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16Sr RNA Gene Oligonucleotide Probes for Genetic Profiling of Hot Spring Cyanbacteria from Saudi Arabia

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Evaluate the phylogenetic diversity of isolated species from hot spring water in Bani Malik on the foot hill of Fifa mountain in Gizan Region of southwestern Saudi Arabia. The identification based on the molecular techniques such as 16Sr RNA, phycocyanine intergenic spacer (PG-IGS) and nitrogenase (nifH) amplification. In addition, the differentiation between all isolated cyanobacterial strain applying restriction fragment length polymorphism (RFLP) and sequencing the gene produced.16Sr RNA-PCR amplification has been performed for all isolated strains; *Fischerella sp., Synechococcus sp., Prochlorothrix sp., Oscillatoria sp., Phormidium sp.,* and the two species of *Leptolyngbya sp.* resulting number of bands which determine the identity of the genomes as prokaryotes. Amplification by PCR of the phycocyanine gene of intergenic spacer region (PG-IGS) observed that is highly conserved within genus, but differ significantly between genera. The results of RFLP applications showed a genetic heterogeneity among some isolates while there is a similarity for the other strains. Similar conclusions with bacteria showed that 16Sr RNA sequence similarly of 96+97%. The phylogenetic tree were conducted from the use of distant out groups, long branch attraction, heterogeneous base composition and site-specific rate variation using Mac lade 4-software. DNA sequences of the 16Sr RNA internal transcribed spacer region (16S-23S ITS) are known to be variable and exhibit enormous differences in sequences and length. Diversity surveying, community fingerprinting, and functional interrogation of natural populations have become common, enabled by a battery of molecular and bioinformatics techniques, some specifically developed for the cyanobacteria, which are reviewed in detail. The ensuring effects on our views of cyanobacterial ecology have been perhaps less revolutionary, because of the special characteristics of cyanobacteria among microbes, but also significant.

Key words: Cyanobacteria, Genetic Profiling, Hot springs, 16Sr RNA.

Cyanobacteria are aquatic and photosynthetic organisms, that they live in the water, and can manufacture their own food. Because they are bacteria, they are quite small and usually unicellular, thought they often grow in colonies large enough to see[7.8.12]. The ability of cyanobacteria to perform oxygenic photosynthesis is thought to have converted the early reducing atmosphere into an oxidizing one, which dramatically changed the life forms on Earth and provoked an explosion of biodiversity. Cyanobacteria are the only group of organisms that are able to reduce nitrogen and carbon in aerobic conditions, a fact that may be responsible for their evolutionary and ecological success.

They can be classified as thermophilic, mesophilic or psychrophilic, according to the temperature of the culture media in which these organisms grows[1.2]. This characteristic provides opportunities for the use of blue-green algae as a probe in the studies of the adaptation of an organism to growth in various environments[7]. Phycocyanin, a major biliprotein extracted from these algae, can be used as a probe in the

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investigation of the effect of variations in environmental stress on the structure and function of proteins. It has been reported that the ability of phycocyanin to resist urea denaturation is in the order of thermophile > mesophilic > psychrophile. Based on the data of apparent free energy of protein unfolding, thermophilic phycocyanin was found to be more stable than that from the mesophilic one[19]. Moreover, it has been reported that denaturated phycocyanin undergoes thermal denaturation at a temperature 5ºC lower than normal phycocyanin[20].

Blue-green algae are particularly concentrated in hot-spring waters with a pH of over 6 where they form conspicuous and often unialgal matlike covers over submerged substrates[5]. Since there is in many hot springs a surface effluent with a thermal gradient ranging from supraoptimal to ambient air temperature, specific differences in growth temperature optima may result in distinct species bands covering different portions of the gradient[21]. Since the component organisms differ in their amounts of chlorophyll-a, biliproteins, and carotenoid pigments, these bands may be quite different in color, but ranging from a dark brown to a yellow or rich green or blue-green. The orange or flesh color of many hot spring mats is often caused by compact masses of heterotrophic, carotenoid-containing, filamentous bacteria[23].

Because of the shallowness and the clarity of most thermal waters, and the exposure of many hot springs to high light intensities, various types of "sun adaptation" have occurred in many thermophilic organisms[24]. Most alkaline hot springs contain between 1,000 and 2,000 mg of total dissolved solids (TDS) per liter; some have salinities higher than that of seawater. Even those of lower salinity may be notably enriched in S (reduced), As, F, Mn, Fe, Al, Li, Si, or in several other elements. Almost nothing is known about the algae or chemistry of the hot springs of Saudi Arabia[14]. Algal communities of the hot springs of North America, Japan and other parts of the world have been studied extensively[25].

However, the blue-green algae, mostly in the form of algal mats, continued to be abundant along the spring length. One of the most interesting cyanobacteria observed is *Chlorogloeopsis fritschii*. This species is reported for the first time in Saudi Arabia although it is not restricted to thermal habitants. Bani Malik on the foot hill of Fifa mountain bordering the Yemen Republic on one side and Gizan Region of southwestern Saudi Arabia as the other has may hot springs water over there. Our study concentrates on the hot spring cyanobacteria of this region.

Within the vicinity of the Bani Malik hot spring there is a pungent odor of sulphur and/or hydrogen sulfide in the air. However, the steam and gas rising up from the drains soon evaporates thereby clearing the odoriferous gases. It is believed by the local inhabitants that this natural hot spring has therapeutic qualities and is helpful in curing skin diseases. Sediments deposited at the floor and sides of the concrete pool encourage prolific thermophilic algal and bacterial communities. These floras tend to establish down the cascade and run downstream further for about nine meters.

Molecular phylogeny has become a powerful tool in elucidating evolutionary pattern, and analyses based on 16Sr RNA sequences indicate that cyanobacteria producing baeocytes, heterocysts, and true-branching filaments are each phylogenetically coherent[3.4.6]. In contrast, phylogenies reconstructed by using *nifH* and *nifD*, structural genes fro nitrogenase, the enzyme that catalyses biological nitrogen fixation, do not support monophyly of the *nifH* phylogeny[9.10.11]. Not all cyanobacteria fix nitrogen and, therefore, genes such as *nifH* or *nifD* cannot be used to analyze non-nitrogen-fixing species[13.15.16]. Because even well resolved molecular phylogenies show only the relative timing of diversification events, direct evidence from the geologic record is required to constrain actual divergence times[17.18.22.23]. To reveal how and when cyanobacteria evolved, molecular phylogentic and paleontological studies in tandem. First, to elucidate the phylogenetic pattern of cyanobacterial diversification. Thus, the specific goals of this present study involving the Isolation and cultivation of thermophilic and evaluate the phylogenetic diversity of isolated species from hot spring water. based on the molecular approaches as 16Sr RNA, phycocyanine intergenic spacer (PG-IGS) and nitrogenase (nifH) amplification. In addition, the differentiation between all isolated cyanobacterial strain applying

restriction fragment length polymorphism (RFLP) and sequencing the gene produced.

