organisms. In some cases the acuity of observation may come to good use by including additional characters, which correlate well with phylogenetic positioning.

Chapter 8

General Discussion

8.1. General Discussion

Traditional methods for identification and taxonomy of cyanobacteria have been based primarily on morphological and biochemical characteristics. However, such methods are limited particularly when applied to newly isolated strains. For example morphology may be altered when environmental isolates are cultured in the laboratory. At present, the names of cyanobacteria, which are based on cultures, are often inapplicable to field material because of the loss of certain morphological characteristics under common culture conditions. There is reason for optimism, however, since it is becoming a more common practice to vary culture conditions to the extent where these characteristics are often exhibited (e.g. Evans *et al.*, 1976; Stulp, 1982; Stulp and Stam, 1984). Analysis of photosynthetic pigments, isozyme variation and differentiated cell structures may be variable depending upon levels of expression of cyanobacterial gene products. Furthermore, organisms may be viable but non culturable. Thus other methods that do not rely on gene expression and/or culturing should be used in addition to traditional methods to identify cyanobacteria.

Two approaches were used in this study for identification of cyanobacteria: one based on morphological characters, the other on molecular characters. Both approaches generally provided similar conclusions (see chapters 5, 6, and 7).

Plasmid profile is a limited genetic marker, it can be used to research the relationship of some strains which contain plasmids, but is not suitable for the establishment of classification schemes.

PCR-based techniques, including RAPD-PCR (chapter 5), PCR-RFLP analysis of the rDNA spacer, and rDNA sequencing (chapter 6), have been developed for identification of cyanobacteria and show promise for the genetic typing of cyanobacteria. Such technology has been assessed along side traditional techniques for rapid identification and for phylogenetic analysis. The applicability of the RAPD technique was restricted by the requirement for axenic cultures and the limitation on number of samples processed, but it can recognise more diversity among the cyanobacteria than PCR-RFLP analysis of the rDNA spacer. The method can be employed to identify rapidly cyanobacteria to the species and strain level of classification in pure culture. It is a good method to combine with morphological methods for identifying cyanobacterial isolates. New isolates could be identified to genus level by using morphological methods before purification, and then identified to strain level by RAPD analysis after purification. This would involve matching the RAPD pattern (see chapter 5).

PCR amplification and RFLP analysis of the 16S-23S rDNA spacer provides a potentially useful approach to the identification of cyanobacteria. Analysis of length polymorphism and RFLPs of the amplified rDNA spacer, using primer pair 17/18, generally permitted discrimination between the cyanobacteria tested to genus and species level without the requirement for axenic cultures. With more data, it should be possible to generate a database of RFLP-patterns, in order to identify unknown isolates by the matching of pattern-type and determining similarity to laboratory strains from phenetic analysis. rDNA sequencing provided a more accurate approach than PCR-RFLP analysis of the rDNA spacer, and identified greater diversity among the cyanobacteria. The rDNA sequencing data can be used to generate a database more easily than the RFLP-patterns and it would be applicable world wide. Furthermore, the

sequencing methodology is suitable for application to a large number strains. There was generally a good correlation between molecular and morphological methods for identifying cyanobacteria, except for a few individuals (chapters 6, 7).

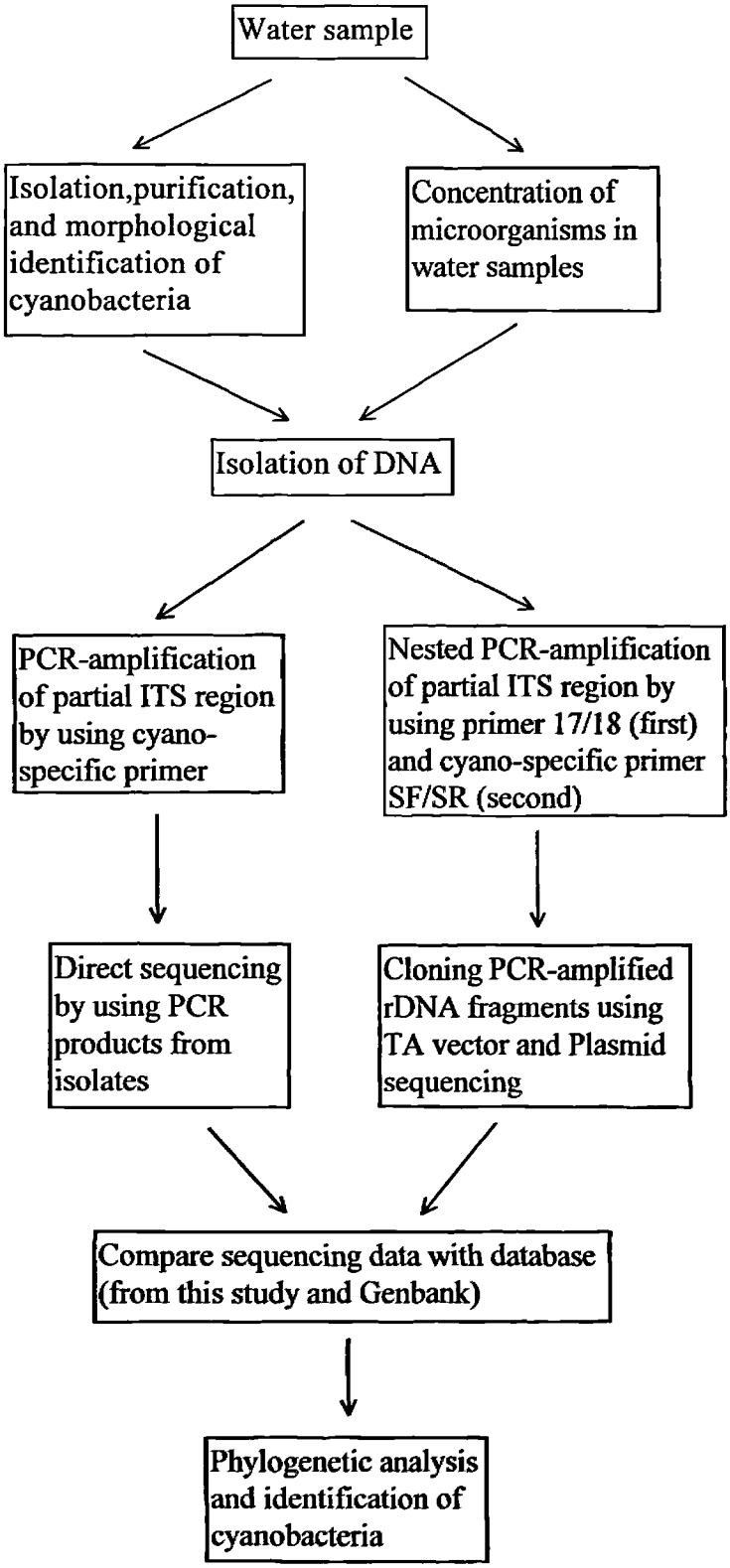
Heterogeneity of the sequence of the 16S-23S rDNA spacer proved useful not only for typing cyanobacteria, but also for developing specific primers. Such specificity opens up possibilities for their use in the rapid identification of cyanobacteria directly from water samples (chapter 6). Furthermore toxigenic species- (or genera-) specific PCR primers/ probes might be developed by the sequencing and analysis of particular toxigenic strains. It should then be possible to detect the present of toxigenic cyanobacteria in water samples more rapidly.

8.2. Recommendations for Further work

The cyano-specific primers developed here, and derivatives there of, could be used to amplify the rDNA spacer of cyanobacteria using DNA extracted from water samples. This would circumvent potential problems associated with culturing of the organisms from water samples. A nested PCR approach (Zhang and Ehrlich, 1994; Zimmermann *et al.*, 1994) could be used: first using primer pair 17/18 to enrich for cyanobacterial DNA, then using SF/SR (or derivatives). The amplified fragments would be cloned and sequenced, following by matching to the sequence database (Fig. 8.1).

