International Scholars Journals

International Journal of Biochemistry and Biotechnology ISSN 2169-3048 Vol. 9 (1), pp. 001-011, January, 2020. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

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

PJ Oberholster[1], A-M Botha[2,3]*, K Muller[2] and TE Cloete[1]

[1]Department of Microbiology and Plant Pathology, [2]Department of Genetics, University of Pretoria, Hillcrest, Pretoria, ZA0002, South Africa.

[3]Department of Soil and Crop Sciences, Colorado State University, Fort Collins, CO80521, USA.

Accepted 15 October, 2019

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 geographical 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 revealed extensive evidence for the applicability of AFLP in cyanobacterial taxonomy, 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, as well as highlighting the potential of this fingerprinting method in evolutionary studies.

Key words: Molecular markers, distribution, cyanobacteria, genetic diversity**.**

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 and 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 and 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

*Corresponding author. E-mail: AMBothaO@postino.up.ac.za or Annamaria.oberholster@FABI.up.ac.za. Tel: +27124203945. Fax: +27124203947.

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 and 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 and Rippka, 1989; Kato and 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. could differ in important characteristics (Cronberg and 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 long-term and contain a wide spectrum of stable types for long periods (Komárek and 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 and Stam, 1984; Stam et al., 1985) and gene sequencing (Masui et al., 1988).

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.

Table 1. Different strains used in the study and their origin.

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

MATERIAL AND METHODS

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 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. 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. 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* (after Smith, 1950); UP03 *Microcystis wessenbergii* (after Teiling, 1941; Wojciechowski, 1971); UP13 *Chroococcidiopsis cubana* (after Komárek and Hindák, 1975) (Figure 1) and the division Chlorophyta: UP26 *Scenedesmus acutus* (after Ettl and Gärtner, 1995). 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 (3000 g) to separate *Microcystis* colonies from the destroyed cells of other cyanobacteria spp.

BG-11, designed by M.M Allen was used as growth media. The liquid BG-11 nutrient medium contains 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 1 L distilled water. Freshly poured petri plates were left to cool, solidify, and dried 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°C for 24 h.

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^o to the previously spread material. The incubation took