MATERIALS AND METHODS

Site Characteristics

Hot springs are known in the Eastern and Central Provinces of Saudi Arabia and the maximum number of these was recorded from Gizan Province[12] . However, one of the least-known and not yet studied hot springs is situated in Bani Malik on the foot-hills of the Al-Fifa Mountains, bordering the Yemen Republic on one side, and the Gizan Region of southwestern Saudi Arabia on the other. Some of the hot springs of the Gizan Area has been previously surveyed by Castenholz[7]. The Gizan Region has many hot springs, unlike the other regions of Saudi Arabia.

Within the vicinity of the Bani Malik hot spring there is a pungent odor of sulphur and/or hydrogen sulfide in the air. However, the steam and gas rising up from the drains soon evaporates thereby clearing the odoriferous gases. It is believed by the local inhabitants that this natural hot spring has therapeutic qualities and is helpful in curing skin diseases. Sediments deposited at the floor and sides of the concrete pool encourage prolific thermophilic algal and bacterial communities. These floras tend to establish down the cascade and run downstream further for about nine meters. Visually distinguishable color zonations between the two dominant mats can be seen through the water column where *Synechococcus lividus* form thin mats (green and/ or turquoise) mostly in a continuous fashion at a higher temperature of 48°C. However, at the far end of the pool their distribution was in discontinuous fashion.

Sample Collections

The samples were collected in sterile plastic sacs from the top (20 - 30 cm) of the soil. A total of 20 soil samples were collected five from each site. There was not marked algal growth evident in the soil at the time of collection. Soil temperatures were measured using a soil thermometer. The pH measurements were made electrometrically on soil paste.

Cultures were incubated at 25°C and 35°C under continuous illumination from cool white fluorescent lamps. When algal growth was observed, cultures were examined microscopically until the algae could be identified. Unialgal cultures of some of the algae were obtained and were maintained at 25°C for the further studies. The algae used in this experiment were grown to mid-exponential growth phase. Growth was measured turbidometrically at 700 nm by a Baush & Lomb spectronic 20 spectrophotometer.

Culture media

A modification of the medium of *BG-11* and *Castenholz*[7] was used in these experiments. It was selected on the basis of comparative tests as the mineral medium of choice for routine maintenance of axenic strains of unicellular and filamentous blue-green algae and also proved very satisfactory for the enrichment of blue-green algae from natural sources.

Cyanobacterial growth

The cultures of *Fischerella sp., Synechococcus sp., Prochlorothrix sp., Oscillatoria sp., Phormidium sp.,* and the two species of *Leptolyngbya sp.* were grown in *Castenholz et al. 1970* media. Where Synechococcus sp. and Oscillatoria sp. are were grown in B.G – 11, 1979. Under constant light intensity (20μ $Mm^{-1}S^{-1}$) for up to 10 days at 45 - 60°C (Appendix I). During an incubaction time growth rate measurement of each strain will take a place using spectrophotometer with 700 nm wavelength (Table 1, 2, 3, 4 &5, Figure 7 to 14). For subculturing, 4 µl of the culture were added to 500 µl of the media.

Direct and indirect investigation

Most algae were seen by direct viewing of samples in the field using a dissecting field microscope and are regarded as direct viewing. The rest of the algae were seen in enrichment cultures and are regarded as indirect viewing (Table 2). All the isolated algal culture were investigated by light microscope.

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.

Amplification of Small Subunt Ribosomal Gene (16Sr RNA)

Oligonucleotide primers (Table .....) were used to amplify an approximately 780 base pair (bp)segment of DNA that included the variable

16SrRNA priming sites had been chosen from completely conserved regions within the (forward primer) and (reverse primer) subunits of the 16SrRNA. PCR was performed in a 25 µl volume reaction containing 2.5 µl of 10X PCR buffer containing 15mM $MgCl_2$, 2 µl of 2.5 mM of each deoxynucleoside triphosphate (dNTPs), 5 pmol of each primer, IU of DNA polymerase, 2 µl of template and enough water to make a final volume of 25 µl. 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 1.5 min, 58°C annealing temperature for 30 s, 72°C for 7 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.

Amplificationof Nitrogenase gene (nifH)

Although, nitrogen is the most abundant gas in Earth's atmosphere, it is extremely unreactive. Before it can be incorporated into biological molecules, nitrogen must be chemically reduced to the equivalent of ammonia.

Microbial nitrogen fixation has traditionally been considered to be of minor importance as a source of fixed nitrogen in oceanic environments. Contemporary mass balance estimates, however, have suggested that there is excess removal of combined forms of nitrogen, particularly from areas of the tropical ocean, and it has been proposed that biological nitrogen fixation may account for the imbalances. This has led to a radically revised view of the quantitative importance of this process in the nitrogen cycle. In almost all cases, *nif* genes are found within one or several extensive, cotranscribed operons or regulons that not only encode the subunits of the functional nitrogenise protein but also code for an expansive suite of proteins involved with regulation, activation, metal transport, and cluster biosynthesis.

Nitrogenase, the enzyme that reduces nitrogen to ammonium, is composed of two multisubunit proteins (the MoFe protein, composed of subunits encoded by nif*D* and nif*K*, and the Fe protein, composed of identical subunits encoded by nif*H*), both of which are conserved among nitrogen-fixing organisms. The amino acid sequence of the Fe protein is very similar among organisms, even those of very different taxonomic groups. Even the alternatives nitrogenise Fe protein amino acid sequences are greater than 63% identical to the conventional nitrogenise Fe protein sequence (91% between *nifH* and *vnfH* and 63% between *nifH* and *anfH*). This conserved feature of the Fe protein gene, nifH, provides a convenient way to develop probes for studying nitrogen fixation genes in divers organisms, as well as for characterizing nitrogen fixation in natural microbial communities. *nifH* gene given a wide utility for investigating the diversity of marine nitrogen fixers. The DNA sequence of the 359-bp segment is sufficiently variable to distinguish cyanobacterial *nifH* genes from other eubacterial and archaeobacterial *nifH* genes. The amplified *nifH* fragments can be used as DNA probes to differentiate between species, although there was substantial cross-reactivity between the *nifH* amplification products of some strains.