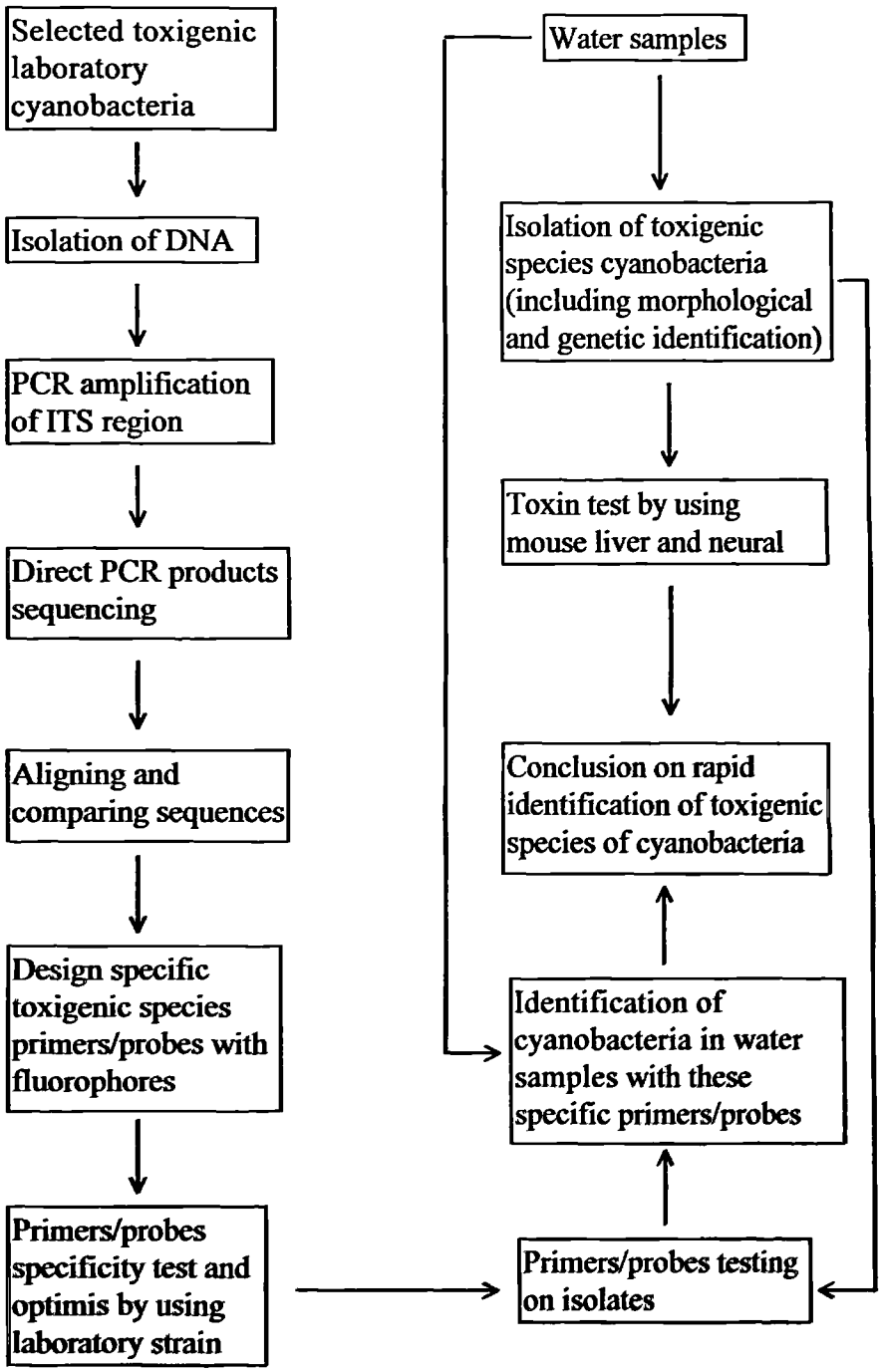
By modifying this approach and developing primers specific for toxigenic species/genera of cyanobacteria, could be identified directly from water samples. Furthermore, by tagging specific PCR primers/ probes with fluorophores, it should

Figure 8.1 Flow diagram summarising the schedule of further work based on this study.



be possible to detect the present of toxigenic cyanobacteria rapidly in water samples by assessing fluorescence, using PCR and hybridisation. Such a study could provide a more rapid and reliable method for identifying toxigenic cyanobacteria in particular. This could prove useful to the water industry in terms of assessing water quality and selecting appropriate water sites. The procedures of this further study are outlined in Figure 8.2.

Figure 8.2 Flow diagram summarising the schedule of further work of detection of the presence of toxigenic cyanobacteria rapidly in water samples.



Summary

- 51 axenic cyanobacterial strains were obtained from several culture collections or purified from freshwater courses, and grown in the laboratory for genetic study. The genus and species investigated were selected to cover a range of taxonomic groups and to reflect different environmental habitats.

- Several medium and culturing condition were used to isolate cyanobacteria from freshwater courses. It was noticed that some species from water samples were visible but non culturable in the medium used here. 16 isolates were purified and identified by using traditional methods. It was found that the traditional methods used for identification and taxonomy of cyanobacteria based on morphological and biochemical characteristics were slow and difficult, particularly at genera, species and strain level (see chapter 3).

- A range of methods for the extraction of plasmid and genomic DNA from cyanobacteria were investigated. It was found that plasmid isolation was effected by isolation method, culture stage and culturing condition. Methods requiring rapid plasmid isolation are limited, as it is difficult to recovery all plasmids in the cyanobacterial strain every time. The results show that plasmid profile is a limited genetic marker and can be used to research the relationship of some strains which contain plasmids, but not for establishment of classification schemes (see chapter 4).

- The randomly amplified polymorphic DNA (RAPD) technique in conjunction with the polymerase chain reaction (PCR) for identification of cyanobacteria was established and optimised. The choice of primers was found useful for optimising the RAPD reaction. Two 10 nucleotides arbitrary primers, ABA-7 and 10 were chosen for generating RAPDs profile. Genomic DNA of fifty different cyanobacterial laboratory strains and isolates was amplified successfully by using primer ABA-7 or ABA-10. The pattern of amplified products was analysed. The results shows that RAPDs profile can be employed to identify rapidly cyanobacteria to the species and strain level of classification in pure culture, but was difficult at genera and order level. It is opening possibilities to identify rapidly cyanobacterial isolates by combining RAPDs technique with morphological method (see chapter 5).

- The 16S-23S rDNA spacer region of selected cyanobacteria was amplified using primers to conserved flanking regions. Single or multiple amplification products were generated depending on the strain and primer pair. Analysis of length polymorphism and RFLPs of products amplified using primer pair 17/18, generally permitted discrimination between the cyanobacteria to genus and species level without requirement of axenic cultures. Four restriction enzymes (*Hinf* I, *Dde* I, *Alu* I and *Taq* I) generated distinguishing restriction profiles. With more data, it should be possible to generate a database of RFLP-patterns, in order to identify unknown isolates by the matching of pattern-type and determining similarity to laboratory strains from phenetic analysis (see chapter 6 and 7).

- Sequences of part of the 16S-23S rDNA spacer of selected cyanobacteria were determined by PCR amplification and direct sequencing of products amplified using primers 17 and 18. Significant variation was generally found in composition and

length of the spacer sequence between cyanobacterial species. A restriction map of the rDNA spacer region was constructed from the sequencing data. Restriction endonuclease sites were found both within the conserved tDNAs and the variable spacers of the region. The map revealed polymorphisms useful in distinguishing between genera and species of cyanobacteria and confirmed the appropriateness of the four enzymes used here in RFLP analysis. A number of phylogenetic trees were generated by using software package RAPDistance and PHYLIP for RFLP profiles and sequences analysis. The clustering of cyanobacterial strains shown from the trees is consistent with that of the 16S rRNA sequence trees (Wilmotte, 1994) and morphological section (see chapter 6 and 7).

- The heterogeneity in the sequence of the 16S-23S rDNA spacer is useful for both typing cyanobacteria and developing specific primers. From rDNA sequence alignment, three short (5-26bp) regions of high sequence homology were found, in addition to the conserved tDNAs in the spacer. Oligonucleotides derived from two of these regions showed promise for use as cyano-specific PCR primers, opening up possibilities for the amplification of cyanobacterial DNA directly from water samples, for rapid species identification. (see chapter 6, 7 and 8).

- Comparing molecular and morphological methods for identifying cyanobacteria, it prove that morphology and genetic of cyanobacteria are related. The morphologically differentiated and complex taxonomic groups appear consistent with phylogenetic information obtained except some individuals.

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Appendix A: Preparation of Media

Medium BG11

Stock solution	g/100ml	ml(stock solution)/L(media)
K_2HPO_4	4.00	1
$MgSO_47H_2O$	7.50	1
$CaCl_26H_2O$	5.34	1
Citric Acid	0,60	1
Ferric Ammonium citrate	0.60	1
Disodium-magnesium EDT A	0.10	1
Na_2CO_3	2.00	1
Trace solution		1
H_3BO_3	0.286	-
$MnCl_24H_2O$	0.181	-
$ZnSO_47H_2O$	0.022	-
$Na_2MoO_42H_2O$	0.039	-
$CuSO_45H_2O$	0.0079	-
$Co(NO_3)_26H_2O$	0.0049	-
Solid	-	-
$NaNO_3$	-	1.5g/L

For media BG11/10 is BG11 with decrease of the $NaNO_3$ concentration (as much as 10-fold).

