Identification of some cyanobacterial strains by molecular methods

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Abstract: Cyanobacteria are reported to be a prolific source of bioactive compounds and enzymes. Two strains of cyanobacteria were identified using a combined morphological and molecular approach. Microscopically one strain was a unicellular coccoid cyanobacterium while the other was a filamentous non-heterocystous one. For the molecular characterization, different protocols were tested for the extraction of DNA of cyanobacterial strains. Partial 23S rRNA gene was used as a molecular marker in the PCR which yielded single PCR amplicon for each cyanobacterial strain tested. The sequences were compared against the deposited sequences in the Genbank. The coccoid cyanobacterium was best matched to *Cyanothece* sp. whereas the filamentous cyanobacterium was best matched to *Spirulina* sp. and the phylogenetic reconstruction confirmed such designation.

[Nermin Adel El Semary, Mohamed Osmanand Heba El Sayed **Identification of some cyanobacterial strains by molecular methods** *Researcher* 2016;8(9):89-91]. ISSN 1553-9865 (print); ISSN 2163-8950 (online).http://www.sciencepub.net/researcher. 11. doi:10.7537/marsrsj080916.11..

Key words: Cyanobacteria, 23S rRNA gene, PCR, phylogenetic reconstruction.

1. Introduction

Cyanobacteria are prokaryotes that lack plastids but possess thylakoids and perform oxygenic photosynthesis. All cyanobacteria contain chlorophyll a as the main photosynthetic pigment in addition to other accessory pigments such as carotenoids and phycobiliproteins (Douglas, 1994). Due to the simple morphological features of cyanobacteria and the lack of diagnostic phenotypic characters, some errors were made in the identification of cyanobacterial strains. This necessitated to the adoption of molecular methods in addition to traditional morphological in a polyphasic identification system (El Semary, 2005). This approach is more reliable as the identification is not based on phenotypic aspects only but it provides an insight into some portions of the genome that are discriminatory between taxa. This would also allow the deduction of phylogenetic relationships of cyanobacteria (El Semary and Abd El Nabi, 2010; El Semary, 2011). At the molecular level, the rRNA genes are the most employed genetic markers due to their conserved and variable regions that are taxonomically discriminatory in addition to their homologous function and ubiquity (Nübel *et al*., 1997; Salomon *et al*., 2003; Sherwood and Presting, 2007). The 16S rRNA gene was largely employed as a genetic marker (Nübel *et al*., 1997; Salomon *et al*., 2003). However, the low discriminatory power of 16S rRNA at the species level (Fox *et al*., 1992) due to the low evolutionary rate variation in its conserved regions makes it less useful for phylogenetic studies of closely-related organisms. As a result, researchers have used other genetic markers that are more discriminatory such as the 16S-23S internal transcribed spacer region (ITS) (Iteman *et al*., 2000; El Semary, 2005) and the intergenic spacer region (IGS) of the phycocyanin genetic locus (Neilan *et al*., 1995; El Semary, 2005).

2. Materials and Methods

Cyanobacterial strains and growth medium

Cyanobacterial cultures

Two strains from cyanobacterial strains from two different localities (Wadi El Natroun and Wadi El Rayan) were initially isolated and kept at Helwan culture collection pending further analysis. The cyanobacterial strains were further purified by streaking on agar plate of solidified BG11 medium (Allen and Stanier 1968; Watanabe *et al*. 2000 modified) (2 % agar, w: v). Microscopic examination was performed periodically to observe the cyanobacterial strains. The pure culture was then moved to 10 ml vials containing 6 ml liquid BG11 medium and incubated until visible growth was obtained then transferred to several 500 ml flasks.

