CHAPTER 6

Assessment of the genetic diversity of geographically unrelated *Microcystis aeruginosa* strains using Amplified fragment length polymorphisms (AFLPs)

The contents of this chapter were published in *African Journal of Biotechnology* 4, 389-399, May 2005.

Abstract

Molecular marker analysis is becoming increasingly capable of identifying informative genetic variation. Amplified fragment length polymorphism markers (AFLPs) are among the recent innovations in genetic marker technologies, and provide a greater capacity for genome coverage and more reproducible results than previous technologies. We have investigated the usefulness of AFLP, which is based on the selective amplification of genomic restriction fragments by PCR, to differentiate between geographically unrelated *Microcystis* strains. In total 23 strains were subjected to the AFLP fingerprinting. After analysis of the data on the basis of the average linkage method, known as the Unweighted Pair Group Method using Arithmetic averages (UPGMA), a dendrogram with four clusters was obtained. Cluster 1 consisted mainly of the NIES strains that originated from Japan, while in cluster 2 the European strains grouped together. The South African strains that originated from the northern part of the country group together in cluster 3, while the strains collected from the central and southern regions group together with the US strains in cluster 4. The study had reveals extensive evidence for the applicability of AFLP in population studies, and furthermore clearly demonstrates the superior discriminative power of AFLP towards the differentiation of geographical unrelated *Microcystis aeruginosa* strains that belong to the same species.

Keywords: Molecular markers, distribution, cyanobacteria, genetic diversity**.**

6.1 Introduction

The phylum Cyanobacteria is large and diverse, containing over 1000 species of oxyphototrophs, and its members are classified by using both botanical and bacteriological taxonomic codes (Castenholz *et al.* 1989; Komárek 1991a, b; Komárek & Anagnostidis 1986, 1989; Rippka 1988; Rippka *et al.* 1979). The taxonomy and classification of cyanobacteria has been under investigation since about the middle of the 19th century using morphological and cellular criteria, similar to other microalgae (Kützing 1849; Nägeli 1849 as cited by Komárek 2003). Because of morphological simplicity of most prokaryotes, their classification was previously based largely on physiological properties, as expressed in pure laboratory cultures (Doers & Parker 1988). While field studies relied mostly on morphological analyses of natural populations, laboratory studies concentrated on culture characterisations. The principle of morphological studies includes the use of characteristics observable and measurable under a light microscope, such as shape of colony, presence of sheaths and envelopes, colour of colonies, differentiation and cell content. Based on these criteria, the traditional taxonomic classification systems of cyanophytes placed a high value on cell division patterns, colony formation and relationship to extracellular envelopes and sheaths. Cell shapes and dimensional differences were used largely to distinguish between species within each genus (Doers & Parker 1988). This method caused difficulties in their classification by introducing organisms with different cell organizations but similar cell arrangements to the same generic identity. Furthermore, it required considerable expertise to identify species since both morphological and developmental characteristics can vary with the growth conditions (Evans *et al.* 1976). The main problems met in applying morphological criteria in cyanophyte classification arise from the considerable variability in morphological features with indifferent environmental conditions (Komárek 1991a, b).

Diversification through ecological acclimation, adaptation and stabilization of diverse morpho- and ecotypes, as well as changes caused by mutation and possibly also genome transfers (Rudi *et al*. 1998) can give rise to new cyanobacterial types in different geographical locations. Nearly all populations of cyanobacteria from different geographical locations differ to some degree from each other, and these deviations may stabilize in long term cultures (Waterbury & Rippka 1989; Kato & Watanabe 1993). This process indicates that new forms continually develop and are stabilized under new constant conditions. Diversification within the cyanobacteria is a continuing process in which new types develop from continually modified cyanobacterial genotypes under different environmental conditions at different geographical locations. Many investigations showed that blooms of *Microcystis* spp. can differ in important characteristics (Cronberg & Komárek 1994). The cyanobacteria *Microcystis aeruginosa* is characterized by the existence of a wide variety of genotypes that differ in their content of secondary metabolites (Martin *et al*. 1993; Moore 1996), plasmid content (Kohl *et al*. 1988; Schwabe *et al*. 1988) and interaction with zooplankton (Hanazato 1996). Recent data also reveal variations within cyanobacterial genomes, such as changes in the toxicity of certain strains (Neilan *et al*. 1997) and possible interchanges of genetic material (Rudi *et al*. 1998). Other well-adapted phytoplankton forms, so called traditional species like *Aphanothece, Chroococcus* and *Hyella* appears to persist over the longterm and contain a wide spectrum of stable types for long periods (Komárek & Anagnostidis 1998). Due to the development of recent molecular techniques new approaches have been introduced to the phylogeny and taxonomy of cyanobacteria. Several methods of molecular biology have to date been successfully applied in aid of cyanobacterial

taxonomy: determinations of DNA base ratios (Herdman *et al*. 1979a, b), DNA-DNA hybridisations (Stam 1980; Wilmotte & Stam 1984; Stam *et al*. 1985) and gene sequencing (Masui *et al*. 1988; Nielan *et al.* 1997; Lyra *et al*. 2001; Gugger *et al*. 2002).

