Molecular Characterization of Some Toxigenic Cyanobacteria by PCR-Based Techniques

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Abstract - The present study proposed different techniques to differentiate some toxigenic cyanobacterial strains applying short tandemly repeated repetitive (STRR), randomly amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), and sequencing the gene produced. In this study, primers corresponding to the STRR sequence used in the PCR were STRRA1, STRRB1 and mixed STRRA1+STRRB1 showed a genetic heterogeneity among isolates from different geographic regions. STRR-PCR indicated to be useful for clustering of even closely related strains. A total of eight primers were initially chosen to generate RAPD patterns. RAPD-PCR was used to generate unique and identify DNA profiles for six strains of cyanobacteria. The strain-specific randomly amplified polymorphic DNA profile made it possible to discriminate among all toxigenic cyanobacteria. RFLP analysis and sequencing of the 16S rRNA gene have revealed a very close relationship between certain strains of cyanobacteria. The PCR products corresponding to the 16S rRNA genes of the six strains were digested with Mspl, Sau3AI, HaeIII, Rsal, EcoRI and HinfI. Several molecular approaches now provide powerful adjuncts to the culture-dependent techniques. One approach in particular that couples PCR and rRNA-based phylogeny has been effective in the exploration of microbial environments and the identification of uncultured organisms. PCR product for cyanobacterial identification (16S rRNA, Nitrogenase, and Phycocyanin genes) have been sequenced, respectively. It is generally accepted for the comparison of phylogenetic characters. RFLP data, corroborated by 16S rRNA sequences show that diversity between strains of cyanobacteria was established. A method based on the combination of two 10-mer oligonucleotides in a single PCR was developed to provide specific and repeatable DNA fingerprints for cyanobacterial isolates [2].

It has been shown that strains can be distinguished by comparing polymorphisms in genomic fingerprints [9,10]. The combination of PCR and random oligonucleotide primers. Current RAPD technology has been optimized for the identification of cyanobacterial cultures to the strain level. The strain-specific randomly amplified polymorphic DNA profiles made it possible to discriminate among all toxigenic cyanobacteria studied to the three [6] taxonomic levels of genus, species, and strain.

This technique is sensitive and specific because the entire genome of an organism is used as the basis for generating a DNA profile. Restriction fragment length polymorphism (RFLP) alone or following the amplification of DNA by polymerase chain reaction (PCR) and gene sequencing are well able to discriminate [7] several morphologically similar species that differ in their physiological or biochemical traits [13]. It therefore seems essential to employ a suitable set of restriction enzymes to produce fragments of different length, which can be chosen by examination of the large number of 16SrDNA sequences now available, in order to permit a higher level of discrimination [7]. RFLP genotypes have been used for phylogenetic characters. RFLP data, corroborated by 16SrRNA sequences, show that diversity between strains of cyanobacteria [2,16].

Toxic and non-toxic cyanobacterial strains were examined by RFLP of PCR-amplified 16S rRNA gene [3]. RFLP analysis of these amplification products, revealed an array of DNA profiles specific for toxin-producing cyanobacteria. RFLP data, corroborated by 16S rRNA sequences [19], show that diversity between marine and freshwater strains. Also, the results showed that readily distinguishable profiles were able to differentiate between filamentous, heterocystous cyanobacteria which were either neurotoxic or hepatotoxic [1]. The technique employed PCR in which one of the two primers used was fluorescently labeled at the 5′ end and was used to amplify a selected region of bacterial genes encoding 16S rRNA from total community DNA. The PCR product was digested with restriction enzymes, and the fluorescently labeled terminal restriction fragment was precisely measured by using an automated DNA sequencer.
Computer simulated analysis of terminal restriction fragment length polymorphisms (T-RFLP) for 1,002 eubacterial sequences showed that with proper selection of PCR primers and restriction enzymes, 686 sequences could be PCR amplified and classified into 233 unique terminal restriction fragment lengths or “ribotypes.” Using T-RFLP, we were able to distinguish all bacterial strains in a model bacterial community, and the pattern was consistent with the predicted outcome. These results demonstrated that T-RFLP is a powerful tool for assessing the diversity of complex bacterial communities and for rapidly comparing the community structure and diversity of different ecosystems [20,21].