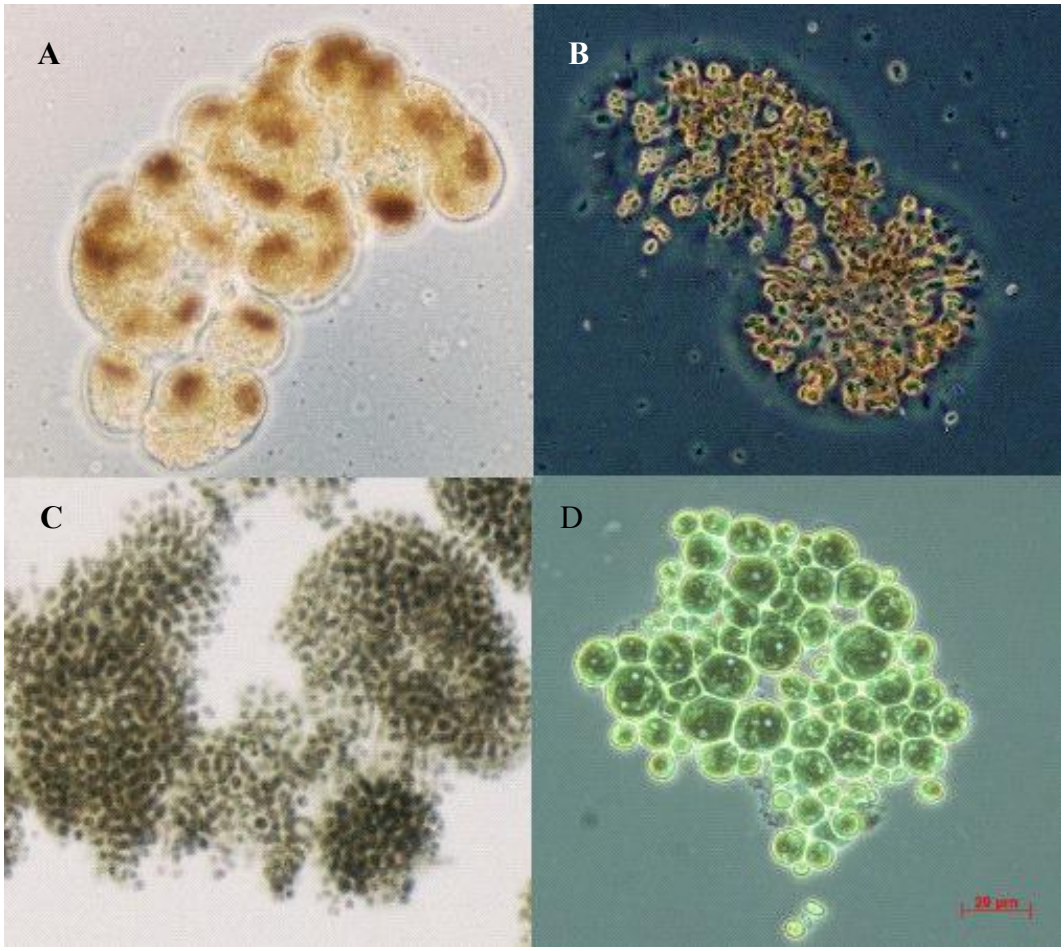

Figure 1. (A) *Microcystis wesenbergii* (after Teiling 1941, and Wojciechowski 1971); (B) *Woronichinia naegeliana* (after Smith 1950); (C) *Microcystis aeruginosa* (after Smith 1950); and (D) *Chroococcidiopsis cubana* (after Komárek & Hindák 1975). Unstained, bright-field microscopy, 200 x.

place at 25°C 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 *M. 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). Long-term 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 (5 ml 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) 2.5 g; glucose, 1.0 g per liter].

The final proof of purity was verified by microscopic examination. Cultures of *M. 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.

DNA extraction

Genomic DNA was extracted according to a modified method of Raeder and Broda (1985). The extraction buffer consisted of 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). A volume of extraction buffer were added to each 1 g of freeze-dried culture, and homogenized in the presence of washed sand. The homogenate was placed at 60°C for 10 min. The homogenate was then centrifuged at 12 000 rpm for 15 min. The supernatant was removed and equal volumes of chloroform:phenol (1: 1) was added, vortexed and centrifuged again at 12 000 rpm for 15 min. The upper layer was carefully removed. The DNA in the aqueous layer was precipitated with two volumes of ice-cold absolute ethanol and stored at –20°C for at least 1 h. Following a centrifugation step (12 000 rpm, 15 min), the resulting pellet was

Table 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	32.30819

washed with 70% ethanol (this step was repeated three times), and dried after removal of the liquid. The DNA was resuspended in distilled water and stored at –80°C.

DNA concentrations were 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) as well as through spectrophotometric measurements at absorbances of 260 and 280 nm, using a Beckman DU650 Spectrophotometer.

AFLP analysis

The AFLP procedure was carried out using the IRDye^{TM} Fluorescent AFLP® Kit (LI -COR Biosciences, Lincoln, USA) following the manufacturer's instructions. Two combinations of restriction endonucleases were used. For the combination *Eco*RI*Mse*I genomic DNA (75 ng) was incubated for 2 h at 37°C with 1.25 U of *Mse*I, 1.25 U of *Eco*RI, 1 U of T4 DNA ligase, 40 pmol of *Mse*I adapters and 10 pmol *Eco*RI adapters. This reaction was done in a volume of 50 l of 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/ l BSA. The reaction was terminated by heating at 70°C for 15 min, and then placed on ice. For adaptor ligation, 25 l of the adapter mix, containing *Mse*I adaptors and *Eco*RI 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, and the reaction was incubated at 37°C for 3 h. A 10- l aliquot 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 a template in the preselective amplification PCR. The remaining portion was used to verify that the digestion was complete.

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

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°C for 3 min and placed on ice for 10 min before loading. A volume of 0.8 l 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 1500 V, 40 mA, 40 W, 50°C, 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 QuantarPro (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.

Data analysis

Polymorphic bands scored as plus (+) and minus (-) and converted to 1 and 0 were compiled in a data matrix (not shown). 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