The use of DNA-based genetic markers (Bodstein *et al*. 1980) has forever changed the practice of genetics. Over the past 20 years since that discovery, many different types of DNA-based genetic markers have been used for the analysis of genetic diversity, as well as for applied diagnostic purposes (Powell *et al*. 1996). The use of modern molecular biological techniques to determine the degree of sequence conservation between bacterial genomes has led to the development of methods based solely on the detection of naturally occurring DNA polymorphisms. These polymorphisms are a result of point mutations or rearrangements (i.e., insertions and deletions) in the DNA and can be detected by scoring band presence versus absence in banding patterns that are generated by restriction enzyme digestion and DNA amplification procedures. The underlying idea is that variation in banding patterns are a direct reflection of the genetic relationship between the bacterial strains examined and therefore, that these banding patterns can be considered as genomic fingerprints allowing numerical analysis for characterization and identification. AFLP markers have been used to scan genome-wide variations of strains, or closely related species, that have been impossible to resolve with morphological features or other molecular systematic characters. Therefore, AFLP has broad taxonomic applicability and have been used effectively in a variety of taxa including bacteria (Huys *et al*. 1996).

AFLP analysis is based on selective amplification of DNA restriction fragments (Vos *et al*. 1995). It is technically similar to restriction fragment length polymorphism analysis, except that only a subset of the fragments are displayed and the number of fragments generated can be controlled by primer extensions. The advantage of AFLP over other techniques is that multiple bands are derived from all over the genome. This prevents over interpretation or misinterpretation due to point mutations or single-locus recombination, which may affect other genotypic characteristics. The main disadvantage of AFLP markers is that alleles are not easily recognized (Majer *et al*. 1998). PCR has proven to be successful in detecting genetic variation amongst plant-pathogenic fungi, as well as bacteria (Majer *et al*. 1996; Janssen *et al*. 1996). The utility, repeatability and efficiency of the AFLP technique are leading to broader application of this technique in the analysis of cyanobacteria populations (Janssen *et al*. 1996). The purpose of this study was to investigate the usefulness of AFLP, which is based on the selective amplification of genomic restriction fragments by PCR, to differentiate between geographical unrelated strains of *Microcystis* spp.

6.2 Material and Methods

6.2.1 Chemicals, Strains and Culture Conditions

Analytical reagent grade chemicals were purchased from various commercial sources and were used without further purification. *Microcystis aeruginosa* strains used in the study represented a wide variety of geographically unrelated strains (Table 6.1). Strains PCC7806 and PCC7813 were obtained from the Pasteur Institute Culture Collection, France; UV027 from the University of the Free State Culture Collection, South Africa; CCAP1450/1 was obtained from the Culture Collection of Algae and

Protozoa, Institute of Freshwater Ecology, UK; NIES88, NIES89, NIES91, NIES99 and NIES299 from the National Institute for Environmental Studies, Japan; and SAG1 from the Pflanzen Physiologisches Institut, Universität Gottingen, Germany. All these strains were received as axenic, maintained as such and microscopically verified prior to further experiments.

Table 6.1 Different strains used in the study and their origin.

Strain	Source	Origin
Microcystis spp.		
M. aeruginosa strain PCC7806	Pasteur Culture Collection, France	The Netherlands
M. aeruginosa strain PCC7813	Pasteur Culture Collection, France	Scotland
M. aeruginosa strain UV027	University of the Free State Culture Collection	Germany
M. aeruginosa strain NIES88	National Institute for Environmental Studies	Japan
M. aeruginosa strain NIES89	National Institute for Environmental Studies	Japan
M. aeruginosa strain NIES91	National Institute for Environmental Studies	Japan
M. aeruginosa strain NIES99	National Institute for Environmental Studies	Japan
M. aeruginosa strain NIES299	National Institute for Environmental Studies	Japan
M. aeruginosa strain SAG1	Pflanzen Physiologisches Institut, Universität Gottingen	Germany
M. aeruginosa strain CCAP1450/1	Institute of Freshwater Ecology	UK
M. aeruginosa strain UP01	University of Pretoria Culture Collection	Rietvlei, ZA
M. aeruginosa strain UP04	University of Pretoria Culture Collection	Hartbeestpoort, ZA
M. aeruginosa strain UP06	University of Pretoria Culture Collection	Paardekraal, ZA
M. aeruginosa strain UP09	University of Pretoria Culture Collection	Hartbeespoort, ZA
M. aeruginosa strain UP10	University of Pretoria Culture Collection	Roodeplaat, ZA
M. aeruginosa strain UP15	University of Pretoria Culture Collection	Bon Accord, ZA
M. aeruginosa strain UP37	University of Pretoria Culture Collection	Krugersdrift, ZA
M. aeruginosa strain UP38	University of Pretoria Culture Collection	Hartbeespoort, ZA
M. aeruginosa strain UPUS1	University of Pretoria Culture Collection	Ft Collins USA
M. wesenbergii strain UP02	University of Pretoria Culture Collection	Rietvlei, ZA
Other genera		
Chroococcidiopsis cubana strain UP13	University of Pretoria Culture Collection	Klipvoor, ZA
Woronichinia naegeliana strain UPUS2	University of Pretoria Culture Collection	Ft Collins USA
Scenedesmus acutus strain UP26	University of Pretoria Culture Collection	Klipvoor, ZA

Unicellular strains UP01, UP03, UP04, UP10, UP13, UP26, UP37 and UP38 were collected by the authors, representatives of the Water Research Commission and Tswhane Metro Council, respectively. The UPUS1 and UPUS2 were collected at Sheldon Lake, Colorado by the authors. Outgroups included in the study were the

division Cyanophyta: UPUS2 *Woronichinia naegeliana* (Smith, 1950); UP03 *Microcystis wessenbergii* (Teiling 1941; Wojciechowski 1971); UP13 *Chroococcidiopsis cubana* (Komárek & Hindák 1975) (Fig.6.1) and the division Chlorophyta: UP26 *Scenedesmus acutus* (Ettl & Gärtner 1995).