Medium C

Stock solutions	g/100ml	ml/L(media)
$Fe_2(SO_4)_3 9H_2O$	0.404	1
Trisodium citrate	16.46	1
$Ca(NO_3)_2 4H_2O$	2.5	1
K_2HPO_4	127.56	1
$MgSO_4 7H_2O$	25.25	1
Trace solution	-	1
H_3BO_3	0.286	-
$MnCl_2 4H_2O$	0.181	-
$ZnSO_4 7H_2O$	0.022	-
$Na_2MoO_4 2H_2$	0.0024	-
$CuSO_4 5H_2O$	0.079	-
Solid	-	-
KNO_3	-	1g/L

Medium ASM-1

Stock solution	g/100ml	ml/L(media)
K_2HPO_4	1.7418	1
Na_2HPO_4	1.4196	1
$MgSO_47H_2O$	4.9296	1
$MgCl_26H_2O$	4.0662	1
$CaCl_2$	2.2198	1
Disodium EDTA	0.7445	1
$FeCl_36H_2O$	0.1081	1
trace solution	-	1
H_3BO_3	0.2487	-
$MnCl_24H_2O$	0.1377	-
$ZnSO_44H_2O$	0.0914	-
$CuSO_45H_2O$		-
$Co(NO_3)_26H_2O$	0.0023	-
Solid		
$NaNO_3$		0.17g/L(media)

CHU 10 D Medium

Stock solution	g/100ml	ml/L(media)
KH_2PO_4	0.780	1
$MgSO_4 7H_2O$	2.500	1
$Ca(NO_3)_2 4H_2O$	5.760	1
$NaHCO_3$	1.585	1
Na_2SiO_3 $5H_2O$	1.087	1
Disodium EDTA	1.335	0.25
$FeCl_3 6H_2O$	0.970	0.25
trace solution	-	1
H_3BO_3	0.0715	-
$MnCl_2 4H_2O$	0.0453	-
$ZnSO_4 4H_2O$	0.0055	-
$CuSO_4 5H_2O$	0.0019	-
$CoSO_4 7H_2O$	0.0010	-
Na_2MoO_4 $2H_2O$	0.0007	-
BUFFER 2.5mM HEPES pH 7.6 (0.6 g/L)		1000ml

ACM medium

Stock solution	g/100ml	ml/L(media)
$K_2HPO_43H_2O$	1.310	1
$MgSO_47H_2O$	5.000	5.0
$CaCl_2$ $2H_2O$	1.986	1
NaCl	2.300	1
KNO_3	10.00	5
Disodium EDTA	0.25	0.2
$FeCl_36H_2O$	0.605	0.2
trace solution	-	1
H_3BO_3	0.2860	-
$MnCl_24H_2O$	0.1810	-
$ZnSO_47H_2O$	0.0220	-
$Na_2MoO_42H_2O$	0.0390	-
$CuSO_45H_2O$	0.0079	-
$Co(NO_3)_26H_2O$	0.0049	-
$NiSO_4$ $7H_2O$	0.0048	-
BUFFER 2.5mM HEPES pH 7.6 (0.6 g/L)		1000ml

AD P(1) Fe(0.4) medium

Stock solution	g/100ml	ml/L(media)
$K_2HPO_43H_2O$	0.736	1
$MgSO_47H_2O$	5.000	4.0
$CaCl_2\ 2H_2O$	6.620	1
NaCl	4.600	5
KCl	1.91	1
Disodium EDTA	1.335	0.2
$FeCl_36H_2O$	0.970	0.2
trace solution	-	1
H_3BO_3	0.2860	-
$MnCl_24H_2O$	0.1810	-
$ZnSO_47H_2O$	0.0220	-
$Na_2MoO_42H_2O$	0.0390	-
$CuSO_45H_2O$	0.0079	-
$Co(NO_3)_26H_2O$	0.0049	-
$NiSO_4\ 7H_2O$	0.0048	-
BUFFER 2.5mM HEPES pH 7.6 (0.6 g/L)		1000ml

MN medium

Stock solution	g/100ml	ml(stock solution)/L(media)
K_2HPO_4	2.00	1
$MgSO_47H_2O$	3.80	1
$CaCl_26H_2O$	2.67	1
Citric Acid	0.30	1
Ferric Ammonium citrate	0.30	1
Disodium-magnesium EDT A	0.05	1
Na_2CO_3	2.00	1
Trace solution		1
H_3BO_3	0.286	-
$MnCl_24H_2O$	0.181	-
$ZnSO_47H_2O$	0.022	-
$Na_2MoO_42H_2O$	0.039	-
$CuSO_45H_2O$	0.0079	-
$Co(NO_3)_26H_2O$	0.0049	-
Solid	-	-
$NaNO_3$	-	0.75g/L
Sea water		750ml
distilled water		250ml

These above media were sterilized by autoclaving at 121°C, 15p.s.i for 20 minutes. After autoclaving VB_{12} , Nystatin and Ampicillin were added if the media was to be used for isolation and purification (section 2.6). For preparation of solid media, 1.5% Difco Bacto-agar should be added in tbe media. Before sterilization, the mineral medium and agar should be separated (as double strength) and mix them only after cooling to about 50 °C.

LB medium:

10g/L Bacto-tryptone
5g/L Bacto-yeast extract
5g/L NaCl

Adjust pH to 7.0 with NaOH.

LB plates with ampicillin:

Add 15g agar to 1 L of LB medium. Allow the medium to cool to 50 °C before adding ampicillin to a final concentration of 100μg/ml. Pour 30ml of medium into 85mm petri dishes. Let the agar harden. Store at 4 °C for up to one month or at room temperature for up to one week.

LB plates with ampicillin/IPTG/X-Gal:

100μl of 100mM IPTG and 20μl of 50mg/ml X-Gal (Appendix B) were spread over the surface of the plate an LB-ampicillin plate and allowed to absorb for 30 minutes at 37 °C prior to use.

Preparation of slide culture for light microscopic examination

A drop of BG11 medium containing Difco agar (1%, w/v) was spread in a thin film over an area of 2×2 cm on a sterile slide and allowed to solidify. A small drop of a liquid culture and medium were placed on the surface of the agar, and a sterile coverslip (2.4×2.4 cm) gently lowered on to the agar surface, the edges then being sealed with sterile vaseline + paraffin (1:1). The slide was examined microscopically, and suitable field for observation selected.

Appendix B: Preparation of Reagents and Buffers

Equilibration of phenol

1. Liquified phenol should be stored at -20C. As needed, melt it at 68C. An equal volume of buffer (usually 0.5M Tris·Cl, PH 8.0 at room temperature) add to the melted phenol. Stir the mixture for 15minutes, when the two phases have separated, aspirate as much as possible of the upper phase using glass pipette.

2. Add an equal volume of 0.1M Tris·Cl (PH 8.0) to the phenol. Stir the mixture for 15minutes. Remove the upper aqueous phase as step1. Repeat the extractions until the pH of the phenolic phase is > 7.8 (with pH paper).

3. After the phenol is equilibrated and the final aqueous phase has been removed, add 0.1 volume of 0.1M Tris·Cl (pH 8.0) containing 0.2% b-mercaptoethanol. The phenol solution may be stored in this form under 100mM Tris·Cl (pH 8.0) in a light-tight bottle at 4C for 1 month.

Phenol:Chloroform:Isoamyl Alcohol (25:24:1)

A mixture consist equal parts of equilibrated phenol and chloroform:isoamyl alcohol (24:1). Neither chloroform nor isoamyl alcohol requires treatment before use. The mixture may be stored under 100mM Tris·Cl (pH 8.0) in a light-tight bottle at 4C for 1 month.

Lysozyme

Prepare a stock solution of lysozyme at a concentration of 50 mg/ml in water. Dispense into aliquots and store at -20°C. Discard each aliquot after use.

Proteinase K

Prepare a stock solution of proteinase K at a concentration of 10 mg/ml in water. Dispense into aliquots and store at -20°C. Discard each aliquot after use.

DNAase Free RNAase

Dissolve pancreatic RNAase (RNAase A) at a concentration of 10mg/ml in 0.01 M sodium acetate(pH 5.2). Heat to 100°C for 15 minutes. Allow to cool slowly to room temperature. Adjust the pH by adding 0.1 volumes of 1 M Tris-Cl (pH7.4). Dispense into aliquots and store at -20°C.

RNAase precipitates when concentrated solutions are heated to 100°C at neutral pH.

Ligase 10× buffer:

300mM Tris-HCL, pH 7.5
100mM $MgCl_2$
100mM DTT
10mM ATP

Store in aliquots at -20 °C. Avoid multiple freeze/thaw cycles.

IPTG stock solution (100mM):

1.2g IPTG, add H_2O to 50ml final volume. Filter-sterilized and store at 4 °C.

X-Gal (2ml):

100mg 5-bromo-4-chloro-3-indolyl-β-D-galactoside, dissolve in 2ml N,N' dimethyl-formamide.

Appendix C: The genera classification of cyanobacteria (after Rippka 1988)

Order Chroococcales (Section I):

Unicellular cyanobacteria, that occur singly or form colonial aggregates held together by additional cell wall layer (sheaths); they reproduce by binary fission or by budding.

I. Reproduction by equal binary fission

A. Thylakoids present

1. Division in only one plane, crosswise, cells rod shaped

a. Sheath layers absent — *Synechococcus* Nageli 1849

b. Sheath layers present — *Gloeothece* Nageli 1849

2. Division in two or more planes, cells coccoid

a. Sheath layers absent — *Synechocystis* Sauvageau 1892

b. Sheath layers present — *Gloeocapsa* Kutzing 1843

B. Thylakoids absent

Division in only one plane, crosswise, cells rod shaped, sheath layers present — *Gloeobacter* Rippka, Waterbury and Cohen-Bazire 1974

II. Reproduction by repeated budding at the apical end of the cells

Thylakoids present

Division in only one plane, producing small spherical daughter cells from ellipsoidal, cylindrical, club-, or pear-shaped mother cells — *Chamaesiphon* Braun and Grunow 1865 emend. Geitler 1925 (sensu Waterbury and Stanier 1977)

Order Pleurocapsales (section II)

Unicellular cyanobacteria that occur singly or form colonial (often pseudofilamentous) aggregates; mature cells are always enclosed by an additional fibrous outer cell wall layer; they reproduce by multiple fission only or by binary fission followed by multiple fission; the baeocytes, at the moment of their release,

are either motile and lack the fibrous outer cell wall layer or possess the latter structure and are immotile.