Amplification of Phycocyanine gene (PC-IGS):

Phycocyanins are accessory pigments in the photosynthetic apparatus of cyanobacteria, rhodophytes, and cryptophytes and they are highly conserved. In cyanobacteria, these proteins are coded by the phycocyanin operon, which consists of five open reading frames (ORFs), separated by noncoding intergenic spacers (IGS) that may be highly variable. In particular, the genetic polymorphisms detected inthe phycocyanin (PC) operon and the 16S – 23S ribosomal RNA internal transcribed spacer (rRNA ITS) readily define genus boundaries and show varying degrees of concordance with traditional classification schemes. Molecular approaches to cyanobacterial identification are not influenced by ecological variables and in many instances do not require axenic or unicyanobacterial cultures for analysis. Indeed, due to the sensitivity afforded by DNA amplification technology, minute samples sizes, containing little cyanobacterial biomass, are required for the generatio of genetic profiles. The sensitivity of the methods described are reflection of the number of restriction endonuclease recognition sites in cyanobacterial specific gene amplification products, in particular, the 16s – 23Sr RNA ITS and the phycocyanin operon. The DNA profiles generated depict genomic polymorphisms and allow the unambiguous identification of toxic cyanobacteria. The gross topology of the

phylogenies inferred from polymorphisms in the phycocyanin and rRNA operons was also supported by RAPD profiles of toxic cyanobacteria. The results of these studies show that genetic relatedness between the genera of bloom-forming cyanobacteria is supported by the described 16S-23S ITS and phycocyanin PCR-RFLP profiles. This system of molecular identification of cyanobacteria, when used in conjunction with traditional morphological evaluation, should continue to assist in the accurate delineation of novel isolates and the presentation of an amended system for cyanobacterial classification. The levels of sensitivity offered by molecular systematic are varied and afforded a continuum of methods for strain discrimination. At the lowest taxonomic level, methods based on whole genome polymorphisms, such as RAPD PCR, readily differentiate between strains of cyanobacteria tested regardless of geographical origins. For example, the *Microcystis* strains formed a monophyletic cluster based on 16Sr RNA gene sequences but comprised two groups with respect to phycocyanin intergenic spacer (PC-IGS0 sequences. The toxigenecities of various strains were contrasted with their positions in phylogenies based on 16S ribosomal DNA (rDNA) and PC-IGS DNA sequences to see if the toxic strains form a distrinct clade. No consistent relationship was found between the (*N*-methyltransferase) NMT genotype, PC-IGS sequence, 16Sr RNA sequence, geographical region of isolation, or morphological species identification.

Restriction Fragment Length Polymorphism (RFLP)

The PCR products corresponding to the 16SrRNA 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 15 mM $MgCl_2$, 10X BSA (Bovin serum albumin acetylated), 15 µl of template 16SrRNA PCR products and IU 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-Trsiacetate-EDTA electrophoresis gels.

Sequencing of PCR Products:

ExsoSAP

Prepare 96 well plate containing 10 µl of PCR and mix it with 4 µl ExoSAP-IT (ExoSAP-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 primes and nucleotides followed by 80°C for 15 min. to inactivate ExoSAP-IT.

Sequencing

Prepare 96 well plates containing 2 µl of ExoSAP-IT PCR and add 1 µl of each forward and reverse primers. 8 µl of Dynamic ET terminator reagent sequencing premix will be added and makeup the volume with 9 µl H_2O 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

Description of Bani Malik Hot Spring and Algal Communities

Hot springs are known in the Eastern and Central Provinces of Saudi Arabia and the maximum number of these was recorded from Gizan Province[7,12]. However, one of the least-known and not yet studied hot springs is situated in Bani Malik on the foot-hills of the Al-Hejjaz Mountains, bordering the Yemen Republic on one side, and the Gizan Region of southwestern Saudi Arabia on the other.The Gizan Region has many hot springs, unlike the other regions of Saudi Arabia.

Within the vicinity of the Bani Malik hot spring there is a pungent odor of sulphur and/or hydrogen sulfide in the air. However, the steam and gas rising up from the drains soon evaporates thereby clearing the odoriferous gases. It is believed by the local inhabitants that this natural hot spring has therapeutic qualities and is

Table 1. Occurrence of Cyanobacteria blue-green algae in different temperature zones (temperature gradients) in the Bani-Malik hot spring

Algae	Occurrence in gradient (Temp.°C)
Fischerella sp.	48
Leptolyngbya sp.	50
Oscillatoria	55
Phormidium sp.	57
Prochlorothrix sp.	55
Synechococcus	60

helpful in curing skin diseases. Sediments deposited at the floor and sides of the concrete pool encourage prolific thermophilic algal and bacterial communities. These floras tend to establish down the cascade and run downstream further for about nine meters. Visually distinguishable color zonations between the two dominant mats can be seen through the water column where *Synechococcus lividus* from thin mats (green and/or turquoise) mostly in a continuous fashion at a higher temperature of 48°C. However, at the far end of the pool their distribution was in discontinuous fashion.

In view of the recreational and medicinal values of the hot springs, the Faifa Development Authority is planning to develop the area as a tourist resort. As a result of such developments, it was considered necessary to explore the water quality and its flora and fauna. . Further, the intent of the present study is to report on thermophilic blue-green algae and their related soft-bottomed biotopes, as these organisms have received little attention in Saudi Arabia. The stratification of the nodular mats and their association with flora and fauna are studied to throw light on their coexistence.

The diversity of algal biomass in relation to their corresponding algal mats can be easily seen on the basis of their distinct zonations that appear through the water column. These forms of mats were recognized in the present study and these were differentiated by their general morphology and color demarcation. A particular nodular mat that has not yet been reported from the thermal springs of Saudi Arabia is interesting and striking. A brief description of the Bani Malik hot spring site in which all the cyanobacteria isolated. Figure 1, 2, 3, 4 & 5 a list of the cyanobacterial algae identified by axenic culture under the light microscope with manufacture 10x is reported in Table 1 along with the temperature gradient.