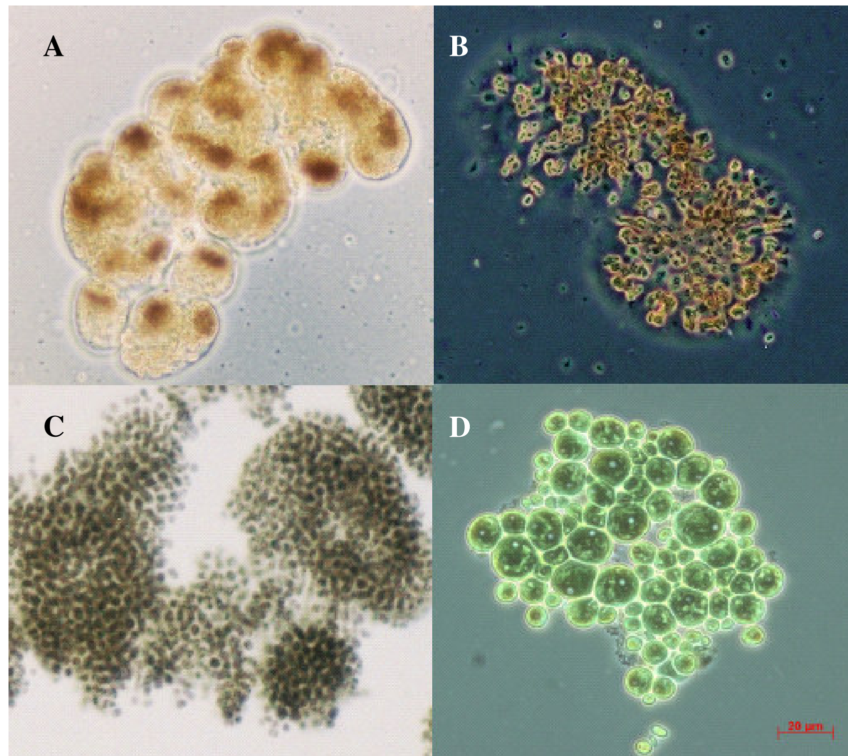

Figure 6.1 (A) *Microcystis wessenbergii* (after Teiling 1941, Wojciechowski 1971); (B) *Woronichinia naegeliana* (after Smith 1950); (C) *Microcystis aeruginosa* (after Smith 1950); and (D) *Chroococcidiopsis cubana* (after Komárek & Hindák 1975). Unstained, bright-field microscopy, 200 x.

After collecting the water samples were placed on ice in a darkened cooler during transport to the laboratory. Holding time for samples was less than 48 h in all cases. An aliquot of the samples were then transferred into 100-mL vessels and treated with overpressure of 0.2 mPa for 2 min. In consequence, gas vesicles, and in some cases, cells of most cyanobacteria (e.g. *Aphanizomenon*) collapsed, whereas *Microcystis* cells were still intact and buoyant. After the pressure treatment, the suspension was

centrifuged (3 000 g) to separate *Microcystis* colonies from the destroyed cells of other cyanobacterial spp. BG-11, designed by M.M Allen was used as growth media. The liquid BG-11 nutrient medium containing 17.65 mM $NaNO_3$, 0.18 mM $K_2HPO_4.3H_2O$, 0.30 mM $MgSO_4.7H_2O$, 0.25 mM $CaCl_2.2H_2O$, 0.03 mM citric acid, 0.03 mM ferric ammonium citrate, 0.003 mM EDTA (ethylenediamine tetra-acetic acid, disodium magnesium), 0.19 mM Na_2CO_3, 0.05 mM H_3BO_3, 9.15 mM $MnCl_2.4H_2O$, 0.77 mM $ZnSO_4.7H_2O$, 1.61 mM $Na_2MoO_4.2H_2O$, 0.37 mM $CuSO_4.5H_2O$ and 0.17 mM $Co(NO_3)_2.6H_2O$ dissolved in 1L distilled water. Freshly poured petri plates were left to cool, solidify, and dry in a laminar flow hood sterilized by UV irradiation. The warm plates were stacked on top of each other to prevent the formation of condensation below their covers. The medium was left to dry at 37 ºC for 24 hr.

When the agar medium was solidified in Petri dishes a bacterial loop was mainly employed in the initial stages of isolation. With the loop, heated in the flame of a bunsen burner and cooled at one edge of the agar plate, the crude cyanobacterial material of the unicellar and axenic strains deposited, was densely spread over the first quarter of agar medium. The loop was then flamed again, cooled and used to perform several parallel streaks traversing at an angle of 90° to the previously spread material. The incubation took place at 25 ºC under continuous illumination of approximately 60 qmol photons/m^2/s^1 from cool white fluorescent tubes for 7 to 10 days (first culture). When colony formation was observed, the *Microcystis aeruginosa* cells in the colonies were identified using Komárek's (1958) criteria of colony morphology and cell diameter. These *Microcystis* cells were than transferred by a sterilized toothpick to fresh agarose medium and incubated again (second culture).

Longterm first culture has been avoided due to the possibility for contamination increase by bacteria and fungi. The cells in fully propagated colonies in the second culture were inoculated into BG-11 medium (5mL per tube) and cultured for 1 to 2 weeks. Then, the culture was examined by a phase-contrast microscope to determine whether any contamination had occurred. If contamination was observed, the culture was returned to the first step of the isolation procedure. The cells from the cultures that were confirmed to be contamination free by microscopic examination were encircle by an autoclaved toothpick and an incision through the agar was made. The tiny piece of agar bearing the microcolony of *M. aeruginosa* was inoculated into 100 mL of BG-11 medium in a 250-mL Erlenmeyer flask and then cultured. The purity of the resulting cultures was verified weekly by the absence of bacterial growth on TYG agar and TYG broth [tryptone (Difco) 5.0 g; yeast extract (Difco) 2.5g; glucose, 1.0 g per liter].