I. Reproduction uniquely by multiple fission

A. Vegetative growth leads to the formation of spherical cells of varying size

1. Baeocytes motile — *Dermocarpa* Crouan and Crouan 1858 (sensu Waterbury and Stanier 1978)

B. Growth and binary fission lead to cubical aggregates

1. Multiple fission yields motile baeocytes — *Myxosarcina* Printz 1921 (sensu Waterbury and Stanier 1978)

2. Multiple fission yields immotile baeocytes — *Chroococcidiopsis* Geitler 1933 (sensu Waterbury and Stanier 1978)

C. Growth and binary fission lead to irregular, pseudofilamentous cell aggregates

1. Multiple fission yields motile baeocytes — *Pleurocapsa* group

a. Baeocytes enlarge symmetrically — Type I

b. Baeocytes develop early polarrity — Type II

Order Oscillatoriales (Section III)

Filamentous cyanobacteria; trichomes composed uniquely of vegetative cells, even in the absence of combined nitrrogen; reproduction by intercalary cell division in only one plane and random transcellular trichome breakage; hormogonia that are structurally distinguishable from parental trichomes are never produced; nonsheathed (or thinly sheathed?) members generally exhibit gliding motility, which may or may not involve rotation around the long axis of the trichomes; firmly ensheathed immotile filaments may release transiently motile trichomes that lack heavy sheaths.

I. Trichome helically coiled

A. Shape of cells not fixed; little or no consteictions between adjacent cells; sheat formation never pronounced; probably transcellular trichome breakage; motility involves rotation around the long axis of the trichomes

1. Cross walls invisible by light microscopy *Spirulina* Turpin 1827

2. Cross walls visible by light microscopy *Arthrospira* Stitzenberger 1852

II. Trichome straight

A. Cells that compose trichome are disk shaparated by shallow constrictuins (if any); end cells different in shape to intercalary cells; transcellular trichome breakage

1. Trichome motile, not heavily ensheathed; motility involves rotation around the long axis of the trichome *Oscillatoria* Vaucher 1803 (sensu Rippka *et al.* 1979)

2. Trichome immotile and heavily ensheathed; motility restricted to sheathless (or thinly sheathed?) trichomal fragments released from the ends of the immotile ensheathed parental filaments; whether motility involves rotation around the long axis of the trichome is unknown LPP group A ("*Lyngbya aestuarii*" type, this proposal)

B.Cells that compose trichome are isodiametric, cylindrical, or barrel shaped; the degree of constriction between adjacent cells varies; inter- or transcellular trichome breakage; motility(if exhibited) may or maynot involve rotation around the long axis of the trichome; sheath production and false branching variable

1. Trichome motile, not ensheathed; cells contain polar gas vacuoles, are cylindrical or barrel shaped, and are generally separated from one another by pronounced constrictions; intercellular trichome breakage' motility not involving rotation around the long axis of the trichome *Pseudanabaena* Lauterborn 1905 (sensu Rippka *et al.* 1979)

2. Properties not as above LPP group B

a. Trichome composed of isodiameteic(or somewhat shorter) cells(2-3μm wide) that are not separated by deep constrictions; probably transcellular trichome breakage; some members produce thin sheaths and may exhibit false branching; motility is never pronounced and does not involve rotation around the long axis of the trichome; thylakoids resteicted to the peripheral regions of the cells; generally hosts to cyanophage LPP-1

LPP group B: type I

("*Plectonema boryanum*"
type, yhis proposal)

b. Properties different to above (this assemblage harbors members that vary greatly with respect to cell morphology, degree of consteictions between cells, sheath peoperties, motility, and genetic complexity; it is probably representative of several different genera)

LPP group B: type X

Order Nostocales (Section IV)

Filamentous cyanobacteria that divide in only one plane; in the absence of combined nitrogen, a certain percentage (~10%) of the vegetative cell populstion differentiates into heterocysts, the specific cellular sites of aerobic N_2 fixation; akinetes (resting cells), resistant to desiccation and cold, are produced by some members; reproduction occurs by random trichome breakage, by germination of akinetes(if produced) or, in addition, by formation of hormogonia (sersu Rippka *et a.* 1979).

I. Hormogonia not produced

A. Trichome never exhibits basal-apical polarity

1. Heterocysts are differentiated from terminal and intercalary cells; the position of akinetes (if produced) is variable

a. Vegetative cells are cylindrical, spherical, or ovoid

Anabaena Bory de St. Vincent 1822

b. Vegetative cells are disk shaped *Nodularia* Mertens 1822

2.Heterocysts are differentiated exclusively from the terminal cells at both ends of the trichome; akinetes are located adiacent to heterocysts

Cylindrospermum Kutzing 1843

II. Hormogonia produced

A. Trichome never exhibita basal-apical polarity

1. Hormogonia give rise to young trichomes that differentiate terminal heterocysts at both ends of the cellular chain

a. Mature trichomes are composed of barrel-shaped, spherical, or ovoid vegetative cells and differentiate heterocysts in both intercalary and terminal

positions; they are immotile and may be enclosed by sheath material of variable consistency; some adult trichomes take on a coiled configuration; akinetes (if produced) differentiate away from the heterocysts and are often formed in chains; hormogonia are composed of cells that are generally smaller in size, different in shape (often cylindrical or isodiametric), and may contain polar or irregularly distributed gas vacuoles; they are sheathless and often exhibit active gliding motility, which (as gas vacuolation) is lost with their maturation toadult trichomes *Nostoc* Vaucher 1803

(sensu Rippka *et al.*1979)

2. Hormogonia give rise to young trichomes that differentiate heterocysts from the terminal cells at only one end of the cellular chains

a. Mature trichomes are composed of disk-shaped, isodiameteic, or cylindrical cells and differentiate heterocysts predominantly in an intercalacy position; they are immotile, heavily ensheathed, and exhibit frequent false branching. (leading on solid media to aerial growth); akinetes not produced; hormogoniaare composed of cells that are not markedly different in size or shape (although they are generally somewhat shorter) than those of the mature filaments and do not contain gas vacuoles; active gliding motility of the sheathlless (or thinly sheathed?) hormogonia is never pronounced or is absent(?)

*Scytonema A*gardh 1824

(sensu Rippka *et al.* 1979)

B. Mature trichome exhibits basal-apical polarity

1. Hormogonia give rise to young trichomes that differentiate a single terminal cell into a heterocyst

a. Mature trichome composed of cells that are disk shaped., isodiametric, or cy-lindrical, and whose size decreases from the base to the apex; young trichomes bear exclusively terminal heterocysts at the basal end; mature trichomes are immotile and may contain intercalary heterocysts; differentiation of akinetes (if produced) is initiated adjacent to basal heterocysts, and they may be formed in chains; sheath production variable; false branching frequent in ensheathed members; hormogonia are sheathless (or thinly sheathed), generally motile, and composed of cells that are smaller in size and different in shape; some hormogonial cells contain gas vacuoles.

Calothrix Agardh 1824

(sensu Rippka *et al.* 1979)

i. Degree of tapering little pronounced, even after growth in the absence of combined nitrogen; hormogonia (normally motile) are composed of isodiametric cells that contain phycoerythrin and adapt chromatically

Calothrix: type I

ii. Properties not as above *Calothrix*: type X

Order Stigonematales (Section V)

Filamentous cyanobacteria capable of divisions in more than one plane; in the absence of combined nitrogen, heterocysts are formed that are in terminal, intercalary, or lateral positions; reproduction by random trichome breakage, germination of akinetes (if produced), or hormogonia production.

I. Hormogonia produced

A. Trichom never exhibits basal-apical polarity

1. Hormogonia give rise to young filaments that differentiate terminal or intercalary heterocysts

Cells of mature trichomes are spherical and divide in more than one plane; subsequent detachment of resulting multicellular groups (derived from the individual cells that constituted the primary trichome) leads to a mode of growth resembling that of chroococcalean cyanobacteria (i.e., *Gloeocapsa,* see Section I); heterocysts in the multicellular aggregates are terminal or lateral (with respect to the long axis of the original trichome); rapid division of some cells in only one plane leads to the production of uniseriate, motile hormogonia, composed of small cylindrical cells; gas vacuoles not produced

Chlorogloeopsis Mitra and Pandey 1966

2. Hormogonia give rise to young trichomes in which heterocyst differentiation occurs exclusively in an intercalary position

Cells of mature trichomes are barrel shaped to spherical and may divide in more than one plane; concomitant division of some cells in only one plane leads to multiseriate primary trichomes with lateral uniseriate branches; heterocysts in the multiseriate trichomes are predominantly terminal or lateral, those of the branches are uniquely in an intercalary position ; hormogonia , composed of small cylindrical cells, are produced by rapid division from the ends of the primary

trichomes or from the lateral branches and are generally motile; gas vacuoles not produced; akinetes, formed in some members, occur in chains

Fischerella Gomont 1895

(sensu Rippka *et al.* 1979)

MOLECULAR COMPARISONS OF CYANOBACTERIAL FREE LIVING AND LABORATORY STRAINS.