Genomic DNA extraction

Multiple displacement amplification (MDA) using Ö29 DNA polymerase was used to amplify whole genomes of all the isolates, subsurface sediments. By first amplifying the genomic DNA (gDNA), biodiversity analysis and g DNA library construction of microbes found in contaminated soils were made possible. The

Fig. 1.

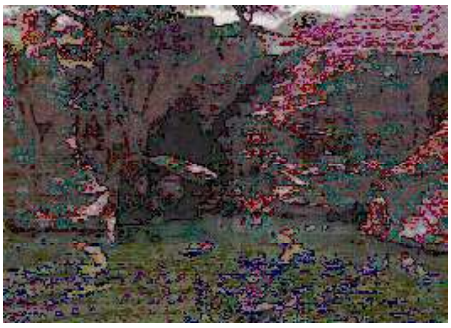
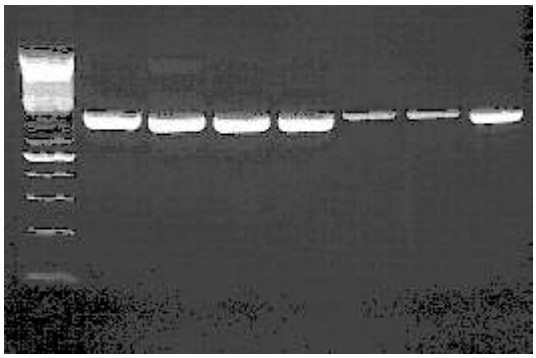


Fig. 3. A view of Bani Malik hotspring water

Fig. 2. A view of Bani Malik hotspring water



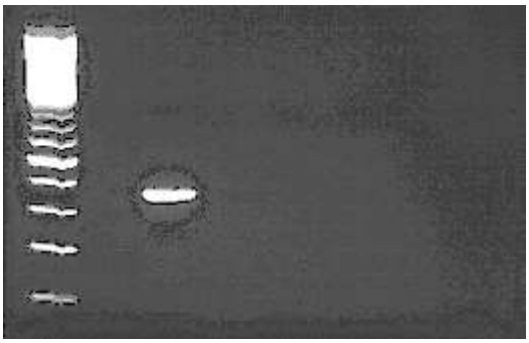
Lane 1: *Synechococcus sp.*, Lane 2: *Fischerella sp.*, Lane 3: *Oscillatoria sp.*, Lane 4: *Phormidium sp.,* Lane 5: *Leptolyngbya sp*., Lane 6: *Leptolyngbya sp*., Lane 7: *Prochlorothrix sp*. 100 bp DNA step ladder as a marker (M).

Fig. 4. Amplification of 16Sr RNA using 27F and 809R primers.

method was further validated by confirming overall representative species coverage and also an amplification bias when amplifying from a mix of seven cyanobacterial strains. In this study, multiple displacement amplification (MDA) using Ö29 DNA polymerase was applied to seven strains of cyanobacteria *Fischerella sp., Synechococcus sp., Prochlorothrix sp., Oscillatoria sp., Phormidium sp.,* and the two species of *Leptolyngbya sp.* The MDA was performed for 16 hours at 30°C using hexamer random primers generated approximately 600 ng of amplified DNA. Increasing the random primers to more than 20 µM decrease the yield of amplified produced. These data indicate that excess primers might prevent the MDA reaction.

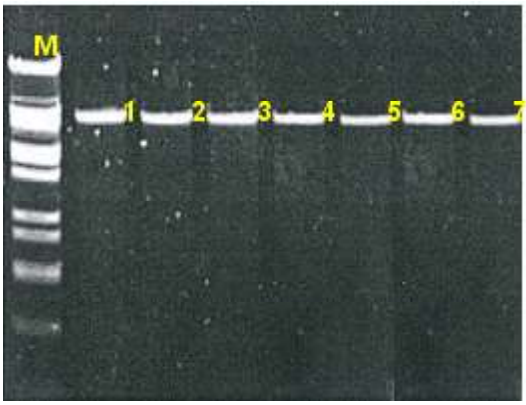
Evaluation of MDA product from the cyanobacteria species

Multiple displacement amplification (MDA) was performed on 20 ng of genomic DNA. Direct genomic amplification from cyanobacterial cells eliminated cutting of DNA during the extraction process. In to validate uniformity of the MDA product, presence number in the amplified DNA was measured by a real-time TaqMan PCR



Lane1: *Synechococcus* sp., Lane2: *Fischerella* sp., Lane3: *Oscillatoria* sp., Lane4: *Phormidium* sp., Lane5: *Leptolyngbya* sp., Lane6: *Leptolyngbya* sp., Lane7: *Prochlorothrix* sp. 100 bp DNA step ladder as a marker (M).

Fig. 5. Nitrogenase gene (nifH) amplification.



Lane 1: *Synechococcus sp.*, Lane 2: *Fischerella sp.*, Lane 3: *Oscillatoria sp.*, Lane 4: *Phormidium sp.,* Lane 5: *Leptolyngbya sp.*, Lane 6: *Leptolyngbya sp.*, Lane 7: *Prochlorothrix sp.* 100 bp DNA step ladder as a marker (M).

Fig. 6. Phycocyanine intergenic spacer (PC-IGS) amplification

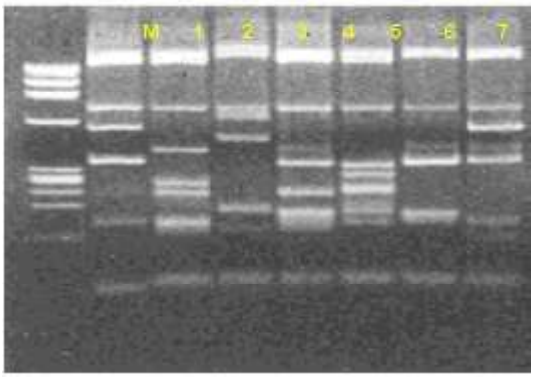


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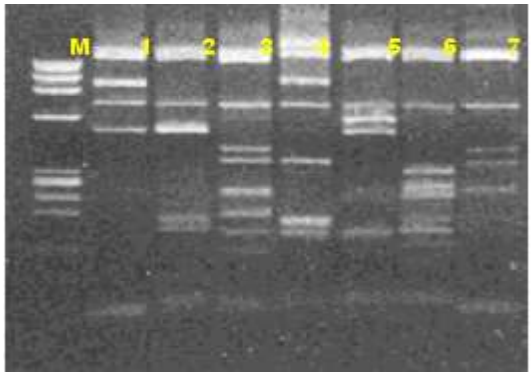
Fig. 7. RFLP analysis of 16Sr RNA digested by *HaeIII*



Lane 1: *Synechococcus sp.*, Lane 2: *Fischerella sp.*, Lane 3: *Oscillatoria sp.*, Lane 4: *Phormidium sp.,* Lane 5: *Leptolyngbya sp.*, Lane 6: *Leptolyngbya sp.*, Lane 7: *Prochlorothrix sp.* 100 bp DNA step ladder as a marker (M).