The final proof of purity was verified by microscopic examination. Cultures of *Microcystis aeruginosa* were harvested at the end of exponential growth phase (three weeks) by centrifugation at 6 000 g for 10 min at room temperature. The cultures were then freeze-dried and stored at –20 °C.

6.2.2 DNA Extraction

Genomic DNA was isolated from a freeze-dried culture according to a modified method of Raeder and Broda (1985). One volume extraction buffer (200 mM Tris-HCl (pH 8.00), 150 mM NaCl, 25 mM EDTA, 0.5 % (w/v) SDS, 1 % (v/v) 2-mercaptoethanol, 1 % (w/v) Polyvinylpyrrolidone, PVP) was added to each gram of freeze-dried culture, and homogenized in the presence of washed sand, thereafter the

homogenate was placed at 60 °C for 10 min. The homogenate was then centrifuged at 6 000 g for 15 min. After removal of the supernatant, the extraction was further purified by the addition of equal volumes of chloroform:phenol (1:1). The resulting mixture was vortexed and centrifuged again at 6 000 g for 15 min, where after the upper layer was carefully removed. This was followed by a DNA precipitation step using two volumes of ice-cold absolute ethanol and stored at –20 °C for at least 1 h. The DNA mixture was centrifuge at 6 000 g for 15 min, and the resulting pellet was washed with 70 % ethanol. The ethanol wash-step was repeated three times, and dried after removal of the liquid. The resulting DNA was resuspended in distilled water and stored at –80 °C until further use (Oberholster 2004).

Quantification of the DNA concentrations was done through spectrophotometric measurements at absorbances of 260 and 280 nm, using a Beckman DU650 Spectrophotometer. The DNA quality was also assessed and the concentration determined by visualisation under UV light, on 1 % TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) agarose gels containing ethidium bromide (Sambrook *et al*. 1989).

6.2.3 AFLP analysis

To determine the usefulness of AFLP analysis to discern between the individual strains, as well as their geographical origin, AFLP analysis was conducted using the the IRDye™ Fluorescent AFLP® Kit (LI-COR Biosciences, Lincoln, USA) following the manufacturer's instructions. Restriction endonucleases were done with primer combinations *Eco*R1/*Mse*1. For this, genomic DNA (75 ng) was incubated for 2 h at 37 °C with 1.25 U of *Mse*1, 1.25 U of *Eco*R1, 1 U of T4 DNA ligase, 40 pmol of *Mse*1 adapters and 10 pmol *Eco*R1 adapters. This reaction was done in a volume of

50 µL restriction–ligase buffer containing 10 mM Tris-HCl (pH 7.4), 50 mM NaCl, 1 mM dithriothreitol (DTT), 0.1 mM EDTA, 50 % (v/v) glycerol, 0.15 % (v/v) Triton X-100 and 200 ng/µL BSA. Termination of the reaction was done by heating at 70 ˚C for 15 min, where after it was placed on ice. For adaptor ligation, 25 µL of the Adapter mix (containing *Mse*1 adaptors and *Eco*R1 adaptors, 0.4 mM ATP, 10 mM Tris-HCl (pH 7.5), 10 mM Mg-acetate, and 50 mM K-acetate, and 1 U of T4 DNA ligase) was added followed by incubation at 37 °C for 3 h. An aliquot (10-µL) of the adapter-ligated DNA was diluted (1:10) with TE buffer (10 mM Tris-HCl pH 8.0, 1.0 mM EDTA) to serve as template in the preselective amplification PCR. The remaining portion was used to verify whether the digestion was complete (Oberholster 2004).

The preselective amplification reaction contained 2.5 µL of adapter-ligated DNA (diluted 1:10), 2.5 U of Taq DNA polymerase (Roche Molecular Biochemicals), 2.5 µL of 10 x PCR reaction buffer (Roche Molecular Biochemicals), 15 mM $MgCl_2$, 500 mM KCl and 100 µM of IRDye700^{TM}-labeled *Eco*R1 or IRDye800^{TM}-labeled *Eco*R1 and *Mse*1 primers (containing dNTPs) with every selective nucleotide, in a total volume of 25.5 µL. The amplification procedure consisted of twenty cycles of 30s at 94 °C, 1 min at 56 °C, 1 min at 72 °C, and soaked at 4 °C. The selective PCR contained 2 µL of the diluted (1:10) product of the preselective PCR, 0.5 U of Taq DNA polymerase (Roche Molecular Biochemicals), 2 µL of 10 x Taq DNA polymerase buffer (Roche Molecular Biochemicals), KCl and $MgCl_2$ as mentioned above, and 100 µM of IRDye700^{TM}-labeled *Eco*R1 or IRDye800^{TM}-labeled *Eco*R1 and *Mse*1 primers (containing dNTPs) with every selective nucleotide, in a total volume of 11 µL. Eight *Eco*R1-*Mse*1 primer pairs (LI-COR Biosciences, Lincoln,

USA) were used for selective amplification (Table 6.2). The first amplification cycle was carried out for 30s at 94 °C, 30s at 65 °C and 1 min at 72 °C. At each of the following 12 cycles, the annealing temperature was reduced by 0.7 °C per cycle. The last 23 cycles of annealing were carried out at 72 °C for 1 min; and then soaked at 4 °C (Oberholster 2004).