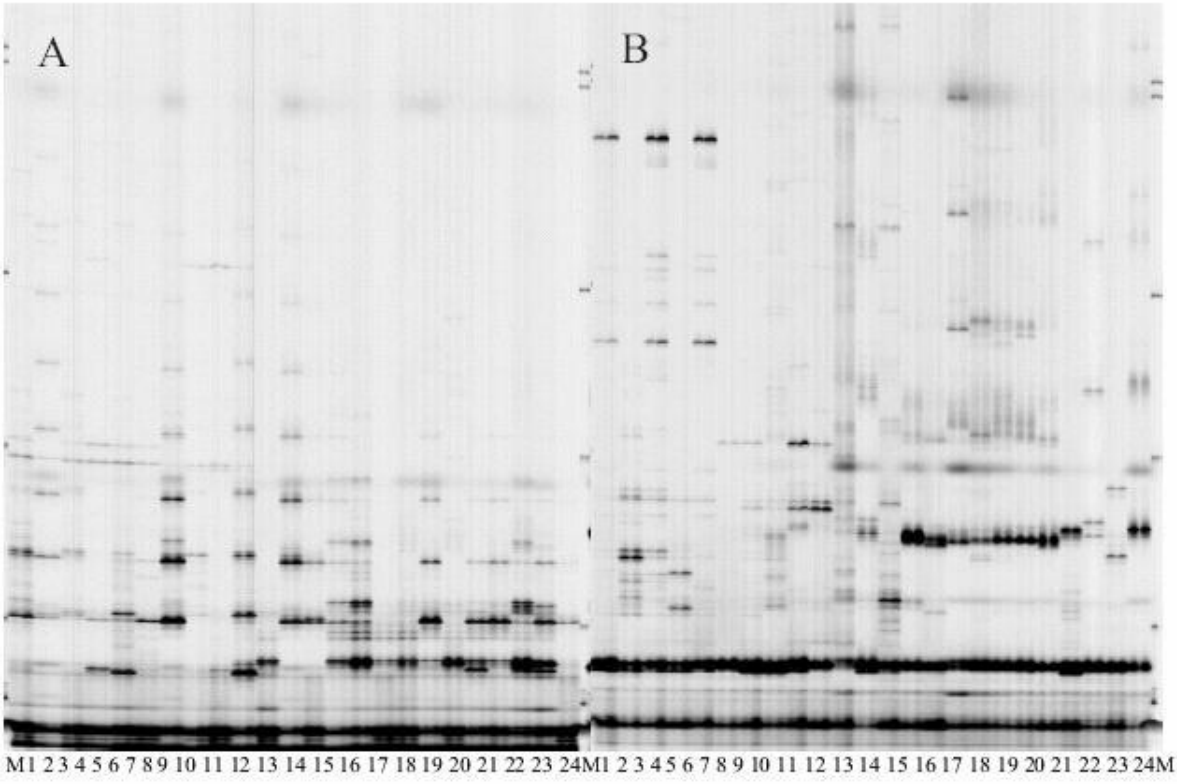

Figure 2. AFLP banding patterns generated using primer combinations E*co*R1-CC/*Mse*1-CT (A) and *Eco*R1-ATC/*Mse*1-CCA (B). M = 100 bp ladder marker; 1 = NIES88; 2 = NIES89; 3 = NIES90; 4 = NIES99; 5 = NIES299; 6 = PCC7806; 7 = CCAP1450/1; 8 = SAG1; 9 = UV027; 10 = PCC7813; 11 = UP01; 12 = UP02; 13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

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 (not shown). 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 and Sokal, 1973; Sneath, 1989).

RESULTS

Fast screening of AFLP primer combinations

After screening 20 primer combinations on a subset of strains using either IRDye700™- labeled *Eco*RI or IRDye800™-labeled *Eco*RI primers, eight IRDye700™-labeled *Eco*RI primer pairs were selected for analysis (Table 2). The generated fingerprints were evaluated for repeatability and overall clearness of the banding pattern. The number of informative fragments was also taken into account (Figure 2). Primer pair EcoRI-ATC/MseI-CCA amplified the largest number of bands (53), but the lowest PIC (0.287) and a MI of 28.156. Primer pair *Eco*RI-AAA/*Mse*I-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). 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.

Genetic diversity as defined by AFLP fingerprinting

The genetic relationship among all the *M. aeruginosa* strains based on the combination of data obtained with