Fig. 8. RFLP analysis of 16Sr RNA digested by Sau3A1

assay in *Fischerella sp., Synechococcus sp., Prochlorothrix sp., Oscillatoria sp., Phormidium sp.,* and the two species of *Leptolyngbya sp.* (Figure 7 to Figure 14) shows DNA optical density after and prior to whole genome amplification almost 10 – 20 fold by MDA.

The result indicted that all genes located in all different positions of the cyanobacterial genome could be amplified by MDA with enough quantity for genome wide study.

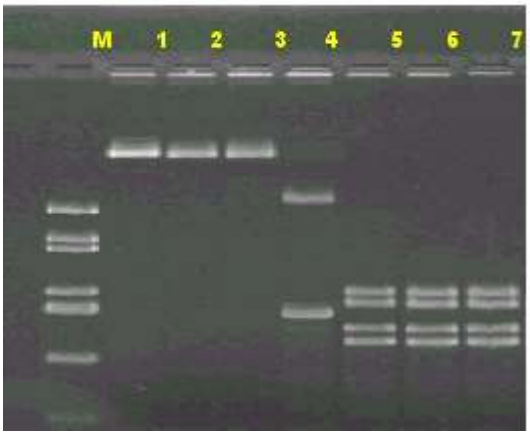
Molecular Identification and Characterization of Isolated Cyanobacteria

Molecular characterization of seven hot spring cyanobacterial isolates was carried out using the 16SrRNA, phycocyanine gene, and nitrogenous (*nifH*) gene. DNA sequences from the 16Sr RNA gene discriminated the seven species, which corresponded to their morphotypes identified by traditional microscopic analysis. PCR–RFLP using restriction enzymes will be available tools to characterize the all samples in term of presence or absence of polymorphism.

Phylogenetic analysis supports the conclusion that the *phormidium and leptolyngbya are not monophyletic*. Based on published 16Sr RNA sequences, we developed a PCR procedure for the selective retrieval of cyanobacterial rRNA gene fragments. This procedure proved useful to visualize the diversity of cyanobacterial 16Sr RNA genes in environmental samples to detect the uniqueness of isolated strains and to assign PCR products derived from cultures to population in the field. PCR products containing a single homogeneous population of DNA molecules are recognized as single bands after gel electrophoresis (Figure 4) and can be directly sequenced, yielding information about approximately 780bp nucleotides of the 16SrRNA genes. Sequence data therefore can be generated without time consuming. Cloning procedures from cyanobacterial cultures containing heterotrophic bacterial allowing the rapid survey of collections of strains for genetic diversity. The cyanobacterial specific 16Sr RNA PCR amplified about 780bp form all strains analyzed. Ribosomal RNA amplified by using the primer set, which is targeted to universally conserved regions. Thus, the information for the small-subunit rRNA gene showed there was relationally good correlation among the strains used.

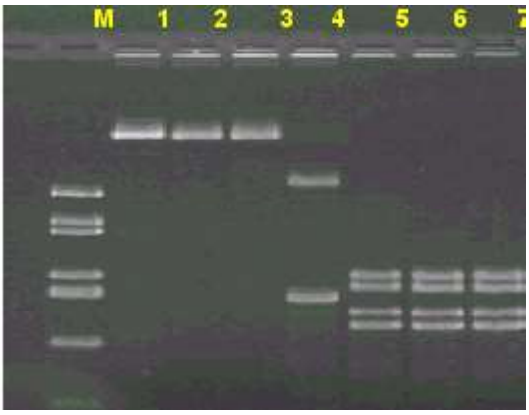
Small sub-unit Ribosomal RNA (16Sr RNA implication)

The 16Sr RNA gene was amplified for all cyanobacteiral seven strains with universal primers produce 780bp product. Based on published 16Sr RNA sequences, we developed a PCR procedure for the selective retrieval of cyanobacterial rRNA gene fragments. This procedure proved useful to visualize the diversity of cyanobacterial 16Sr RNA



Lane1: *Synechococcus* sp., Lane2: *Fischerella* sp., Lane3: *Oscillatoria* sp., Lane4: *Phormidium* sp., Lane5: *Leptolyngbya* sp., Lane6: *Leptolyngbya* sp., Lane7: *Prochlorothrix* sp. 100 bp DNA step ladder as a marker (M).

Fig. 9. RFLP analysis of 16Sr RNA digested by *Hinf1*



Lane 1: *Synechococcus sp.*, Lane 2: *Fischerella sp.*, Lane 3: *Oscillatoria sp.*, Lane 4: *Phormidium sp.,* Lane 5: *Leptolyngbya sp*., Lane 6: *Leptolyngbya sp*., Lane 7: *Prochlorothrix sp*. 100 bp DNA step ladder as a marker (M).

Fig. 10. RFLP analysis of 16Sr RNA digested by *Hinf1*

genes in environmental samples to detect the uniqueness of isolated strains and to assign PCR products derived from cultures to population in the field. PCR products containing a single homogenous population of DNA molecules are recognized as single bands after gel electrophoresis (Figure 4) and can be directly sequenced, yielding information about approximately 780 bp nucleotides of the 16Sr RNA genes. Sequence data therefore can be generated without time consuming. Cloning procedures from cyanobacterial cultures containing heterotrophic bacterial, allowing the rapid survey of a collection of strains for generic diversity. The cyanobacterial specific 16Sr RNA PCR amplified was about 780 bp from all strains analyzed. Ribosomal RNA amplified by using the primer set, which is targeted universally conserved regions. Thus, the information for the small-subunit rRNA gene showed there was relationally good correlation among the strains used. The conclusions from these studies helped to distinguish the genetic abundance profiles.