6.2.4 Gel electrophoresis and scoring

An equal volume of loading solution (LI-COR Biosciences, Lincoln, USA) was added to each selective amplification reaction. Samples were denatured at 95 ºC for 3 min and placed on ice for 10 min before loading. A volume of 0.8 µL was loaded with an 8-channel syringe (Hamilton, Reno, Nevada) onto 25-cm 8% Long Ranger gels (BMA, Rockland, ME, USA). Electrophoresis and detection of AFLP fragments were performed on LI-COR IR2 (model 4200S) automated DNA analysers. The electrophoresis parameters were set to 1 500V, 40mA, 40W, 50 ºC, and a scan speed of 3. The run-time was set to 4 h and gel images were saved as TIF files for further analysis.

The gel images were scored using a binary scoring system that recorded the presence and absence of bands as 1 and 0, respectively. Semi-automated scoring was performed with AFLP Qauntar Pro (Version 1,0, LI-COR) and followed by manual editing to make adjustments to the automated score where necessary. A locus was scored as polymorphic when the frequency of the most common allele (band present or absent) was less than 0.97 (absent or present in at least two individuals). Bands with the same

mobility were considered as identical products (Waugh *et al*. 1997), receiving equal values regardless of their fluorescence intensity.

6.2.5 Data analysis

Polymorphic bands scored as plus (+) and minus (-) and converted to 1 and 0 were compiled in a data matrix. The data matrix was used to perform cluster analysis on the basis of the average linkage method, known as the Unweighted Pair Group Method using Arithmetic averages (UPGMA) and PAUP 4.0 software (Hintze 1998). The average polymorphic information content (PIC) was calculated across each primer combination according to Riek *et al*. (2001) as follows:

$$PIC = 1-[f^2+(1-f)^2]$$

Where f is the frequency of the marker bands in the data set. Marker index (MI) was determined by multiplying PIC values with percentage polymorphism for each primer combination (Lübberstedt *et al*. 2000). The raw data matrix was used to estimate genetic similarities among different geographical cyanobacterial strains. Estimates of genetic similarity between all pairs of strains were calculated in the form of dissimilarity and expressed as Euclidean genetic distance (Jacoby *et al*. 2003). The 'goodness of fit' of the clustering to the data matrix was determined by calculating the cophenetic correlation coefficient between the dissimilarity matrix and the cophenetic matrix derived from the dendrogram (Sneath & Sokal 1973; Sneath 1989).

6.3 Results

6.3.1 Fast screening of AFLP primer combinations

After screening 20 primer combinations on a subset of strains using either IRDye700^{TM}-labeled *Eco*R1 or IRDye800^{TM}-labeled *Eco*R1 primers, eight IRDye700^{TM}-labeled *Eco*R1 primer pairs were selected for analysis (Table 6.2).

Table 6.2 Degree of polymorphism and average polymorphism information content (PIC) and marker index (MI) for the eight AFLP primer combinations used to analyse the 23 strains.

No	Primer combination	Total number of bands	Number of polymorphic bands	% of polymorphic bands	PIC	MI
1	*Eco*R1-ATC / *Mse*1-CCA	53	52	98.11	0.287	28.156
2	*Eco*R1-AAA / *Mse*1-CCC	18	17	94.44	0.293	27.671
3	*Eco*R1-ACG / *Mse*1-CCC	34	33	97.05	0.324	31.444
4	*Eco*R1-ACG / *Mse*1-CAC	19	18	94.73	0.363	34.387
5	*Eco*R1-ACT / *Mse*1-CAC	47	45	95.74	0.342	32.743
6	*Eco*R1-AT / *Mse*I-CG	28	25	89.28	0.326	29.105
7	*Eco*R1-AT / *Mse*1-CT	43	42	97.67	0.360	35.161
8	*Eco*R1-CC / *Mse*1-CT	34	34	100.00	0.397	39.700
	Total	276	266	NA	NA	
	Mean	34.5	33.25	95.87	0.337	32.308

Primer pair *Eco*R1-ATC/*Mse*1-CCA amplified the largest number of bands (53), but the lowest PIC (0.287) and a MI of 28.156. Primer pair *Eco*R1-AAA / *Mse*1-CCC amplified the lowest number of bands (18) and had the lowest MI (27.671). While primer pair *Eco*R1-CC / *Mse*1-CT had the highest PIC (0.397) and MI (39.700). The generated fingerprints were evaluated for repeatability and overall clearness of the banding pattern. The number of informative fragments was also taken into account (Fig. 6.2). We found no significant difference in MI through the inclusion of primer

pairs with two rather than three selective nucleotides, since the mean MI of the primer pairs with three nucleotides was 30.881 ± 3.506 and the MI of primers with two selectives was 34.655 ± 5.044. A total of 276 bands were amplified from the eight primer combinations, of which 266 were informative, 10 non-informative, with an average of 95.87 polymorphic bands per primer combination.