DNA extraction methods & PCR

There were several methods used to extract total genomic DNA from cyanobacterial strain. The one that yielded best quality of DNA was according to Smoker and Baruum(1988). One ml of culture was pelleted by centrifugation at 6000 rpm for 20 min at room temp then the medium was decanted. The pellet was re-suspended in 500 µl of (50 mM tris-HCL, pH 8 -5 mM EDTA – 50 mM NACl) . Lysozyme was added (1 mg/ ml). The solution was incubated at 37 °C for 2 hr with occasional mixing. After the addition of 10 µl of proteinase k (10 mg/ ml) and 20 µl of (w/v) sodium dodecyl sulfate (SDS). The mixture was incubated at 55 °C for 1hr or until the solution was clear. The solution was chilled on ice & extracted with an equal volume of (phenol – chloroform – isoamyl alcohol) (25: 24: 1).The previous organic extraction was repeated until obtaining a clear upper layer after centrifugation at 6000 rpm for ten minutes. The supernatant was added to one tenth of its volume of 3M sodium acetate. Total genomic was precipitated by incubation with an equal volume of isopropanol at -20°C for one hour followed by centrifugation at 4.500 x g for 10 min at room temperature. The pellet was washed with 70% ethanol, centrifuged then the ethanol was decanted and the pellet was left to dry in the air. The pellet was dissolved in 25 µl TE buffer (10 mM tris -1mM EDTA) & kept at 4°C overnight. The DNA was detected by running in gel electrophoresis using 0.8% agarose gel then visualized under UV-transilluminator.

Polymerase chain reaction

The extracted DNA was used in PCR to amplify partial 23S rDNA. The characterisation of the partial ssu rDNA locus was performed using the highly-specific universal algal forward and reverse 23S rDNA primers (Sherwood and

Presting, 2007). The reaction mixture contained the following: 12.5 µl of master mix (One PCR™, Genedrix), 1µl of DNA template, 1µl of 23S forward primer (100 µM) and 1µl 23S reverse primer (100 µM) and 9.5 µl double distilled water. The amplification protocol was as follows:

Initial cycle: 94ºC for 5 min, Amplification cycles (35 cycles): 94 ºC for 1 min; 55 ºC for 1 min, 72 ºC for 1.5 min, Last cycle: 72 ºC for 10 min.

The PCR products were purified and sequencing was performed using sequencing service (Macrogen, South Korea).

Phylogenetic analysis

Cyanobacterial gene sequences were imported from GenBank and aligned. The alignment was performed using Mega 4 phylogenetic package. The bootstrap consensus tree was inferred from 500 replicates (Felsenstein, 1985).

3. Results

Morphological characterization:-

Microscopic examination revealed that the first isolate was a unicellular cyanobacterium. Unicellular sometimes appears in pairs after division, with no thick mucilaginous envelope. The isolate was identified initially as *cyanothece* sp.

On the other hand, the second isolate had a distinctive morphological characteristics as regular helical coiling of the trichome, which is composed of shorter and broader cells with cross-walls**.** This morphological description fits well that of *Spirulina* sp.

Partial 23S rRNA gene and phylogenetic analyses

The amplification reaction using partial 23S rRNA gene specific primers yielded single PCR amplicon for each cyanobacterium tested (Figure 3)**.** The sequence retrieved was compared to other sequences deposited at GenBank using nucleotide BLAST search.

Figure(1): *Cyanothece* strain under light microscope (magnification 100x).

Figure (2): *Spirulina* sp. under light microscope (magnification 100 x).

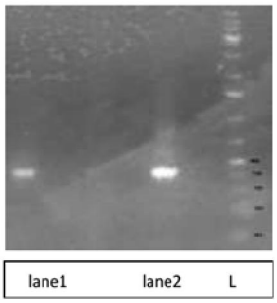
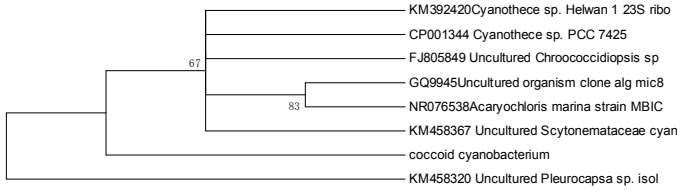


Figure (3): Gel electrophoresis image of ssu 23S rDNA amplification from two different strains. Bands appeared at about 460 bp (Lane 1&2). L is 100 bp DNA marker.

Phylogenetic analysis

The sequences obtained were used for Phylogenetic reconstruction. The evolution history was performed using the Maximum Parsimony method (Eck and Dayhoff, 1966). The phylogenetic tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The MP tree was obtained using the Close-Neighbor-Interchange algorithm (Nei and Kumar, 2003, pg. 128) with search level 3 (Felsenstein, 1985; Nei and Kumar, 2003) in which the initial trees were obtained with the random addition of sequences (10 replicates). All positions containing gaps and missing data were eliminated from the dataset (Complete Deletion option). There were a total of 82 positions in the final dataset, out of which 5 were parsimony informative. Phylogenetic analyses were conducted in MEGA4 (Tamura *et al*., 2007).