Materials and Methods: Cyanobacterial Strains

The strains used are species of the filamentous genus Anabaena, Nodularia, Cylindrospermopsis and the unicellular genus Microcystis, which are commonly found in cyanobacterial blooms and which produce neurotoxins and hepatotoxins, respectively. Toxic strains (Microcystis aeruginosa flos-aquae, Anabaena circinalis, Nodularia spumigena and Cylindrospermopsis raciborskii) were chosen from the culture collections (CSIRO) Australia’s Commonwealth Scientific and Industrial Research Organization. Domestic Anabaena sp. has been collected from King Saud university culture collection and King Abdullah University culture collection kindly provided domestic Microcystis sp.

Cyanobacterial Growth

The cultures of Mictocystis aeruginosa fbs-aqual, Anabaena circinalis, Nodularia spumigena, cylindrospermopsis raciborskii, Microcystis sp. and Anabaena sp. were grown in MLA media [2], whereas Microcystis aeruginosa PCC7880 and Microcystis aeruginosa PCC 7813 were growth in BG-11 media 22 under constant light intensity (20 mmol·S⁻¹) for up to 8-21 days at 60°C. During an incubation time growth rate measurement of each strain will take a place using spectrophotometer with 700nm wavelength.

Genomic DNA Extraction

Total genomic DNA was extracted from lyophilized samples of the six isolates grown under stock culture conditions by using a modification of a technique for purification of DNA from gram negative bacteria [6]. The integrity and concentration as the extracted genomic DNA were determined spectrophotometrically at 260 and 280 nm [6].

Differentiation of Cyanobacterial Strains

Differentiation between six cyanobacterial strains has been accomplished by the following assays: Short Tandemly Repeated Repetitive (STRR), Randomly Amplified Polymorphic DNA (RAPD), Restriction Fragment Length Polymorphism (RFLP) and Sequencing of identification and toxicity genes.

Short Tandemly Repeated Repetitive (STRR)

PCR products were carried out in a 25 μl volume containing 10X PCR buffer containing 15 mM MgCl₂, 50 pmol of each oligo nucleotide primer (table 1), 1.25 mM deoxynucleotide triphosphate, 2 μl of template DNA and 1U of DNA polymerase. A PTC-100 Programmable Thermal Controller (MJ Research Inc., MA, USA) was used to carry out the PCR, with a program of 1 cycle at 95°C for 6 min; 30 cycles of 94°C for 1 min, 56°C for 1 min, and 65°C for 5 min; 1 cycle at 65°C for 16 min; and a final step at 4°C. To visualize PCR products on agarose gel, 8 μl of the PCR mixture was loaded. Ethidium bromide-stained 2% agarose-Tris-acetate-EDTA electrophoresis gels [17].

Randomly Amplified Polymorphic DNA (RAPD)

PCR was performed in a total volume of 50 μl containing 3 mM MgCl₂, 200 mM each deoxynucleotide triphosphate (dATP, dCTP, dGTP, and dTTP), 10 pmol of each PCR primer (table 1), 1U of Taq DNA polymerase. A PTC-100 Programmable Thermal Controller (MJ Research Inc., MA, USA) was used to carry out the PCR, with a program of denaturation step of 95°C for 15 min, followed by 40 cycles of 95°C for 1 min, 56°C annealing temperature for 1 min, 65°C for 5 min, and ending with an additional extension step of 65°C for 16 min. To visualize PCR products on agarose gel, 8 μl of the PCR mixture was loaded. Ethidium bromide-stained 2% agarose-Tris-acetate-EDTA electrophoresis gels [2].

Restriction Fragment Length Polymorphism (RFLP)

The PCR products corresponding to the 16S rRNA genes of the cyanobacterial strains digested with restriction enzymes. RFLP technique was performed in a total volume of 25 μl containing 10X PCR buffer containing 15mM MgCl₂, 10X BSA (Bovin serum albumin acetylated), 15 μl of template 16SrRNA PCR products and 1U of restriction enzyme. Incubate mixture overnight in 37°C. To visualize RFLP patterns on agarose gel, 8 μl of the products mixture was loaded. Ethidium bromide-stained 3% agarose-Trisacetate-EDTA electrophoresis gels [7].