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Taxonomic studies on cyanobacteria have traditionally used methods such as morphological characteristics and DNA G/C content. Recently molecular studies based on the use of polymerase chain reaction (PCR) techniques and RFLP (restriction fragment length polymorphism) analysis have become widely used. Comparisons of the techniques and their efficacy in strain differentiation is needed, in particular to enable analysis of the strains involved in the recent increase in free living, cyanobacterial growth.

We have isolated and purified a number of free living cyanobacteria from local ponds. These ponds varied in size and aspect but did not appear to be associated with high nutrient levels. From these ponds both unicellular and filamentous strains were obtained and a number of techniques are being used to characterise these stains. Comparisons with laboratory strains are also being performed. So far the isolated strains have mainly been characterised by morphological characteristics whilst both genomic and plasmid DNA has been isolated and analysed from the laboratory strains. RAPD (randomly amplified polymorphic DNA) analysis of the genomic DNA gave very distinct banding patterns using both the normal 10 mer primers as well as 17 mer primers for PCR amplification. This anallysis is now being applied to the isolates. However, it must be noted that careful choice of conditions and thermostable enzyme used was required for consistent results. Results from our analysis so far performed will be presented and discussed along with the associated difficulties in comparing molecular analysis results of the RAPD type from different laboratories.

P-13-018

ALTERATIONS OF THE PHOTOSYNTHETIC APPARATUS IN A *SYNECHOCYSTIS* SP. PCC 6803 ORF184 MUTANT

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We cloned and sequenced a gene of the unicellular cyanobacterium *Synechocystis* 6803 that is homologous to the conserved chloroplast open reading frame (ORF) 184. The function of the putative gene product remained unknown till present. *Synechocystis orf*184 mutants were constructed by insertional inactivation and deletion. We have investigated the effects of this mutation on kinetics and composition of components of the linear electron transport chain. Besides extensive changes in photosystem (PS)I- to PSII ratio, antenna size and quantum yield, we found also alterations in kinetic parameters as light dependent slope of PSII fluorescence yield and PSI donor and acceptor limitation, respectively. Further analyses concerning effects of photosynthetic and respiratory inhibitors as well as excitation energy transfer efficiency elucidated the role of the putative ORF184 protein in photosynthesis.

P-13-019

SIGNAL TRANSDUCTION DURING CHROMATIC ADAPTATION

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We have used a combination of site directed mutagenesis (SDM) and complementation of novel mutants to extend our understanding of the mechanisms controlling complementary chromatic adaptation (CCA). Found in a number of cyanobacteria, CCA is an adjustment of the phycobilisomes' composition in response to changes in ambient light quality. Previous work demonstrated that one of the components controlling CCA, RcaC, shows strong sequence similarity to response regulator proteins of two component regulatory systems. The activity of such proteins is controlled by the reversible phosphorylation of an aspartate (D) residue found within a domain of highly conserved amino acids. RcaC contains two such putative D phosphorylation domains, at the amino- and carboxy-terminal ends. SDM of the D within each of these domains demonstrated that the amino-terminal D is primarily responsible for the control of CCA and established the relationship between the phosphorylation state of this D and the physiological state of the cells. We have also gained insights into photoperception and signal transduction during CCA by complementing a number of new mutants.

P-13-020

CLONING AND CHARACTERIZATION OF *SEC* AND *FFH* GENES FROM THE CYANOBACTERIUM *Synechococcus* PCC7942

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Cyanobacteria have an internal thylakoid membrane network in addition to the cell envelopes that consist of the outer and the inner cytoplasmic membranes. Distinct protein compositions between the cytoplasmic and the thylakoid membranes suggest the presence of a mechanism of protein sorting between the two membranes in cyanobacterial cells. We have cloned *secY* and *secA* gene homologs from the genome of cyanobacterium *Synechococcus* PCC7942; SecA, a peripheral membrane protein, and SecY, an integral membrane protein, are known to play essential roles in the protein translocation across the cytoplasmic membrane in *E. coli*. The cyanobacterial *sec* genes are likely single-copy genes and their gene products are localized in both the cytoplasmic and thylakoid membranes, suggesting that the same Sec proteins probably mediate protein translocation across both the cytoplasmic and thylakoid membranes in the cyanobacterium. Furthermore, we have cloned a cyanobacterial *ffh* gene which encodes a component of putative bacterial homolog of signal recognition particle (SRP). Intracellular localization of the Ffh protein and its possible involvement in protein transport in the cyanobacterial cells are under investigation.

P-13-021

Several Methods for Isolation and Characterisation of Plasmid DNA in Cyanobacteria

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Plasmid distribution among four different cyanobacteria was studied using six simplified method which involved direct agarose gel electrophoresis of heat-treated, ethanol-precipitated, plasmid preparations from the cleared lysates without requiring ultracentrifugation and plasmid screening from different growth period culture. Two of these methods, based on lysis by lysozyme and lysis by SDS, were successful. The latter method proved more sensitive. All of strains examined possessed one or two plasmids, and mid-exponential phase culture was optimal for all plasmids and large plasmid yield obtained. These procedures permitted the selective isolation of plasmids DNA that can be used directly in electrophoretic analysis and restriction endonuclease analysis.

P-13-022

GENERATION AND CHARACTERIZATION OF A PHOTOHETEROTROPHIC MUTANT OF *Synechocystis* sp. PCC 6803

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Cyanobacterium *Synechocystis* sp. PCC 6803 performs oxygenic photosynthesis analogue to that of higher plants. The reaction center of photosystem I (PSI) is composed of a heterodimer of homologous polypeptides, PsaA and PsaB. In order to investigate the structure and function of the PSI, cartridge mutagenesis technique was used to inactivate the *psaB* gene which encodes subunit Ib (PsaB protein) of PSI. The *psaB*⁻ mutants were generated by transforming wild type cells with the *psaB* gene of *Synechococcus* 7002 which was interrupted by a chloramphenicol acetyltransferase gene. Unexpectidly, many kinds of mutants having different color were obtained. One of the mutants, B2, was selected for further dissection. The B2 mutant would not grow photoautotrophically, which is the common characteristics of photosystem mutants. The amount of chlorophyll of the mutant was greatly reduced. Oxygen uptake rate of the mutant was ca. 60% of wild type. Immunodetection analysis indicated that the mutan has reduced amount of PsaA/PsaB, but a normal range of PsaC and PsaD. This mutant will be used to study structure/function relationship and biogenesis of PSI.

P-13-023

SOME STUDIES WITH MUTANT STRAINS IN WHICH SPECIFIC GENES ENCODING SUBUNITS OF PHOTOSYSTEM I WERE INACTIVATED.

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In order to clarify functions of subunits of photosystem I complex (PSI), we have conducted biochemical analysis of PsaD-, E-, F-, and I-less mutant strains of Synechocystis sp. PCC6803.

We observed slow photoautotrophic growth of PsaD-less mutant at all temperatures examined and of PsaE-less mutant under 22°C. Western blot analysis showed the absence PsaC in thylakoid membranes from PsaD less mutant. Thus, PsaC is unstable in PSI in the absence of PsaD. Photoautotrophic growth of PsaD- and PsaE-less mutants were severely inhibited at high light intensity (1200 uE/m²/s). This photoinhibition was further increased under low temperature. Possible fuctions of PsaD and PsaE in relation to photoinhibition will be discussed.

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Identification of cyanobacteria by polymorphisms of PCR-amplified ribosomal DNA spacer region

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Abstract

The 16S-23S ribosomal DNA spacer region of selected cyanobacterial strains was amplified by the polymerase chain reaction using primers to conserved flanking sequences. Single or multiple rDNA amplification products were generated depending on the strain and primer pair. Species could generally be distinguished on the basis of size heterogeneity of the products. Analysis of restriction digests of the amplified rDNAs indicated polymorphisms useful in identification. Four enzymes (*Hinf*I, *Dde*I, *Alu*I, *Taq*I) generated restriction fragment length patterns that could discriminate between the cyanobacteria to the taxonomic levels of genus and species. This approach should prove useful in the rapid identification of cyanobacteria.

Keywords: Ribosomal DNA; Spacer region; Cyanobacteria; Polymorphism

1. Introduction

The cyanobacteria are one of the largest subgroups of Gram-negative photosynthetic prokaryotes, containing a number of diverse genera and species [1]. Traditional techniques for identification and systematics of cyanobacteria have relied primarily on observed morphological characteristics. However, morphology may change depending on environmental conditions (e.g. [2]) and the diversity of strains within a culture may be suppressed by selective culturing conditions [3]. Photosynthetic pigment content, lipid composition, differentiated cell structures and other characters may also be subject to change because of the variable expression of cyanobacterial gene products in culture [4,5]. The limitations of such methods have prompted the development of molecular biological techniques for identification of cyanobacteria.