Nitrogenase gene is conserved among nitrogen-fixing cyanobateria. Thus, the nitrogenase gene provide a convenient way to develop probes for studing nitrogen fixation gene in diverse organisms, as well as for characterizing nitrogen fixation in natural microbial communities. Investigated the possibility of distinguish different types of cyanobacteria on the basis of DNA sequence of an amplified fragment of nif gene. Testein g the use of the amplified as DNA probes to distinguish individual species or groups of species. We also used the DNA sequence data to

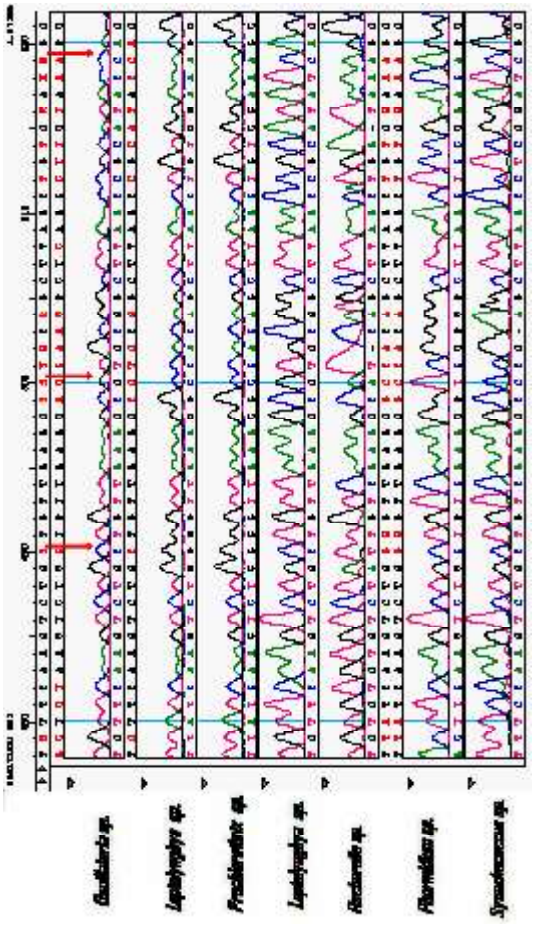


Fig. 11. The chromatogram analysis for 16Sr RNA sequence of cyanobacterial strains shows various differentiation regions in this gene through different strains

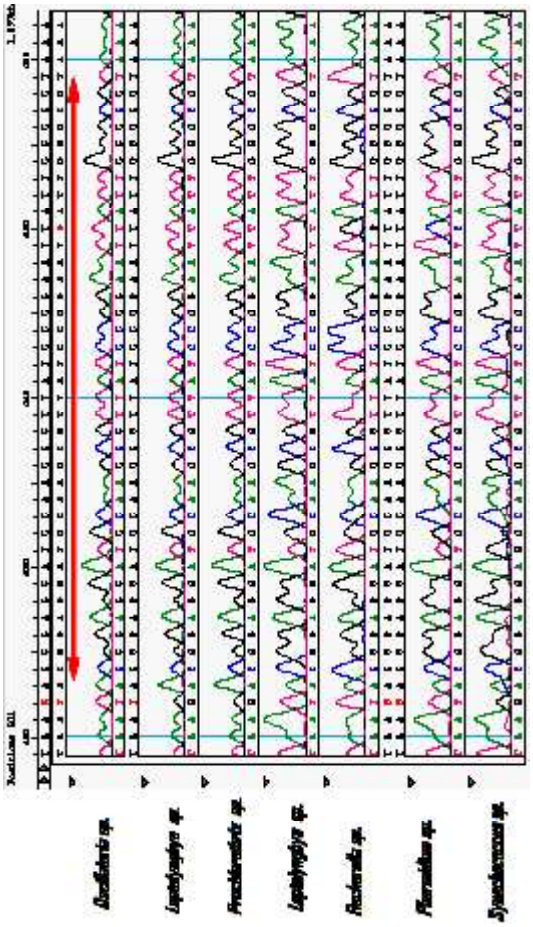


Fig. 12. The chromatogram analysis for 16Sr RNA sequence of cyanobacterial strains shows the similarity region in this gene through different strains

design oligonucleotides that may he useful for characterizing cyanobacterial strains. The amplified DNA of heterocystous cyanobacterial species are identical to each other and more similar to the amplified DNA of other cyanobacterial species. A degenerate oligonucleotide primer was used for the PCR amplification of the nifH gene resulting 359 bp fragment (Figure 5).

Sequence data have limited analyses to comparisons among genes from rather distantly related organisms. Previously, there have been few cyanobacterial nitrogenase sequences available to determine whether the phylogeny of nitrogenase is consistent with a phylogeny of cyanobacteria based on other genes (e.g. 16Sr RNA) sequences of a portion of *nifH*, the gene encoding the two identical subunits of the Fe protein of nitrogenase, were compared for three reasons. First, the comparison of phylogenies based on two genes (16Sr RNA and *nifH*) may provide insight into the phylogeny of the cyanobacteria. Second, the phylogeny of nifH within the cyanobacteria will provide additional information regarding the evolution of nitrogenase. Finally, the phylogenetic relationships of cyanobacterial nifH can be used as the basis for a powerful approach to the study of uncultivated nitrogen-fixing cyanobacterial strains in the environment. In the past, we had to prepare specific culture media eliminated of nitrogen source and incubation for days to determine either the strain is able or not to fix atmospheric nitrogen. Nowadays, this biological operation has been detectable during an hour using primers able to amplify the gene of nitrogenase enzyme, they key of nitrogen fixation. We could get 359bp amplified fragment for seven strains which indicate only fischerella perform the nitrogen fixation (Figure 5).