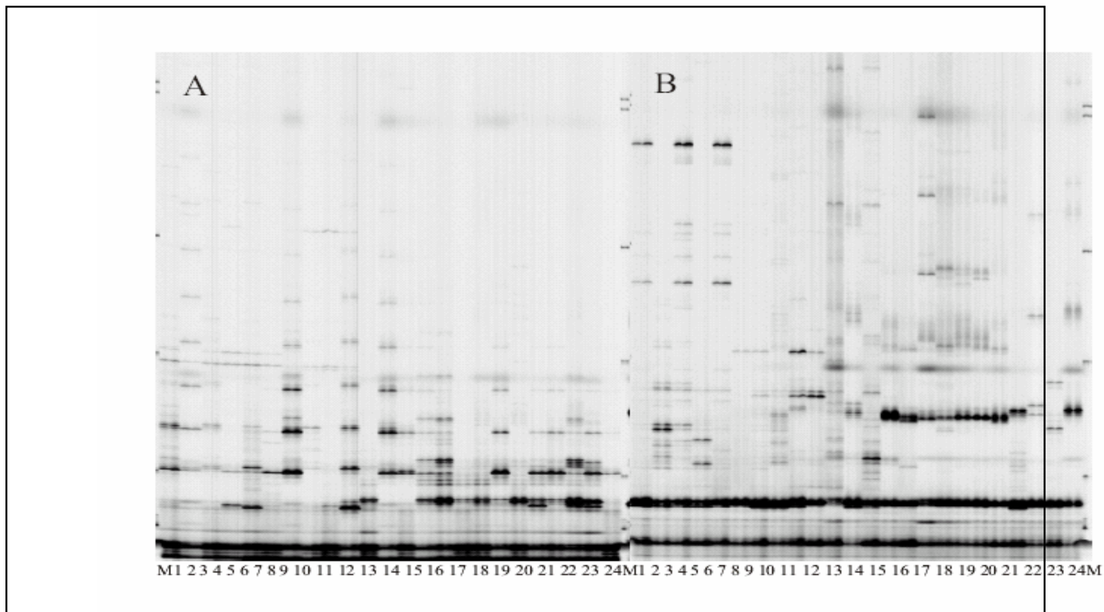

Figure 6.2 AFLP banding patterns generated using primer combinations E*co*R1-CC/*Mse*1-CT (A) and *Eco*R1-ATC/*Mse*1-CCA (B). M = 100 bp ladder marker; 1 = NIES88; 2 = NIES89; 3 = NIES90; 4 = NIES99; 5 = NIES299; 6 = PCC7806; 7 = CCAP1450/1; 8 = SAG1; 9 = UV027; 10 = PCC7813; 11 = UP01; 12 = UP02; 13 = UP09, 14 = UP04, 15=UP04, 16 = UP10, 17 = UP13, 18 = UP15, 19 = UP26, 20 = UP37, 21 = UP38, 22 = UPUS1, 23 = UPUS2, 24 = UP06.

6.3.2 Genetic diversity as defined by AFLP fingerprinting

The genetic relationship among all the *Microcystis aeruginosa* strains based on the combination of data obtained with the eight primer combinations is represented in the dendrogram (Fig. 6.3).

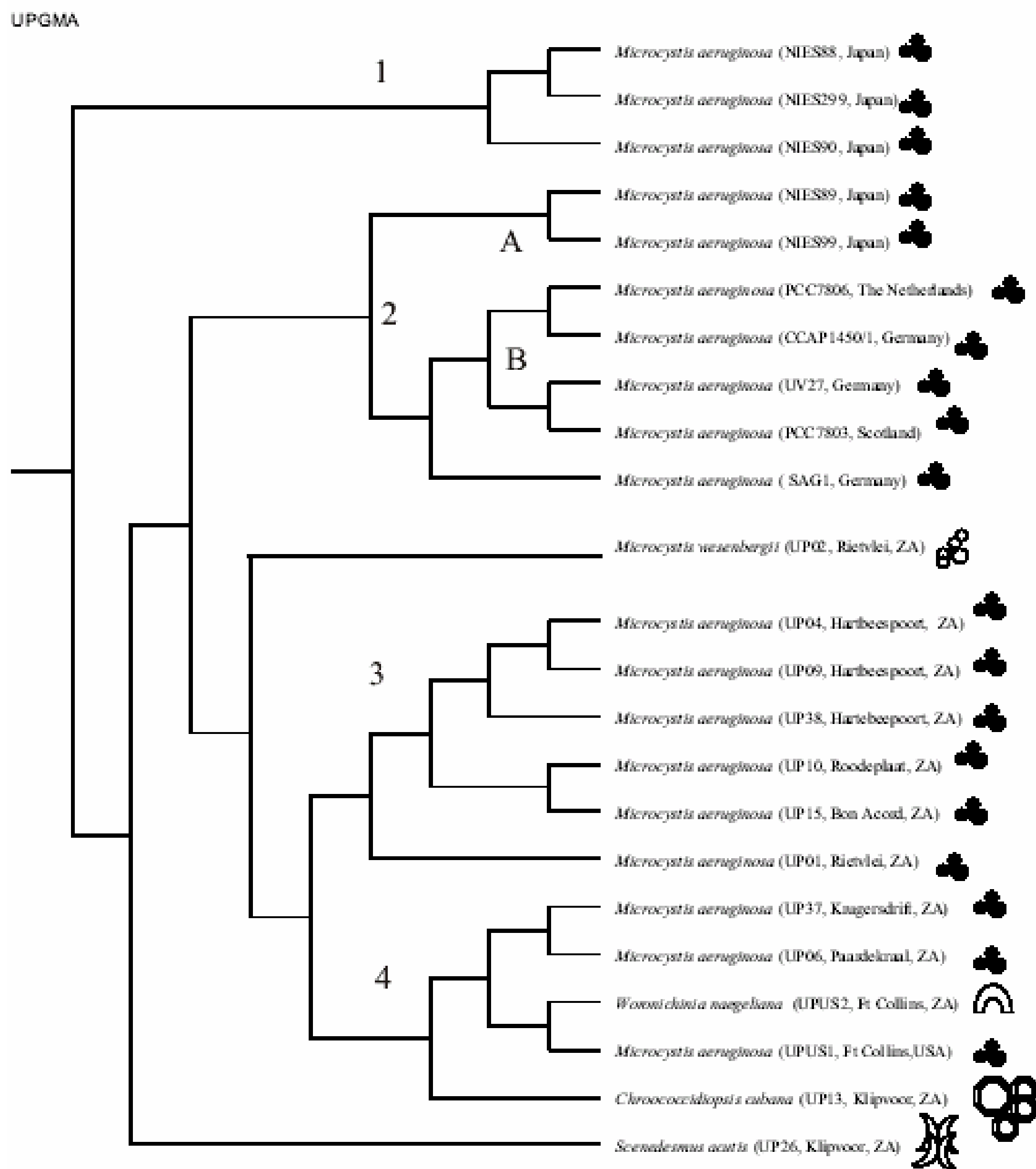

Figure 6.3 Combined cluster analysis derived from AFLP analysis of 23 *Microcystis aeruginosa* and outgroup strains using eight AFLP primer combinations.