Sequencing of PCR Products

1. ExsoSAP

Prepare 96 well plate containing 10 μl of PCR and mix it with 4 μl ExsoSAP-IT (ExsoSAP-IT treats PCR products ranging in size from less than 100 bp to over 20 kb with absolutely no sample loss by removing unused primers and nucleotides). Spin down at 4000 rpm for 1 min at 20°C. Incubate at 37°C for 15 min to degrade remaining primers and nucleotides followed by 80°C for 15 min to inactivate ExsoSAP-IT.

2. Sequencing

Prepare 96 well plate containing 2 μl of each forward and reverse primers. 8 μl of Dyenamic ET terminator reagent sequencing premix will
be added and makeup the volume with 9 µl H2O up to 20 µl. the cycles were as follows: 40 cycles of 95°C for 20 sec, 50°C for 15 sec, and 60°C for 1 min.

Results:
Cyanobacterial Growth

The strains used are species of the filamentous genus, *Anabaena circinalis*, *Nodularia spumigena* and *Cylindrospermopsis raciborskii* and the unicellular genus *Microcystis aeruginosa flos-aquae* which are commonly found in cyanobacterial blooms. During an incubation time growth rate measurement of each strain will take a place using Spectrophotometer and the morphologic examination was performed using ConFocal microscope. We can obviously see that *Microcystis*, whether the domestic or Australian strain could reached the log-phase in a shorter time comparing to other strains specially *Cylindrospermopsis raciborskii* which takes more than two weeks to reach late log-phase. Domestic *Anabaena sp.* and *Nodularia spumigena* had the densest growth. (Figure. 1)

Differntiations of Cyanobacterial Strains
Short Tandemly Repeated Repetitive (STRR)

The presence of repeated DNA short tandemly repeated (STRR) sequences in the genome of cyanobacteria was used to generate a fingerprint method to distinguish between cyanobacterial strains [18]. In this study, primers corresponding to the STRR sequence used in the PCR were STRRA1, STRRB1 and mixed STRRA1+STRRB1. The above method resulted in generating specific fingerprints for each individual isolated. The results showed a genetic heterogeneity among isolates from different geographic regions.STRR-PCR indicated to be useful for clustering of even closely related strains, and this method can be applied to nonheterocystous cyanobacteria from which a fingerprint pattern will be obtained. Amplification of cyanobacterial genomic DNA with PCR primers derived from repetitive sequence including bacterial; *Escherichia coli* as a positive control. The use of the primer STRRA1 (or A1) in the PCR on the strain used yielded multiple distinct DNA products ranging in size from approximately from 50- 1000bp.(Figure. 2). Only minor PCR products were obtained from bacterial *E.coli* included as central. However when the inverted primer STRRB1 (B1) was used, few PCR products were generated from some species whereas multiple distinct bands were obtained from *Ecoli* (Figure. 3). The amplified PCR products obtained when combination of primers STRRA1 (A1) and STRRB1(B1) at an annealing temperature ranged in of 56°C size from approximately 50-1000 bp(Figure.4). No fingerprints were obtained from *E.coli* whereas one product was generated from *Microcystis aeruginosa flos-aquae*.

Randomly Amplified Polymorphic DNA (RAPD)

RAPD-PCR technology was used for the detection of genetic heterogeneity among axenic cultures of cyanobacteria. The methods reported are a modification of standard RAPD protocols, which use a single 10-mer primer, and, because of the increased stringency of the method for amplification reaction, provide a highly reproducible RAPD pattern for each strain analyzed. RAPD-PCR was used to generate unique and identify DNA profiles for six strains of cyanobacteria. A total of eight primers were initially chosen to generate RAPD patterns. The strain-specific randomly amplified polymorphic DNA profile made it possible to discriminate among all toxigenic cyanobacteria[9]. The criteria for choosing these primers were their availability and the generally accepted bias toward oligonucleotides of high G+C. This number of 10-mer oligonucleotides was reduced to eight, these being the primers which produced informative and reproducible genetic markers for the cyanobacteria. Primers which produced consistently even product intensities throughout a pattern were favored because of the high reproducibility of markers in these reactions. Of the primers selected, three had a G/C content of 70%