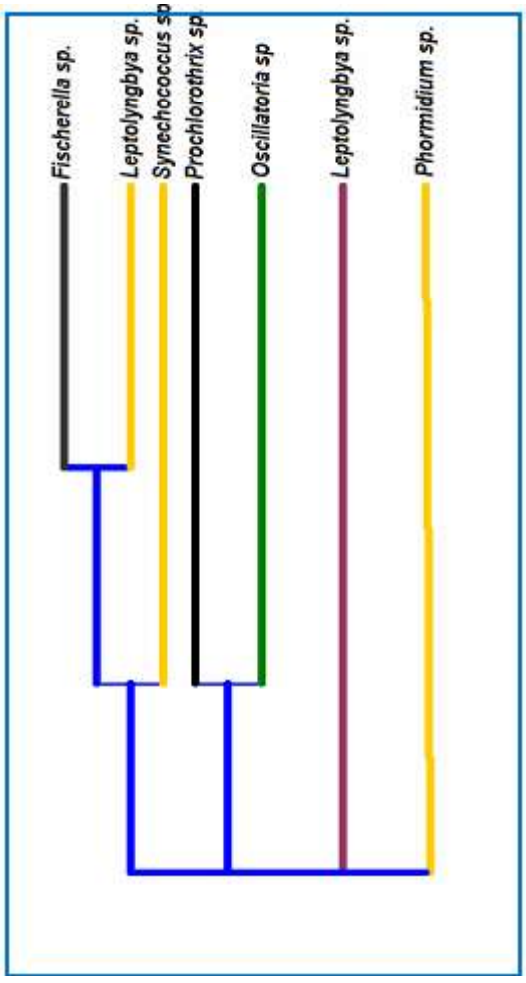


Fig. 13. Phylogenetic tree constructed from 16Sr RN*A* nucleotide sequence alignments of the seven cynobacterial strains

Phycocyanin Intergenic Spacer (PC-IGS) Amplification

Identification of cyanobacteria strains (Figure 6) used the polymerase chain reaction (PCR) based on the phycocyanin operon especially sequence homology of the intergenic spacer region (IGS) between the â- and á- phycocyanin subunits (PC-IGS). Amplification by PCR of the PC-IGS region based on the extraction of DNA samples from cultured cells, has been used to differentiate cyanobacteria. This approach relies on the culturability of the isolates to supply DNA samples.

Examining the PC-IGS region in reference cultures and environmental samples, let to the discovery that PC-IGS length are highly conserved within a genus. Multiple alignment of the sequences of the PC-IGSs of samples from this study and database entries shows that the nucleotide sequence is highly conserved within genus but differs significantly between genera and thus can be used for genus identification. Entries identification by sequence homology will become more reliable as the database expands. The complete identity of the PC-IGS sequence of morphologically identical samples from widely separated sampling sites is remarkable. The 610bp

PCR product of intergenic spacer between the â- and á-phycocyanin subunits (PC-IGS) region ensures that all our strains belong taxonomically to cyanobacterial group.

The distribution of PC in cyanobacterial microorganisms makes the study of PC operon that contains genes coding for two subunits and three linker polypeptides. The intergenic spacer (IGS) between the two subunit genes, of the PC operon was choosen as a potentially highly variable region of DNA sequence useful for the identification of cyanobacteria to the strain level. In addition, amplification of PC-IGS sequence via PCR can be used in genetic analysis of cyanobacterial strains. Oligonucleotide primers were used to amplify an approximately 610 base pair (bp) segment of DNA that included the variable phycocyanin intergenic spacer (PC-IGS).

Differentiations between Cyanobacterial Strains by Restriction Fragment Length Polymorphism (RFLP)

RFLP analysis of 16Sr RNA known has been used for several years as a method for rapid comparison of 16Sr RNAs. RFLP analysis and sequencing of the 16Sr RNA gene have revealed a very close relationship between certain strains of cyanobacteria. Briefly, 16Sr RNAs 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 in performed on relatively low-resolution agarose gels. After digestion with any of 6 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 terminal restriction fragment (T-RFs) or ribotypes observed in digests of 16Sr RNAs 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 with unique lengths.

The PCR products corresponding to the 16Sr RNA genes of the seven strains were digested with *HaeIII, Sau3A1 and HinfI*.

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 16Sr RNA 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 16Sr RNA 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 16Sr RNA gene are not distributed along the sequence but located in specific sites and the remaining sequences keep their similarity between some of strains genomes (Figures 11 and 12), a part of 16Sr RNA gene sequence the differences in nitrogen bases among our strains especially between the *Oscillatoria* and *Phormidium*. Also this informative gene sequence provides specification of the cyanobacteria genome. In phylogenetic analysis tree of 16Sr RNA (Figure 13) displayed a relation between each cyanobacterial strains in our study which it can provide obvious view of the differentiations between an *Oscillatoria* and the *Phormidium* cyanobacterial strains which could prove the genetic diversity among the strains depending on the geographical regions. Figure 11, 12 regarding calculated values of the nitrogen bases contents of the tree and the shifting between particular bases.

Isolation and cultivation of cyanobacterial isolates

Cyanobacterial strains of our isolates are usually stored as living cultures, by transferring cells to fresh media when needed. The living cultures and frequent transfers increase the risk of mixing cultures as well as possibility for the phenotypic changes of cyanobacterial isolates, which seem to be common during prolonged cultivation (Christian G Klatt et al 2011). However, some cyanobacterial strains such as *Oscillatoria* do not survive in preservation methods commonly used for other bacteria such as lyophilisation or storage in liquid nitrogen[8]

Molecular characterization of cyanobacterial DNA

Molecular characterization of seven hot

spring cyanobacterial isolates belonging to the order *Oscillatoriales* was carried out using the phycocyanin locus and the 16Sr RNA. DNA sequences from the phycocyanin operon discriminated ten genotypes, which corresponded to seven morphotypes identified by traditional microscopic analysis. Phylogenetic analyses support the conclusion that the *Phormidium* and *Leptolyngbya* genera are not monophyletic. The nucleotide variations were heterogeneously distributed with no or minimal informative nucleotides. Our results suggest that the discriminatory power of the phycocyanin region varies across the cyanobacterial species and strains. The DNA sequence analysis of the 16Sr RNA internally transcribed spacer region also supports the polyphyletic nature of the studied oscillatorian cyanobacteria[1]. This study demonstrated that morphologically very similar strains might differ genotypically. Thus, molecular approaches comprising different gene regions in combination with morphological criteria may provide better taxonomical resolution the order *Oscillatoriales*.