The dendrogram consists of four clusters. Cluster one contains NIES88, NIES299 and NIES90 basal to this group. The mean character difference between the NIES88 and NIES299 strains were 0.163, while they differ in pairwise distance from NIES90 with a mean of 0.243. The mean character difference of 0.163 is also the lowest obtained mean value in the data set. The largest mean character differences of 0.558 were obtained between the strains *Scenedesmus acutis* (UP26) and *M. aeruginosa* (NIES88), and *M. wesenbergii* (UP02) and *M. aeruginosa* (NIES90), respectively.

In cluster 2 there are two groupings. In cluster 2A, the NIES strains 89 and 99 group together with a mean character difference of 0.243, while in 2B strains PCC7806 and CCAP1450/1 fall into a group with a mean character difference of 0.207, and UV27 group with PCC7813 (mean character difference of 0.192), with SAG1 basal to this group.

Cluster 3 consists exclusively of *Microcystis aeruginosa* strains from the geographical area of Gauteng and North West provinces in South Africa, and includes strains UP04, UP09, UP38, UP09 and UP15, with UP01 basal to this group. The strains from Hartbeespoort reservoir (UP04, UP09 and UP38) also group together, while UP10 (Roodeplaat) and UP15 (Bon Accord) forms a subgroup. The geographical distances between the Hartbeespoort reservoir and Bon Accord and Roodeplaat reservoirs are 36.6 km and 53.8 km, respectively (Table 6.3). UP01 was collected from the Rietvlei reservoir that is located in a south-eastern direction to all the above reservoirs. The mean character difference between UP01 and the Hartbeespoort strains was 0.361.

Table 6.3 Geographical distance in kilometers between the reservoirs in Gauteng and North West Provinces, South Africa.

Reservoir	Klipvoor	Hartbeespoort	Bon Accord	Rietvlei	Roodeplaat
Klipvoor	0.0	65.9	66.6	96.5	78.4
Hartbeespoort	65.9	0.0	36.3	48.1	53.8
Bon Accord	66.6	36.3	0.0	30.7	18.1
Rietvlei	96.5	48.1	30.7	0.0	30.0
Roodeplaat	78.4	53.8	18.1	30.0	0.0

In cluster 4, UP37 and UP06 of the geographical area of the Free State and Western Cape province of South Africa group together with a mean character difference of 0.275. The strains UPUS1 and UPUS2 of Colorado U.S. fall in a separate group, while strain UP13 is basal to cluster 4.

6.4 Discussion

AFLP fragments have been used to unravel cryptic genetic variation for a wide range of taxa, including plants (Mackill *et al.* 1996), fungi (Majer *et al.* 1996, 1998) and bacteria (Huys *et al.* 1996), which have previously been impossible to resolve with morphological characters. In the present study, complex AFLP banding patterns were obtained. Janssen *et al.* (1996) have showed that the choice of the restriction enzymes, and the length and composition of the selective nucleotide will determine the complexity of the final AFLP fingerprint. Primer selectivity is good for primers with one or two selective nucleotides in simple genomes such as fungi, bacteria and some plants, and still acceptable with primers having three selective nucleotides, but is lost with the addition of a fourth nucleotide (Vos *et al.* 1995). We used a combination of primers with two and three selective nucleotides on 23 *Microcystis aeruginosa* and outgroup strains, and a total of 276 bands were amplified, constituting

95.87 % informative bands and 4.13 % monomorphic bands (Table 6.2). However, we did not gain advantage in number of amplified loci when using two rather than three selective primers sets.

In the dendrogram, the Japanese strains (NIES88 NIES90, NIES299) group separate from the other NIES strains (cluster 1), with NIES88 and NIES299, genetically closest to each other, while NIES strains 89 and 99 group together with the European strains (cluster 2). In cluster 3, all the *Microcystis aeruginosa* strains of Gauteng and North West province of South Africa group together, except for the outgroup strain *Chroococcidiopsis cubana* from Klipvoor dam. This phenomenon may be due to the fact that geographical distance between the *Microcystis* strain locations in cluster 3 is close and are located in the northern geographical region of South Africa (Table 6.3).

The position of the UPUS1 and 2 strains in their respective cluster is surprising, since these strains originated from Colorado, U.S. However, they group together with the unrelated strains from the central and southern geographical areas of South Africa (i.e., the Western Cape and the Free State provinces), but not with the *Microcystis* strains from the Gauteng and North West provinces or the European strains. There are two plausible explanations for this observation. Firstly, this occurrence is most likely the result of a reflection of ease of dispersal; yet for the majority of species there is little information on their mechanism of transport (Round 1981). Among the factors shaping the geographical distribution of cyanobacterial species, temperature is usually considered to be of prime importance. Other factors that also play an important role are migratory barriers for example oceans and mountain ranges, as well as passive dispersal agents involving water, air and different animals. The probability of

successful dispersal to other geographical areas depends strongly on the effectiveness of the carrier, and the ability of cyanobacteria to tolerate the transport conditions (Kristiansen 1996a, b). Water beetles and aquatic mammals may be effective over a distance of a few kilometres, whereas dragonflies have been shown to transport viable algal material almost 1 000 km. Atkinson's (1980) reported in his experiments that viable remains of *Asterionella* was found in the faeces of waterfowl after a maximum of 20 hours, perhaps corresponding to a flight of 220 km. Atkinson (1980) has also shown that transport by the digestive tract of birds may be more efficient because desiccation is ruled out. Specialised life cycle forms for example spores, cysts and akinetes, as well as thick, resistant cell walls like in the case of *Microcystis* spp. will better survive longer transport, either externally or internally. Many ducks migrate up to 4 800 km, but with many stops. Nonstop flight distances of the order of 3 200 km in 48 hours have been noted for the white-fronted goose (*Anser albifrons*) (Owen & Black 1990). For airborne transport, it is very difficult to give maximal distances. Since viable *Melosira* were found at 3 000 m altitude, this dust containing diatom frustules can certainly be blown across the Atlantic Ocean (Kristiansen 1996b) and with it, possible some species of cyanobacteria.