Primers with a G/C content of 60%, which did not produce RAPD patterns with 10 pmol of primer, were successful with 100 pmol per reaction. Higher concentrations of primers with 70% G/C resulted in complicated and unrepeatable PCR patterns. Single primers which gave similar patterns across a range of PCR annealing temperatures up to 42°C were chosen for further optimization of the reaction. Two primers, CRA22 and CRA23, were combined in equimolar ratios and used at 10 pmol per primer per 50-ml reaction mixture. RAPD patterns generated from this multiplex reaction were identical over a range of PCR annealing temperatures between 35°C and 45°C and template DNA levels from 100 pg to 100 ng. (Figure. 5).The annealing temperature of 45°C provided the most stringent thermal cycling conditions that provided reproducible PCR products. Increasing the annealing temperature to 50°C led to no amplification in the multiplex reaction but produced an altered pattern in the single-primer PCR. RAPD patterns in the dual-primer PCR were also highly reproducible with neat and diluted cyanobacterial cell lysate as the PCR template (Figure. 5).

Reproducibility was also achieved for this multiplex RAPD in a number of other areas previously considered drawbacks of RAPD analyses. First, no change in pattern was seen among reactions from different regions of the thermal cycling heating blocks used. Similarly, patterns among various thermal cycling instruments were identical, although cycling times were extended for machines which do not employ an in tube thermocouple to monitor the actual reaction temperature (such as Perkin-Elmer Cetus model 480). Second, identical patterns were obtained with thermostable DNA polymerases from different suppliers, e.g., AmpliTaq (Perkin-Elmer, Norwalk, Conn.). Assuming similar unit activity values for different enzymes, reproducible results should be achieved when this parameter is altered. The concentration of MgCl2 in the PCR was held constant at 3 mM for all experiments, as was the thermostable polymerase at 1U per 50-ml reaction mixture, based on previously reported RAPD reaction optimizations [2].

Restriction Fragment Length Polymorphism (RFLP)
RFLP analysis of 16S rRNA known has been used for several years as a method for rapid comparison of 16S rRNAs. RFLP analysis and sequencing of the 16S rRNA gene have revealed a very close relationship between certain strains of cyanobacteria. Briefly, 16S rRNAs are obtained by PCR amplification by using universal primers, and the product is digested with restriction enzymes with the typical analysis of restriction digests for isolates or clones is performed on relatively low-resolution agarose gels. After digestion with any of sex different restriction enzymes, the sizes of the terminal fragments amplified with the PCR primers were determined. The species richness of natural communities was estimated by determining the number of unique T-RFs or ribotypes observed in digests of 16S rRNAs amplified by PCR from total community DNA. The T-RFLP pattern observed (referred to as the “community fingerprint”) is a composite of the number of fragments pattern observed (referred to as the “community fingerprint”) is a composite of the number of fragments with unique lengths. The PCR products corresponding to the 16S rRNA genes of the six planktonic strains were digested with MspI, Sau3A1, HaeIII, Rsal, EcoRI and HinfI, according to NebCutter website. (Figures. 6, 7, 8, 9, 10 and 11).

DNA Sequencing Study

Several molecular approaches now provide powerful adjuncts to the culture-dependent techniques. One approach in particular that couples PCR and rRNA-based phylogeny has been effective in the exploration of microbial environments and the identification of uncultured organisms. The stepwise strategy of this approach is to isolate total community DNA and use this DNA as a template for PCR amplification of 16S rDNA genes with universal or domain-specific primers. PCR product for cyanobacterial identification (16S rRNA[12,18]), (Figures. 12, 13 and 14). It is generally accepted for bacteria that a 16S rDNA sequence similarity of 96±97% and DNA-DNA hybridization values of 70% relative binding (RB) with a ΔTm of 5°C represent the lower boundary of a species and that a genus may be defined by species with 95% or greater sequence similarity. However, it has been suggested that values as low as 50% RB and a ΔTm of 7°C may be employed for the delineation of species, and showed that sequence similarity is often greater than 98±99% when total DNA similarity is as low as 10±40% RB. Similar conclusions were drawn that the relationship between RB and sequence similarity varies even within the same subphylum. It therefore seems that 16S rDNA sequence similarity does not always (Fig 14,15 and 16) adequately reflect total genomic relationships. A comparison of cyanobacterial sequence similarity with DNA hybridization values determined is consistent with this proposition. (Figure. 12) The data was analysis by SeqMan software (DNA star lasergene V.6, Madison, WI, USA). Phylogenetic artifacts are also known to result from the use of distant outgroups, longbranch attraction, heterogeneous base composition, and site-specific rate variation.