The randomly amplified polymorphic DNA (RAPD) technique in conjunction with the polymerase chain reaction (PCR) has been used to characterise cyanobacteria in pure culture and in symbiotic associations [6,7]. This approach has been employed to identify many organisms to the strain level of classification [8,9], but the requirement for axenic cultures restricts the applicability of the RAPD technique. Genetic characterisation of cyanobacterial strains has also been undertaken using restriction fragment length polymorphisms of the intergenic spacer of the genes encoding phycobilisome subunits (*cpc BA*) [10].

In this study we have developed a PCR-based

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technique that requires only small quantities of relatively impure DNA, and can distinguish between the various genera and species of cyanobacteria by using the variability of the ribosomal DNA spacer region. A number of cyanobacterial 16S rRNA genes and the internal transcribed spacer (ITS) region between 16S and 23S rRNA genes have been sequenced for taxonomic purposes [11 16]. The high interspecific variability reported for the spacer makes it an appropriate candidate for restriction fragment length polymorphisms (RFLPs). The approach we have therefore taken has been to amplify the 16S-23S rDNA spacer from cyanobacteria by PCR and test various restriction enzymes to find those producing RFLPs useful in identification. The primers used recognise conserved regions of cyanobacterial rDNA in the flanking 16S and 23S genes [12].

2. Materials and methods

2.1. Cyanobacterial cultures

The cyanobacterial strains used are described in

Table 1
Cyanobacteria used and the sizes of rDNA fragments amplified by primer pairs 1/18, 14/18 and 17/18

Strain	Source[a]	Amplified rDNA (bp)[b]		
		Primer 1/18	Primer 14/18	Primer 17/18
Unicellular				
Chroococcus prescottii	BSM	1865	555	310
Gloeocapsa sp	BSM	1910	560	322
Microcystis aeruginosa	BSM	1905	585	320
Synechococcus PCC 7942	LJMU	2100	735	425
Synechococcus CCAP 1479/7	LU	2400	1010	665
Synechococcus WH 7803	LU	2300	930	625
Synechocystis PCC 6803	UW	1940	665	390
Filamentous non-heterocystous				
Oscillatoria animalis	BSM	2200	840	575
Phormidium autumnale	BSM	2200	800	535
Pseudanabaena sp LJM 001	LJMU	2075	735	450
Filamentous heterocystous				
Anabaena ATCC 27892	LU	1875,2100 (2300,3642)	515,730 (665,857,960,1100)	435
Anabaena ATCC 27892 R3 2	LU	1875,2100 (2300,3642)	515,730 (665,857,960,1100)	435
Anabaena cylindrica	UW	1890 (2150,2230)	575 (723,797,948,1036,1217)	485
Anabaena flos-aquae D0848	UD	1836 (2065)	535 (797,887,995)	430
Anabaena PCC 7120	UW	1875,2100 (2300,3642)	515,730 (665,857,960,1100)	435
Anabaena sp D0697	UD	1836 (2065,2230)	530 (695,750,887,995)	430
Aphanizomenon gracile	BSM	1780,2350	450,858 (329)	440
Chlorogloeopsis Chlorogloea fritschii	LJMU	2018,2185 (2300)	593 (730,980)	480
Fischerella muscicola	BSM	2018 (2105,2340)	530 (730,930)	475
Gloeotrichia sp	UW	1940,2300 (2465)	494,845 (1029,1170)	586
Nostoc commune	BSM	1970,2000,2190 (2460,3943)	560,790 (705,985,1230)	535
Nostoc ellipsora	UW	1875,2100 (2300,3642)	515,730 (665,857,960,1100)	435
Nostoc linckia D0734	UD	1930 (2190,2400)	530,686,790 (930,1050,1182)	445
Nostoc MAC PCC 8009	UW	2090 (1979, 2320)	710 (630, 960)	495
Nostoc MAC R1	LU	2090 (1979,2320,1876)	710 (630,960,540)	495
Nostoc muscorum	BSM	2195 (1875,2005,3642)	780 (510,675,756,857,960,1100)	435
Nostoc piscinale D0201	UD	1890,2190 (2410,3743,4133)	490,675,760 (880,985,1182)	490
Nostoc planctonicum	BSM	1875 (2050)	515 (675,740,867)	520
Nostoc sp D0740	UD	1890 (2190,2500)	490,745 (675,930,1100)	445
Tolypothrix distorta	BSM	2175 (1821)	682 (602)	440

[a]LJMU, UW, BSM, LU, UD: Liverpool John Moores University, University of Warwick, Biobred Sciento Manchester, Lancaster University and University of Durham, respectively.

Data are the means of three determinations. Figures in parentheses are secondary fragments.

Table 1. Strains were grown in BG11 liquid medium or MN liquid medium for *Synechococcus* WH 7803 [17] in an illuminated orbital incubator at 36 ± 1°C bubbling with CO_2 enriched air. Strains were maintained on BG11 or MN agar as appropriate.

2.2. DNA preparation for PCR

Total genomic DNA was prepared according to a modified method of Porter [18]. A pelleted 10 ml aliquot of mid to late exponential phase culture was washed and resuspended in 0.5 ml of sucrose buffer (10% (w/v) sucrose, 100 mM EDTA pH 8.0, 50 mM Tris-HCl pH 8.0). Lysozyme (to 5 mg ml^{-1}) was added and, following incubation at 37°C for 30 min, 6 μl of proteinase K (10 mg ml^{-1}) and 60 μl of 10% (w/v) sodium dodecyl sulfate were added. The mixture was incubated at 50°C for 2 h, chilled on ice and extracted with an equal volume of phenol-chloroform-isoamylalcohol (25:24:1), followed by chloroform-isoamylalcohol (24:1). The supernatant was added to 1/3 volume of 10.5 M ammonium acetate. DNA was precipitated by the addition of isopropanol (−20°C) followed by centrifugation for 5 min at room temperature. The pellet was washed with 70% (v/v) ethanol, dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at −20°C. In a second method, a 1 ml aliquot of mid to late exponential phase culture was pelleted by centrifugation, the cells were washed, resuspended in 0.1 ml of TE buffer and used directly for PCR.

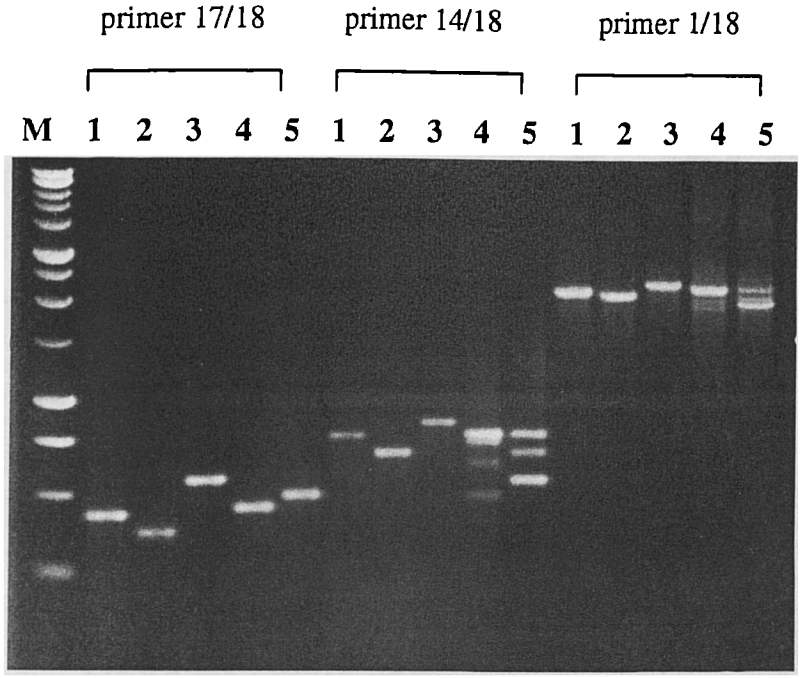
2.3. PCR and digestion of amplified DNAs

Each 100 μl reaction mixture for PCR contained 40 pmol of each primer, 2.5 units of *Taq* polymerase (Bioline), 0.2 mM deoxyribonucleoside triphosphates (dATP, dCTP, dGTP and dTTP), 3 mM $MgCl_2$ and 0.1 μg cyanobacterial DNA or 1 μl TE-buffered culture. The reaction mixture was overlaid with mineral oil, and thermal cycling was achieved in a Perkin Elmer Cetus thermal cycler according to the following programme: initial denaturation at 94°C for 3 min, 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 3 min. Primers 1, 14, 17 and 18 as described by Wilmotte [12] were used. DNA negative controls consisted of the standard PCR reaction mixture, but with the DNA template replaced by an equal volume of water. Amplified DNA (10 μl, approximately 0.5–0.8 μg) was digested without further purification with various restriction enzymes. *Dde*I, *Taq*I, *Alu*I, *Sau*3A, *Hae*III, *Pvu*I, *Xba*I, *Sal*I, *Sac*I, *Ava*I, *Bam*HI, *Hin*dII, *Hin*dIII, *Pst*I, *Bgl*II, *Eco*RV (from Boehringer Mannheim, Germany), and *Hin*fI, *Hha*I, *Hpa*II, *Rsa*I (from Sigma, UK) according to the manufacturers' instructions. The products were separated on gels containing 2% (w/v) agarose NA (nucleic acids) +1% (w/v) agarose MP (multi purpose). Appropriate molecular mass markers (123 bp) (Sigma, UK) were run on each gel. Each band visualised on a gel was measured using the Phoretix ID (version 2.51) package of Phoretix International Ltd, UK. Analysis of these data was performed by calculating pairwise genetic distances from a binary matrix. Distances were calculated using the 14 algorithms supplied by the RAPDistance package (version 1.03) of Armstrong et al. [19]. The resulting dendrogram was constructed using the neighbour-joining method.