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 work describes molecular methods which provide descriptive DNA profiles, composed of phylogenetic characteris, which were appropriate for the inference of relatedness and evolution of cyanobacterial taxa. Phylogenetic characters provided differing levels of strain discrimination[2]. The sensitivity of the methods described here a reflection of the number of restriction endonuclease recognition sites in cyanobacterial – specific gene amplification products, in particular, the 16Sr RNA. The DNA profiles generated depict genomic polymorphisms and allow the unambiguous identification of cyanobacteria.

Ribosomal RNA (rRNA) sequence homology (as determined by comparisons of T1 oligonucleotide catalogs of P-labelled 16Sr RNAs) has been used to assess phylogenetic relationships within in the filamentous and unicellular blue-green bacteria, and to identify regions of evolutionary conservatism within blue-green bacterial 16Sr RNAs. *Lyptolyngbia* and *Fischerella,* representatives of two morphologically distinct and highly differentiated orders, are shown to be as closely related (on the basis of RNA sequence homology). They are further shown to be indistinguishable from typical unicellular members of a subgroup of the unicellular blue-green bacterial order Chroococcales. These results have general implications for studies of the origin of differentiated prokaryotes and of evolutionary change in prokaryotic macromolecules. In particular, they provide indirect evidence that the divergences of contemporary major prokaryotic groups are truly ancient ones.

Phylogeny of cyanobacteria based on the 16Sr RNA gene

The phylogenetic analysis of the 16Sr RNA gene has revealed close relationships among cyanobacteria, indicating that the diversification of cyanobacteria happened within a short period of time.

The 16Sr RNA gene, the most commonly used marker gene, has a central role in inferring phylogenetic relationships and in identification of bacteria. The 16Sr RNA gene sequence similarities of bacteria were shown to correlate well with genome relatedness, expressed as DNA: DNA reassociation values or as the average nucleotide or amino acid identity (ANI/AAI) of shared genes These correlations support the robustness of the 16Sr RNA gene–based microbial phylogeny

The 16Sr RNA gene has a universal distribution in prokaryotes, functional consistency, both variable and conserved regions, and large size and thus, rather high information content–characteristics needed for a good phylogenetic marker gene[14]. In addition, the 16Sr RNA gene sequences are relatively easy to align, and a large database has accumulated (currently over 6000 cyanobacterial sequences), allowing comparisons between strains However, the resolution power of the 16Sr RNA gene is at or above species level. The 23Sr RNA gene is longer than the 16Sr RNA gene and consequently, contains more informative sites and leads to a better resolution, but the sequence database of the 23Sr RNA gene is small in comparison to the 16Sr .

Horizontal gene transfer (HGT) and the

presence of multiple heterogeneous rRNA gene copies have raised concern about the reliability of relationships of bacterial strains determined on the basis of the 16Sr RNA genes. The bacterial genome can contain up to 15 copies of 16Sr RNA genes. Although intragenomic divergence of the 16Sr RNA genes can be as high as 11.6%, generally it seems to be low, less than 1% . Among cyanobacteria, the observed intragenomic divergence of the 16Sr RNA genes has been rather low (<1.3%) and related to heterocytous cyanobacteria. A few heterocytous cyanobacterial strains, for which either information or whole genomes were available, contain several (4-5) copies of the 16Sr RNA gene, whereas unicellular cyanobacteria have only one to two identical copies.

Characterization of 16Sr RNA

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[22], has provided an opportunity to design and develop rapid and sensitive protocols for detection and delineation of cyanobacterial strains. This work 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 a reflection of the number of restriction endonuclease recognition sites in cyanobacterial – specific gene amplification products, in particular, the 16Sr RNA. The DNA profiles generated depict genomic polymorphisms and allow the unambiguous identification of cyanobacteria.

Characterization of cyanobacteria by RFLP

Although the 16Sr RNA molecule contains variable regions, it is too well conserved for studying species identity or intraspecies variation. In our study, different species and strains were compared using restriction fragment length polymorphism (RFLP) of amplified fragments of 16Sr RNA digested with *HaeIII, Sau3A1* and *Hinfl*, according to NebCutter website which was sufficient to accomplish a differentiation between each strain. *HaeIII* enzyme revealed similarity between all strains at the highest bands compared to the marker, while there is also a similarity between *Synechococcus* and *Prochlorothrix* in the numbr of bands isolated.

Sau3AI enzyme was effective with *Oscillatoria sp.* and *Leptolyngbya sp.* and *Prochlorothrix sp.* distinguishes with similar patterns, comparing to the remaining strains. With *HinfI* it reveals high similarity between *Fischerella sp., Synechococcus sp.* and *Oscillatoria sp.* strains. However, united all various genomes in our strains in the same size of single band. *HaeIII and Sau3AI* was more useful than shows a multi bands describe a variation in genetic components in between *Fischerella sp., Synechococcus sp., Prochlorothrix sp., Phormidium sp.,* and the two species of *Leptolyngbya sp.* but still unable to distinguish from *Synechococcus sp.*, *Prochlorothrix sp.* and *Leptolyngbya sp.* while *Phormidium sp.* has an own patterns. These variations between the strains indicate that 16Sr RNA gene has a different construction from cyanobacterial strain to another which confirms the value of this gene to use a remarkable tool in cyanobacterial differentiation, taxonomy and classification

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 16Sr RNA 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 16Sr RNA 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 16Sr RNA gene are not distributed along the sequence but located in specific sites and the remaining sequences keep their similarity between our strains genomes 16Sr RNA (Figure 11, 12). We clearly notice in Figures

16, 23 and a part of 16Sr RNA gene sequence the differences in nitrogen bases among our strains especially between *Oscillatoria* and *Prochlorothrix*. Also this informative gene sequence provides specification of the genome cyanobacteria. In phylogenetic analysis tree of 16Sr RNA (Figure 13) displayed a relation between each cyanobacterial strains in our study which it can provides obvious view of the differentiations between cyanobacterial strains which could proof the genetic diversity among the strains depending on the geographical regions.

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 â- and á-subunit ORFs (PC-IGS), which encode for phycocyanin *b* and *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 16Sr RNA (Figure 12, 13). This highly variation we noticed in 16Sr RNA 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 N_2 fixation and photosynthesis with spatial heterogeneity appears in several trees as an early branch, suggesting a very ancient past. Yet, in other threes it shows a later divergence. Moreover, organisms with the same strategy (e.g., *Oscillatoria* and *Leptolyngbya*) do not always cluster together. Whether this is due to parallel evolution or is an artefact of the phylogenetic reconstruction remains to be elucidated. 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.

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