Discussion

Differentiations between Cyanobacterial Strains

Sensitive methods for the detection and genetic characterization of cyanobacteria have been developed based on DNA amplification techniques. DNA amplification technology, combined with sequences of cyanobacterial genes, has provided an opportunity to design and develop rapid and sensitive protocols for detection and delineation of cyanobacterial strains. This study describes molecular methods which provide descriptive DNA profiles, composed of phylogenetic characters, which were appropriate for the inference of relatedness and evolution of cyanobacterial taxa. These phylogenetic characters provided differing levels of strain discrimination. The sensitivity of the methods described here are a reflection of the number of restriction endonuclease recognition sites in cyanobacterial specific gene amplification products, in particular, the 16S rRNA. The DNA profiles generated depict genomic polymorphisms and allow the unambiguous identification of cyanobacteria.

Short Tandemly Repeated Repetitive (STRR)

Repetitive sequences constitute an important part of the prokaryotic genome. The repetitive extragenic palindromic (REP) and enterobacterial repetitive intergenic consensus (ERIC) sequences were originally described for the family Enterobacteriaceae but later found in several gram-negative bacteria and close relatives in the same phyla. For cyanobacteria, a distinct family of repetitive sequences, the short tandemly repeated repetitive (STRR) sequences, has been described [17]. Short tandemly repeated repetitive (STRR) sequences found to occur at high frequency in the genomes of filamentous, heterocystous cyanobacteria have also been used to establish strain-specific DNA fingerprints. STRR sequences have been used either as oligonucleotide probes or as primers in the generation of PCR amplified DNA profiles. The STRR sequences have been identified in a number of cyanobacterial genera and species, all belonging to the heterocystous cyanobacteria. The function of repetitive sequences is still unclear. It has been suggested that they may regulate transcription termination or be the target of DNA binding proteins responsible for chromosomal maintenance in the cell. In this study we have performed STRR in heterocystous and non-heterocystous cyanobacteria (Microcystis). All our strains have the three different STRR sequences amplified in their genomes which allow multi different patterns distinguish each strain from another. (Figure. 2). STRR A1 provides multi bands with Australian Microcystis aeruginosa flos-aquae more than domestic Microcystis sp. which make a significant genomic variation between the two closest genus. For the Anabaena, we can see in lower degree the differentiation between the two species, Australian and domestic strain. Remarkably, E.coli genome shows a multi sharp patterns which indicates that STRR A1 is appropriate for E.coli strains fingerprint (Figure. 2). STRR B1 oligonucleotide also shows a clear differentiation among a closet strains and an obvious multi bands for Nodularia spumigena but it doesn't provide multi bands for Cylindrospermopsis raciborskii (Figure. 3) whilst STRR A1 is more effective with this strain(Figure. 1). Significantly, Cylindrospermopsis raciborskii genome does distinguish from others with this oligo and multi patterns shown with
**Randomly Amplified Polymorphic DNA (RAPD)**

RAPD markers are well suited for genetic mapping, for plant and animal breeding applications, and for DNA fingerprinting, with particular utility for studies of population genetics. RAPD markers can also provide an efficient assay for polymorphisms, which should allow rapid identification and isolation of chromosome-specific DNA fragments. RAPD assay may in some instances detect single base changes in genomic DNA. Most single nucleotide changes in a primer sequence caused a complete change in the pattern of amplified DNA segments. The most significant advantage to this method is that the determination of genotype can be automated. Genetic maps consisting of RAPD markers can be obtained more efficiently, and with greater marker density [2,8].