3. Results and discussion

A number of primer pairs were tested for amplification of the 16S-23S rDNA spacer with a view to generating products useful for discriminating between cyanobacterial strains. The PCR was performed either directly on cyanobacterial cultures or on DNA extracted from them. In each case identical results were obtained. The choice of primers was critical to the use of spacer variation for species identification. In line with the work of Nelissen et al. [16] we have found that primer 18 is very cyanobacteria-specific, preferentially amplifying cyanobacterial DNA, even in the presence of contaminating eubacterial DNA (data not shown). Primer pair 1/18 (which amplifies 16S rDNA, ITS region, $tDNA^{ile}$ (or $tDNA^{ile}$ and $tDNA^{ala}$) and 5′ end of 23S rDNA) and 14/18 (which amplifies 3′ end of 16S rDNA, ITS region, $tDNA^{ile}$ (or $tDNA^{ile}$ and $tDNA^{ala}$) and 5′ end of 23S rDNA) amplified the rDNA to give multiple DNA bands of different intensities for a number of the species tested. The intense bands have been classified as primary products, the weaker variable bands as secondary products (Table 1 and Fig. 1A). Such classification of product

A


primer 17/18
primer 14/18
primer 1/18
M 1 2 3 4 5 1 2 3 4 5 1 2 3 4 5

B

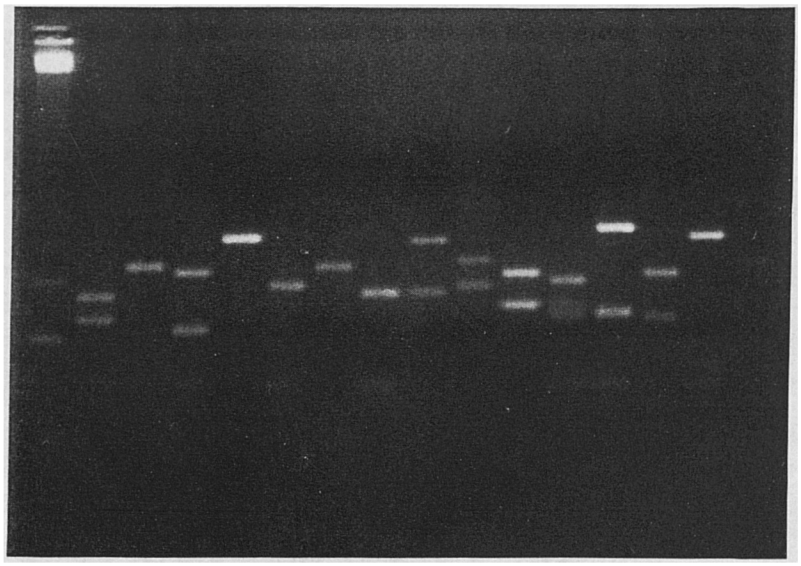

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Fig 1 Agarose electrophoresis gels of amplified rDNA spacer products and RFLP patterns of selected cyanobacterial strains. A: PCR amplification using primer pairs 17/18, 14/18 and 1/18. Lanes: M, 1 kb DNA ladder; 1, *Synechococcus* PCC 7942; 2, *Synechocystis* PCC 6803, 3, *Oscillatoria animalis*, 4, *Nostoc muscorum* and 5, *Anabaena cylindrica*. B: RFLP patterns of rDNA spacer amplified using primers 17 and 18 and digested by *Taq*I. Lanes. M, 123 bp DNA ladder; 1, *Synechococcus* PCC 7942; 2, *Synechococcus* CCAP 1479/7; 3, *Synechococcus* WH 7803 4, *Synechocystis* PCC 6803; 5, *Microcystis aeruginosa*; 6, *Gloeocapsa* sp.; 7, *Chroococcus prescottii*; 8, *Oscillatoria animalis* 9, *Phormidium autumnale*; 10, *Pseudanabaena* sp LJM 001; 11, *Aphanizomenon gracile*; 12, *Gloeotrichia* sp.; 13, *Tolypothrix distorta*, 14, *Chlorogloeopsis fritschii*; 15, *Fischerella musicola*.
←

profiles has been reported in the amplification of the 16S-23S rDNA spacer of other bacteria (e.g. [20]). Weak bands were common in the heterocystous genera *Nostoc* and *Anabaena*, which may reflect rDNA heterogeneity for heterocyst differentiation. An explanation for the secondary amplification products could be that heteroduplexes form, containing conserved homologous sequences at the 3′ and 5′ ends separated by highly variable ITS sequences. These heteroduplexes would migrate more slowly in gels than the expected homoduplexes because of the single-stranded regions [21].

The pattern and length heterogeneity of the amplified bands was generally sufficient to resolve the different genera and species with the exception of *Nostoc ellipsora*, *Anabaena* PCC 7120, *Anabaena* ATCC 27892 and *Anabaena* ATCC 27892 R3.2 (which carries cyanophage AN23 DNA), which all shared the same sized fragments. However, a different RAPD DNA profile was generated in each case (data not shown). The same primary fragment of 2090 bp (with primer 1/18) or 710 bp (with primer 14/18) was generated for both strains of *Nostoc MAC*, with two weak secondary fragments in each case. However, strain R1 showed a further weak fragment, using either primer pair. For the unicellular and non-heterocyst-forming filamentous cyanobacteria a single amplified rDNA product was obtained using either of these primer pairs. Such variability in products generated may be due to differential specificity of primers, variation in copy number and microheterogeneities of the 16S-23S rDNA spacer regions

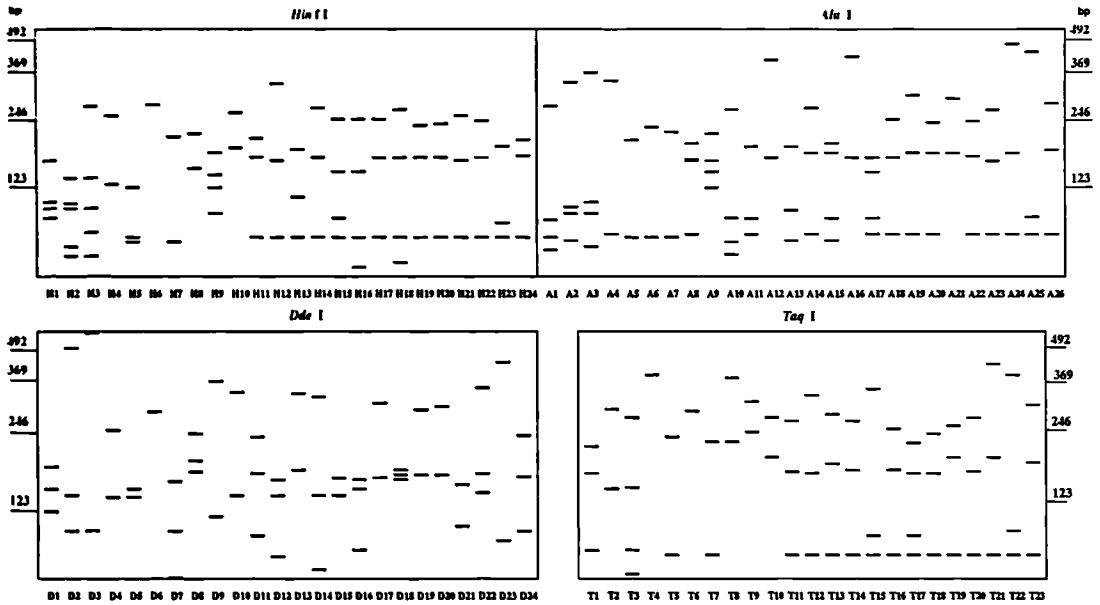


Fig 2. Diagrammatic representation of RFLP patterns of amplified rDNA spacer products (using primers 17 and 18) from selected strains of cyanobacteria with the restriction enzymes *Hin*fI, *Dde*I, *Alu*I and *Taq*I. The numbers below the tracks correspond to the RFLP patterns in Table 2.