A second explanation for the grouping of UPUS1 and 2 together with the South African strains, rather than with the European and NIES strains, may be due to the fact that the so called traditional cultured strains PCC, NIES, CCAP1450/1, UV and SAG1 are decades in prolonged subcultures. In contrast, the UP and UPUS are much shorter in subculture and thus 'wild-type' strains. This phenomenon is most likely due to the fact that although nearly all populations of cyanobacteria from different locations differ to some degree from each other, they may stabilize in longterm

cultures (Waterbury & Rippka 1989; Kato & Watanabe 1993; Van der Westhuizen & Eloff 1982). Carr (1999) has argued convincingly about the bias introduced to research by use of strains selected for their ability to grow exponentially. There exists abundant evidence that morphological changes take place during prolonged subculture, with almost as much evidence for physiological changes (Doers & Parker 1988). Fray (1983) stated that records of mutations (i.e., permanent changes of the genome) are well substantiated in laboratory cultures. These include longterm subculture strains that fail to differentiate heterocysts and to synthesize nitrogenase (*Anabaena variabilis*); which lack the ability to produce akinetes (*Anabaena cylindrical*); which are incapable of forming gas vesicles (*Anabaena flos-aquae*); synthesizing toxin (*Microcystis aeruginosa*) or depositing sheath material. Such mutants generally outgrow the 'wild type' strains because energy previously invested in the formation of structures, which confer no advantage in particular conditions, can be diverted to cell growth.

We have also noted two outgroups in cluster 4, namely *Chroococcidiopsis cubana* (UP13) belonging to the division Cyanophyta (Fig. 6.1D), which cells are solitary or aggregated in irregular groups, enveloped by thin, firm, colourless sheaths. This species is non-heterocystous but able to fix N_2. The cells are spherical, oval to irregular rounded of varying sizes between 1.5-20 µm in diameter and are usually attach to stony substrata in the aquatic habitat. The other outgroup in this cluster is *Woronichinia naegeliana* which comprises of spherical or irregular, free-living colonies surrounded by a fine, colourless mucilage (Fig. 6.1B). Cells are at or within the ends of mucilaginous stalks and radially arranged with dimensions of (1)2.5-7 x 1-4(5) µm. Stalks are densely packed, causing radial lamellation, but sometimes are

diffuse near the center. Of the 15 described species, 3 contain gas vesicles of which *Woronichina naegeliana* is one, and may form cyanobacterial surface blooms in eutrophic North American waters (Komárek & Anagnostidis 1998). The other outgroups that don't fall in any cluster is (UP3) *Microcystis wessenbergii* (Fig. 6.1A) and the green algae (UP26) *Scenedesmus acutis* which belong to the division Chlorophyta. Colonies of *Scenedesmus* are 2-4-8-16-32 celled, flattened, with long axes of cells parallel, laterally adjoined and arranged in single linear or alternating series. Cells are ellipsoidal or tapering toward each end, while the cell wall is smooth and spines are absent (Shubert 2003).

Nielan *et al.* (1995) assessed the usefulness of AFLPs and restriction fragment length polymorphism (RFLP) analyses on members of the genera *Microcystis*, *Anabaena*, *Aphanizomenon*, *Cylindrospemopsis*, *Nodularia*, *Nostoc* and *Planktothrix* with regard to cyanobacterial systematics. In their study, they were able to discern the individual strains when using AFLP analysis, but not always when using RFLPs. They observed delineation of the *Microcystis* strains with regard to geographical origin when using both AFLP and RFLP analyses, but the technologies were not able to discriminate between strains on the basis of their levels of toxicity. But more importantly, they found no relationship between the PCR amplicons and restriction fragment profiles obtained with the respective genera and the taxonomic placement of the respective genera in cyanobacterial systematics.

In view of the present study, AFLP analysis is useful for the identification of genetic diversity of geographical unrelated strains and analysis of *Microcystis aeruginosa* strains. AFLPs seem to overcome the major pitfalls present in other PCR based

methods, e.g. DAF or RAPD analysis, and appear to be as reproducible, heritable and intraspecific as RFLPs (Law *et al*. 1998). The use of the AFLP fingerprinting method resulted in a high degree of discrimination and identification of geographically unrelated *Microcystis aeruginosa* strains, and was found useful and practical. The results from this study is concurrent with the results by Nielan *et al.* (1995) that AFLP analysis is not suitable if evolutionary phylogeny is the objective, since the data did not support the taxonomic placing of the genera, but rather the geographical origin.

Acknowledgements

The authors would like to express their sincere gratitude to Karin van Ginkel (Department of Water Affairs and Forestry) and Leanne Coetzee (Tshwane Metro Council) for the collection of some of the strains. Also, we wanted to thank the National Foundation of Research, and the Water Research Commission, South Africa for provision of funding. We especially want to thank Dr. Jiří Komárek for his assistance with the identification of some of the strains.

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