**Generation of DNA Profiles and Measurement of Genetic Relatedness:**

The electrophoretic patterns for strains of toxigenic cyanobacteria derive from eight single primer reactions and one double primers reaction were analyzed to provide diagnose fingerprints for each culture and genetic distances between strains based on RAPD markers. The multiplex RAPD-PCR was chosen, because of its greater degree of reproducibility and its stringency, for the accurate differentiation of cyanobacteria to the strain level and the inference of cyanobacterial phylogeny. Successive analyses of cultures have shown that these RAPD profiles are consistent over periods up to 18 months and after several PCR template extraction protocols. The results of single multiplex RAPD-PCR like the results shown for the two primers RAPD-PCR were also able to distinguish among strains of *Microcystis* *spp.* Isolated from different geographical locations and exhibiting various levels of microcystin production. The results also show the ability of the RAPD method to identify *Microcystis* species isolated and purified from a common freshwater habitat. Genetic diversity among strains tested was determined with banding patterns from the multiplex RAPD reaction. Each band was considered a genetic marker for the strain from which it was amplified. Comigrating bands were recorded as similar markers but were not sequenced or probed for further identification. The multiplexing of primers CRA22 and CRA23 generated a total of 33 RAPD markers and 29 RAPD markers when analyzed on polyacrylamide and agarose gels, respectively.

The data produced used to calculate genetic distances allowing to construct the phenogram. The tree illustrates the similarity of RAPD patterns seen on the gels. The phenogram clearly supports the delineation of the genera *Anabaena* and *Microcystis* [2]. The method has used in this study provide a highly reproducible RAPD pattern for each strain analyzed by using a multiplexing of random eight oligonucleotide primers which produced informative and reproducible genetic markers for the cyanobacteria. This technique is sensitive and specific because the entire genome of an organism is used as the basis for generating a DNA profile. Our result indicates that this technique could distinguish between even a closet genus and between closet species (Australian and domestic *Microcystis* and *Anabaena*). Obviously, we can notice that the only one band appeared with *E.coli* genome indicates these oligonucleotide primers do not provide a multi patterns which specify these primers to cyanobacterial genome (Figure. 4).

**Restriction Fragment Length Polymorphism (RFLP)**

Although the 16S rRNA molecule contains variable regions, it is too well conserved for studying species identity or intraspecies variation[3]. In our study, Different species and strains were compared using restriction fragment length polymorphism (RFLP) of amplified fragments of 16S rRNA digested with *MspI*, *Sau3A1*, *HaeIII*, *RsaI*, *EcoRI* and *Hinfl*, according to NebCutter website which were sufficient to accomplish a differentiation between each strain. *MspI* enzyme revealed similarity between Australian *Microcystis aeruginosa flos-aquae* and domestic *Microcystis* *sp.* and between domestic *Anabaena* *sp.* and *Cylindrospermopsis raciborskii* whereas it shows variation between Australian *Anabaena cirinalis* and domestic *Anabaena* *sp.*. Furthermore, *Nodularia spumigena* has five specific patterns. As an opposite of *MspI*, *Sau3A1* restriction enzyme distinguishes between *Microcystis aeruginosa flos-aquae* domestic *Microcystis* *sp.*, and the variation still shown between Australian *Anabaena* cirinals and domestic *Anabaena* *sp.* while the similarity remains between domestic *Anabaena* *sp.* and *Cylindrospermopsis raciborskii* and within *Nodularia spumigena* and domestic *Microcystis* *sp*. *HaeIII* enzyme was effective with domestic *Anabaena* *sp.* and distinguishes *Cylindrospermopsis raciborskii* from others whereas its show similar patterns with remaining strains. With *RsaI* it reveals high similarity between all strains except Local *Anabaena* *sp.* However, *EcoRI* united all various genomes in our strains in the same size of single band. *Hinfl* was more useful than *EcoRI* shows a multi bands describe a variation in genetic components in between *Anabaena cirinalis* and local *Anabaena* *sp.* but still unable to distinguish *Microcystis aeruginosa flos-aquae* from local *Microcystis* *sp.*. *Nodularia spumigena* and *Cylindrospermopsis raciborskii* has an own patterns. These variations between the strains indicate that 16S rRNA gene has a different construction from cyanobacterial strain to another which confirms the value of this gene to use as a remarkable tool in cyanobacterial differentiation, taxonomy and classification (Figure. 6 7,8,9,10and 11).

**DNA Sequencing Study**
The use of DNA sequences for the taxonomic and phylogenetic analysis of cyanobacterial isolates has been reviewed. A number of genes have been used as evolutionary markers in the delineation of cyanobacterial taxonomy, with the 16SrRNA gene analyzed most extensively because of its ubiquitous distribution throughout prokaryotic phylogenetic groups. DNA amplification technology, combined with sequences of cyanobacterial genes, has provided an opportunity to design and develop rapid and sensitive protocols for detection and delineation of cyanobacterial strains. Sequencing of 16SrRNA gene is becoming more efficient and provides the most accurate method for determining relatedness and inferring evolution within this group of Prokaryotes.