Table 2
RFLP patterns and pattern types obtained for selected cyanobacterial strains

Strain	Pattern type[a]	*Hin*fI	*Dde*I	*Alu*I	*Taq*I
Unicellular					
Chroococcus prescottii	A	H7	D7	A7	T7
Gloeocapsa sp	B	H6	D6	A6	T6
Microcystis aeruginosa	C	H5	D5	A5	T5
Synechococcus PCC 7942	D	H1	D1	A1	T1
Synechococcus CCAP 1479/7	E	H2	D2	A2	T2
Synechococcus WH 7803	F	H3	D3	A3	T3
Synechocystis PCC 6803	G	H4	D4	A4	T4
Filamentous non heterocystous					
Oscillatoria animalis	H	H8	D8	A8	T8
Phormidium autumnale	I	H9	D9	A9	T9
Pseudanabaena sp LJM 001	J	H10	D10	A10	T10
Filamentous heterocystous					
Anabaena ATCC 27892	K	H17	D17	A18	T16
Anabaena ATCC 27892 R3 2	K	H17	D17	A18	T16
Anabaena PCC 7120	K	H17	D17	A18	T16
Anabaena cylindrica	L	H18	D18	A19	T17
Anabaena flos-aquae D0848	M	H19	D19	A21	T18
Anabaena sp D0697	N	H19	D19	A20	T18
Aphanizomenon gracile	O	H20	D20	A22	T19
Chlorogloeopsis Chlorogloea fritschii	P	H23	D23	A25	T22
Fischerella musicola	Q	H24	D24	A26	T23
Gloeoetrichia sp	R	H22	D22	A24	T21
Nostoc commune	S	H12	D12	A12	T12
Nostoc ellipsora	K	H17	D17	A18	T16
Nostoc linckia D0734	T	H14	D15	A15	T14
Nostoc MAC PCC 8009	U	H11	D11	A11	T11
Nostoc MAC R1	U	H11	D11	A11	T11
Nostoc muscorum	V	H16	D17	A17	T16
Nostoc piscinale D0201	W	H13	D13	A13	T13
Nostoc planctonicum	X	H15	D16	A16	T15
Nostoc sp D0740	Y	H14	D14	A14	T14
Tolypothrix distorta	Z	H21	D21	A23	T20

Details of the patterns (H1 H24, D1 D24, A1 A26 and T1–T23) for amplified (primer pair 17/18) rDNA digests are given in Fig. 2. Pattern type derives from the pattern combination with the four test restriction enzymes.

among cyanobacteria. The pattern of amplified products with primer pair 14/18 enabled clearer discrimination between species than that with primer pair 1/18.

Analysis of length polymorphism and RFLPs of the amplified rDNA spacer using primer pair 17/18 has generally permitted discrimination between the cyanobacteria tested to genus and species level. This primer pair (which amplifies $tDNA^{ile}$ (or $tDNA^{ile}$ and $tDNA^{ala}$), ITS region and 5′ end of 23S rDNA) amplified rDNA from all the strains tested, generating a single band (Table 1 and Fig. 1A). This amplified DNA was digested with 20 different restriction enzymes to identify RFLPs. Most of the enzymes did not cut the DNA or only very small fragments were cut from the ends. However, *Hin*fI, *Dde*I, *Alu*I and *Taq*I showed potentially useful discrimination among the different genera and species (Figs. 1B and 2). In most cases, a different RFLP pattern was generated (generally of three bands) for each species tested, with each restriction enzyme used. The organism could be assigned an RFLP pattern type on the basis of the four enzymes used (Table 2). For strains of the same species the same pattern type was obtained. Different species and genera had different pattern types, with the exception of *N. ellipsora*, *Anabaena* PCC 7120, *Anabaena* ATCC 27892 and *Anabaena* ATCC 27892 R3.2.

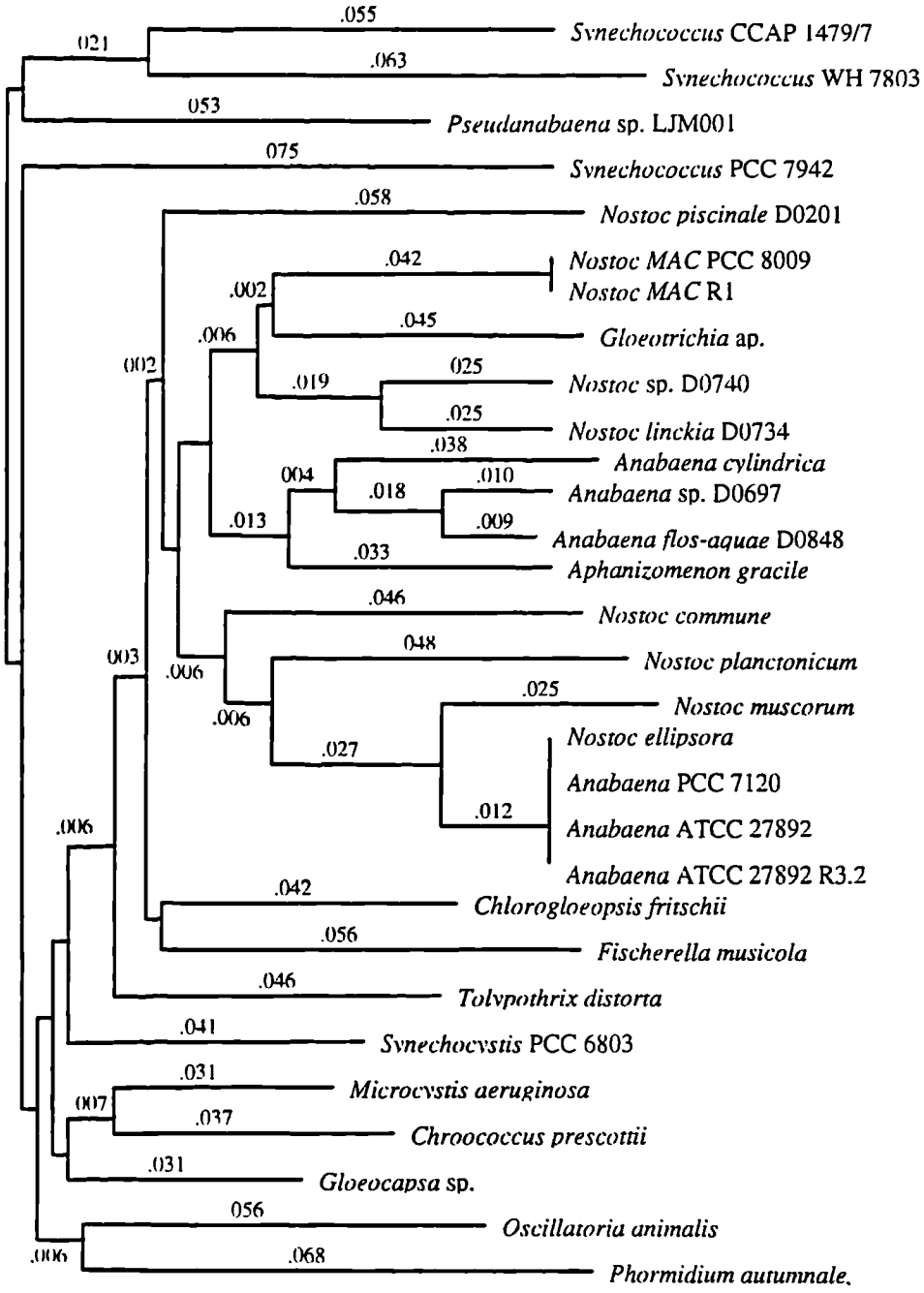


Fig. 3. Dendrogram indicating genetic distances among cyanobacterial strains. The genetic distance from the node, based on the RFLP profiles of 30 strains, is indicated on each branch.

An identical length (435 bp) and RFLP pattern type (K) were obtained for these cyanobacteria, indicating that they may be in the same species group. This is consistent with results using primer pairs 1/18 and 14/18 where identical product profiles were obtained for these strains. In addition pattern type K showed

very high similarity to pattern type V for *Nostoc muscorum*. Strains of the genus *Nostoc* are closely related to, and often confused with, those of the genus *Anabaena* [22], particularly when morphological characteristics are used for identification. From this study, morphologies of *N. ellipsora* and *Anabaena* PCC 7120 were very similar (data not shown), although previously PCC 7120 had been reported to be closer to *Anabaena* than to *Nostoc*, when using this criterion [17]. By contrast, on the basis of DNA-DNA hybridisation data and hybridisation patterns with highly repetitive (STRR) DNA sequences the strain has been renamed *Nostoc* sp. PCC 7120 [23,24]. This confusion highlights some of the difficulties in identification and taxonomy of cyanobacteria, which is exacerbated by continued use of traditional nomenclature and the botanical code. A dendrogram (Fig. 3) constructed on the basis of pairwise genetic distances using the combined RFLP data from the four restriction enzymes further indicates the similarity between *Anabaena* and *Nostoc* strains. In addition a low similarity is revealed between the freshwater *Synechococcus* PCC 7942 and the marine *Synechococcus* WH7803, in line with hybridisation data [25]. Since detailed biochemical and morphological studies were not carried out on some of the strains here, it is not possible to conclude on the correspondence between the genotypic characterisation and other properties of the organisms. However, it is noteworthy that the heterocystous strains form a cluster, excluding all the other strains. This is consistent with 16S rRNA sequence trees (e.g. [16]).

Thus PCR amplification and RFLP analysis of the 16S-23S rDNA spacer yield potentially useful markers for the identification of cyanobacteria. In addition more than one cyanobacterial strain can be detected in a mixed culture (data not shown). With more data it should be possible to generate a database of band patterns, in order to identify unknown isolates by the matching of pattern type and determining similarity to laboratory strains from genotypic analysis. Furthermore, the use of cells as the starting material for the PCR reaction, a procedure already reported for cyanobacteria (e.g. [15]) and confirmed here, should facilitate the development of a rapid and reproducible method for distinguishing between cyanobacteria.

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