The accession numbers are written to the right hand side of each taxon. Our isolate was arbitrarily designated as *Cyanothece* sp. Helwan 1



Figure(4). Evolutionary relationships of the coccoid cyanobacterium.

The evolutionary history was inferred using the Maximum Parsimony method (Eck and Dayhoff, 1966). The consensus tree inferred from 22 most parsimonious trees is shown. Branches corresponding to partitions reproduced in less than 50% trees are collapsed. The MP tree was obtained using the Close-Neighbor-Interchange algorithm (Felsenstein, 1985 pg.128) with search level 2 (Eck and Dayhoff, 1966; Felsenstein, 1985) in which the initial trees were obtained with the random addition of sequences (10 replicates). All positions containing gaps and missing data were eliminated from the dataset (Complete Deletion option). There were a total of 89 positions in the final dataset, out of which 33 were parsimony informative. Phylogenetic analyses were conducted in MEGA4 (Tamura *et al*., 2007). The strain was arbitrarily designated *Spirulina* Helwan.

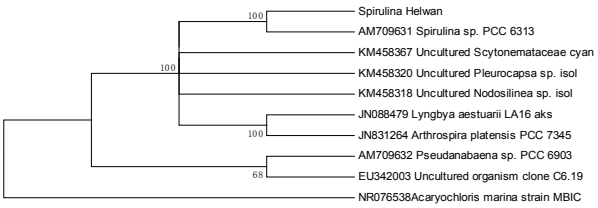


Figure (5). Evolutionary relationships of the Filamentous non-heterocystous cyanobacterium

4. Discussion

The accurate identification of cyanobacteria is facilitated by the combination of different morphological and molecular methods. Currently, cyanobacteria are regarded as prokaryotes and classified as oxygenic bacteria under Bacteriological code but because of their photosynthetic system and the possession of chlorophyll a they are classified as algae under Botanical code. Historically, classification of cyanobacteria was firstly reliant upon their morphological attributes (Geitler, 1925 until Bourrelly, 1970). However Komárek and Anagnostidis (1989) have estimated that as many as 50% of cyanobacterial strains that are found in culture collections have been misidentified leading to erroneous organism phylogenies. To overcome the errors in the old morphological system that resulted from the lack of diagnostic phenotypic features that are taxonomically discriminatory, molecular methods were used in cyanobacterial classification. This system depends upon amplifying some portions of the genome in order to characterize and deduce phylogenetic relationships of cyanobacteria (Neilan *et al*., 1995; Garcia-Pichel *et al*., 1996; Orcutt *et al*., 2002; Taton *et al*., 2003). The rRNAgenes are the most widely used markers for the identification of bacteria and cyanobacteria due to their conserved function and universal presence. 16S rRNA gene is the most widely-used genetic locus for phylogenetic analysis of cyanobacteria (Nübel *et al*., 1997; Crosbie *et al*., 2003; Salomon *et al*., 2003). Nevertheless, it is reported to be taxonomically less discriminatory at the species level due to the low evolutionary rate of its conserved regions (Fox *et al*., 1992). Recently, Sherwood and Presting (2007) employed primers that are specific for the 23S rDNA locus and found them to be highly discriminatory. We used those primers and obtained positive results.

The PCR amplification yielded single amplicon for each isolate and the amplicons were purified and sequenced. The verified sequences retrieved from macrogen company, South Korea were compared to other sequences deposited at GenBank using nucleotide BLAST search. The two strains were best- matched to *Cyanothece* sp.with (92.%) similarity percentage Which might suggest that the strain belongs to a new species of *Cyanothecesp.* The second strain was best matched to *Spirulina* sp. with (94%) similarity which again maight belong to a new species of *Spirulina*. The 97% of sequence similarity is an arbitary value suggested by Madigan *et al*. (1997) to confirm species identity but values that are 90% and above do confirm the generic identity. Overall, the combined use of traditional morphological identification alongside the molecular characterization of cryptic microorganisms has proven to be more accurate and informative than using a single taxonomic parameter for identification.

9/25/2016

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