Surprisingly, variations within nitrogen bases sequence in 16SrRNA gene are not distributed along the sequence but located in specific sites and the remaining sequences keep their similarity between our strains genomes. We clearly notice in figures 11 and 12a part of 16SrRNA gene sequence the differences in nitrogen bases among our strains especially between the domestic and Australian Microcystis and Anabaena. Also this informative gene sequence provides specification of the genome in a toxic and non-toxic cyanobacteria. In phylogenetic analysis tree of 16SrRNA (Figure. 12) displayed a relation between each cyanobacterial strains in our study which it can provides obvious view of the differentiation between an Australian and the domestic cyanobacterial strains which could proof the genetic diversity among the strains depending on the geographical regions. Figure.13 regarding calculated values of the nitrogen bases contents of the tree and the shifting between particular bases. (Transitions and Transversions).

Phycocyanin intergenic spacer (PC-IGS) sequence among cyanobacteria has been described. PC operon consists of five open reading frames (ORFs), separated by noncoding intergenic spacers (IGS) that may be highly variable. Studies have shown that the IGS sequence between the β- and a-subunit ORFs (PC-IGS), which encode for phycocyanin b and phycocyanin a, respectively, can be used to differentiate cyanobacterial species. Sequence of the PC-IGS is capable of predicting the genus accurately, but not the species. In our study, we confirmed this information by the variations appeared in PC-IGS sequence between each of our cyanobacterial strain which could candidates this operon as another identification and differentiation tool for cyanobacterial beside 16SrRNA (Figure. 15).

This highly variation we noticed in 16SrRNA and PC-IGS sequences haven’t shown with nitrogenase gene sequence (nifH) in our study. Tracing the phylogeny of cyanobacteria using nifH is problematic. The reconstructed trees are very sensitive to small changes in sequence. For example, Trichodesmium’s unique biological solution of combining a semi-temporal separation of N2 fixation and photosynthesis with spatial heterogeneity appears in several trees as an early branch, suggesting a very ancient past. Yet, in other trees it shows a later divergence. Moreover, organisms with the same strategy (e.g., Lyngbya and Cyanathoe) do not always cluster together. Whether this is due to parallel evolution or is an artifact of the phylogenetic reconstruction remains to be elucidated.[5]

Our understanding of the phylogenetic history of nif genes has been advanced significantly by extensive efforts in sequencing nitrogenase genes, primarily the highly conserved nifH gene but also the larger but less conserved nifD, nifK, nifE, and nifN genes. Noteworthy, despite our result shown clear variations among nifH gene sequences of some strains, however a similar sequences between a toxic heterocystous Nodularia spumigena and a toxic nonheterocustous Microcystis aeruginosa flos-aquae has been recorded (Figure 16).

References:


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**Figure 1** Growth curve measurements of cyanobacterial strains

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**STRR A1**

Figure 2. STRR amplification using STRR A1 primer. **Lane1**: *Microcystis aeruginosa flos-aquae*, **Lane2**: Local *Microcystis sp.*, **Lane3**: *Anabaena circinalis*, **Lane4**: Local *Anabaena sp.* **Lane5**: *Nodularia spumigena*, **Lane6**: *Cylindrospermopsis raciborskii*. **Lane7**: *Escherichia coli*. 100 bp DNA step ladder as a marker (M).

**STRR B1**

Figure 3. STRR amplification using STRR B1 primer. **Lane1**: *Microcystis aeruginosa flos-aquae*, **Lane2**: Local *Microcystis sp.* **Lane3**: *Anabaena circinalis*, **Lane4**: Local *Anabaena sp.* **Lane5**: *Nodularia spumigena*, **Lane6**: *Cylindrospermopsis raciborskii*. **Lane7**: *Escherichia coli*. 100 bp DNA step ladder as a marker (M).
Figure 4. STRR amplification using STRR A1+STRR B1 primers.  
Lane1: *Microcystis aeruginosa* flos-aquae, Lane2: Local *Microcystis* sp.  
Lane3: Anabaena circinalis, Lane4: Local Anabaena sp. Lane5: Nodularia spumigena, Lane6: Cylindrospermopsis raciborskii. Lane7: *Escherichia coli*. 100bp DNA step ladder as a marker (M).