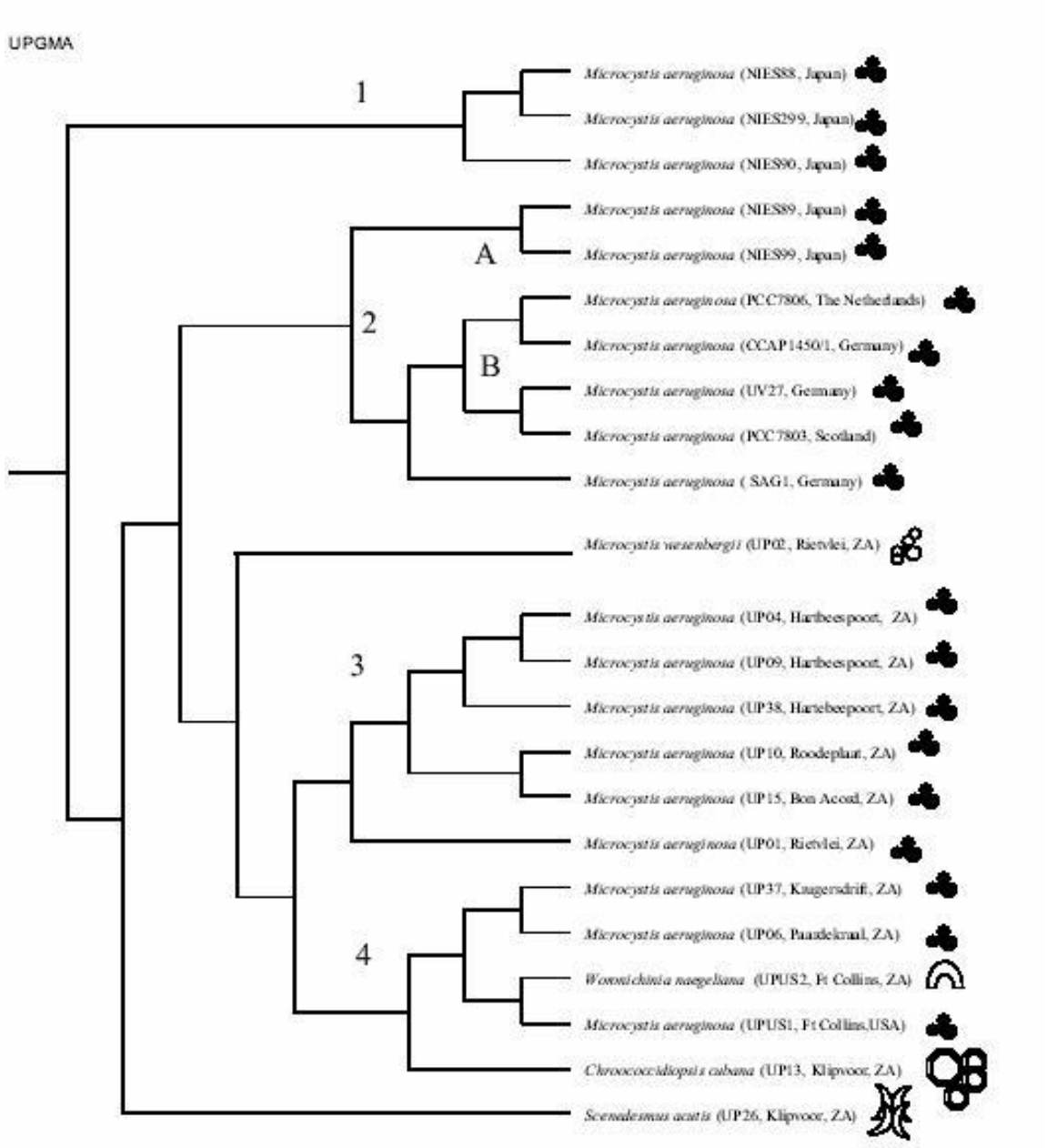

Figure 3. Combined cluster analysis derived from AFLP analysis of 23 *Microcystis aeruginosa* and outgroup strains using eight AFLP primer combinations.

Table 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

the eight primer combinations is represented in the dendrogram (Figure 3).

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 *M. 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 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.

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.

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 *M. aeruginosa* and outgroup strains, and a total of 276 bands were amplified, constituting 95.87% informative bands and 4.13% monomorphic bands (Table 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 *M. 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 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 1000 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 h, 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 h have been noted for the white-fronted goose (*Anser albifrons*) (Owen and Black, 1990) . For airborne transport, it is very difficult to give maximal distances. Since viable *Melosira* were found at 3000 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 long-term cultures (Waterbury and Rippka, 1989; Kato and Watanabe, 1993; Van der Westhuizen and 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 and Parker, 1988). Fray (1983) stated that records of mutations (i.e. permanent changes of the genome) are well substantiated in laboratory cultures. These include long-term 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 (*M. 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 (Figure 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 attached 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 (Figure 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 and Anagnostidis, 1998). The other outgroups that did not fall in any cluster is (UP3) *Microcystis wessenbergii* (Figure. 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).

In view of the present study, AFLP analysis is useful for the identification of genetic diversity of geographical unrelated strains and analysis of *M. 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 *M. aeruginosa* strains, and was found useful and practical.

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. Ji í Komárek for his assistance with the identification of some of the strains.

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