Figure 5. RAPD Amplification of cyanobacterial strains and *E.coli*.  
Lane1: *Microcystis aeruginosa* flos-aquae, Lane2: Local *Microcystis* sp. Lane3: Anabaena circinalis, Lane4: Local Anabaena sp. Lane5: Nodularia spumigena, Lane6: Cylindrospermopsis raciborskii. Lane7: *Escherichia coli*. 100bp DNA step ladder as a marker (M).
Figure 6. 16SrRNA gene digested by *MspI*. 
Lane 1: *Microcystis aeruginosa flos-aquae*, Lane 2: Local *Microcystis* sp. 
Lane 3: *Anabaena circinalis*, Lane 4: Local *Anabaena* sp. Lane 5: *Nodularia spumigena* 
Lane 6: *Cylindrospermopsis raciborskii*. Lane 7: undigested PCR product. 100bp DNA step ladder as a marker (M).

Figure 8. 16SrRNA gene digested by *HaeIII*. Lane 1: *Microcystis aeruginosa flos-aquae*, Lane 2: Local *Microcystis* sp. Lane 3: *Anabaena circinalis*, Lane 4: Local *Anabaena* sp. Lane 5: *Nodularia spumigena*. Lane 6: *Cylindrospermopsis raciborskii*. Lane 7: undigested PCR product. 100bp DNA step ladder as a marker (M).
**RsaI.**

*Figure 9.* 16srRNA gene digested by RsaI. **Lane1:** Microcystis aeruginosa flos-aquae, **Lane2:** Local Microcystis sp. **Lane3:** Anabaena circinalis, **Lane4:** Local Anabaena sp. **Lane5:** Nodularia spumigena, **Lane6:** Cylindrospermopsis raciborskii. **Lane7:** undigested PCR product. 100bp DNA step ladder as a marker (M).

**EcoRI**

*Figure 10* 16srRNA gene digested by EcoRI. **Lane1:** Microcystis aeruginosa flos-aquae, **Lane2:** Local Microcystis sp. **Lane3:** Anabaena circinalis, **Lane4:** Local Anabaena sp. **Lane5:** Nodularia spumigena, **Lane6:** Cylindrospermopsis raciborskii. **Lane7:** undigested PCR product. 100bp DNA step ladder as a marker (M).
Figure 11 16s rRNA gene digested by *HinfI* enzyme. **Lane1**: *Microcystis aeruginosa flos-aquae*, **Lane2**: Local *Microcystis* sp. **Lane3**: *Anabaena circinalis*, **Lane4**: Local *Anabaena* sp. **Lane5**: *Nodularia spumigena*, **Lane6**: *Cylindrospermopsis raciborskii*. **Lane7**: undigested PCR product. 100bp DNA step ladder as a marker (M).
16SrRNA Gene Sequence

Figure 12 The chromatogram analysis for 16SrRNA sequence of cyanobacterial strains: Cylindrospermopsis raciborskii, Mic aeruginosa flos-aquae, Nodularia spumigena, Anabaena circinalis, Microcystis sp. and Anabaena sp. respectively. (A-F) Adenine, Cytosine, Thiamine and Guanine

Figure 13 Multiple sequence alignment of consensus sequences of 16S rRNA of cyanobacterial strains: *Microcystis aeruginosa*, *Anabaena circinalis*, *Nodularia spumigena*, *Cylindrospermopsis raciborskii*, *Microcystis sp.*, and *Anabaena sp.* respectively. 

A. The beginning of the gene (1-51 bp). 
B. The middle of the gene (501-537 bp).
Figure 14. Phylogenetic tree constructed from 16s-RNA nucleotide sequence alignments of six cyanobacterial strains under study.

Microcystis aeruginosa flos-aquae

Nodularia spumigena

Microcystis sp.

Anabaena circinalis

Cylindrospermopsis raciborskii

Anabaena sp.

Sequence changes in almost all positions on branch

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**Figure 15** Calculations of some characters of 16S rRNA phylogenetic tree

**Figure 16.** Calculations of some of the 16S rRNA gene changes shown in the tree
### Primer Target Sequence P.S* T(°C) References

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*P.S PCR product size. *M.B Multiple bands.