https://doi.org/10.33472/AFJBS.1.4.2019.24-31 ISSN: 2663-2187

African Journal of Biological Sciences

Journal homepage: http://www.afjbs.com

Research Paper Open Access

Phylogenetic and morphological diversity of culturable cyanobacteria from Lake Magadi in Kenya

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Article Info

Volume 1, Issue 4, October 2019
Received : 08 July 2019
Accepted : 06 September 2019
Published : 01 October 2019
doi: 10.33472/AFJBS.1.4.2019.24-31

Abstract

Lake Magadi is an alkaline saline Lake in Kenya, which lies on the Great Eastern Rift valley. Although the lake is characterized by extremes of salt, pH and temperature, it supports diverse groups of cyanobacteria. In this study, we used different media to isolate novel groups of cyanobacteria. We recovered 11 isolates affiliated to the orders *Chroococcales, Oscillatoriales, Pleurocapsales and Nostocales.* Isolates affiliated to *Chroococcidiopsis* species had similarity values below 90% to currently characterized taxa indicating that these could be completely new phylotypes. This taxon has not been isolated before from the soda lake indicating the power of molecular techniques in identifying novel cyanobacterial taxa. Only two of the recovered isolates had 99% similarity to known organisms. Previous studies have mainly relied on microscopic examination and identification, which can lead to misidentification and subsequent assignment of an organism to the wrong taxon. The recovered isolates are a useful resource of more studies on taxonomy and secondary metabolite production.

Keywords: *Cyanobacteria, Soda lakes, Extremophiles, Lake Magadi*

1. Introduction

The phylum cyanobacteria is one of the largest taxon amongst gram-negative prokaryotes, with about 56 genera (Hossain *et al*., 2016). Their distribution is influenced by environmental and physiochemical conditions (Flombaum *et al*., 2013). In haloalkaline habitats, temporal blooms comprised of *Arthrospira, Spirulina* and *Anabaena* species have been observed (Dadheech *et al*., 2009) while the marine ecosystem is dominated by species of *Prochlorococcus* and *Synechococcus* (Flombaum *et al*., 2013). Cyanobacteria play an important role of primary production (Mazard *et al*. 2016). Some species (e.g., *Oscillatoria* and *Arthrospira*) are capable of photosynthesizing under both aerobic and anaerobic conditions. Under anaerobic conditions, in the presence of sulphur, the photoautotrophic blue green algae derive electrons by reduction of sulphur (Thajuddin and Subramanian, 2005). In the East African soda lakes, cyanobacteria serve as food for lesser flamingos (Krienitz and Kotut, 2010). At times they form dense blooms dominated by species of *Arthrospira, Synechococcus, Microcystis* and *Synechocystis* (Bell, 2012; Muruga *et al*., 2014; Kotut *et al*., 2010; Ballot *et al*., 2004; and Dadheech *et al*., 2009). However, they have been implicated in the production of secondary metabolites, or bioactive compounds

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(collectively referred to as cyanotoxins) which are toxic to the environment and humans (Pearson *et al.*, 2016). Cyanobacterial studies have relied on morphological characterization and cell counting, which are traditional methods suited for the study of the phytoplankton community (Spiegelman *et al.*, 2005). However; morphology alone cannot always properly reflect the ecological entities found in a system. Molecular characterization of cyanobacterial communities using partial sequence of 16S rRNA gene may be a more powerful tool in the study of microbial communities (Innok *et al.*, 2005). In this study we combined both morphological and molecular approaches to identify cyanobacteria taxa isolated from the haloalkaline Lake Magadi.

2. Materials and methods

2.1. Isolation of cyanobacteria

Samples used in this study were collected from the hypersaline Lake Magadi (1° 52'S 36°16'E; 1.867 °S 36.267 °E). Physiochemical parameters (pH, salinity, temperature, conductivity, O_2 concentration, salinity, and alkalinity were recorded for each sampling point. Water samples were collected in sterile 500 ml containers. Five types of media were used in the isolation efforts as follows: Blue green medium (BG11) by Niemela *et al*., (1979), *Synechococcus* medium (A+) (Niemela *et al*., 1979), artificial lake water medium (ALW) and artificial sea water medium (ASW) described by Wyman *et al.* (1985) and enriched lake water medium (M) described by Mikhodyuk *et al.* (2008). Sodium nitrate was added to BG11 media to facilitate the growth of cyanobacterial species that could not fix their own nitrogen. Bacterial growth was suppressed using streptomycin (50 mg / L) while Vitamin B12 (20 mg / L) was added as a supplement to support species such as *Spirulina.* Two techniques were used in the isolation process: spread plating on agar and dilution to extinction in deep well plates. For the liquid cultures, 100 μl of the lake water was inoculated onto 15 ml of the respective medium and held under continuously lighted chamber. Once growth was observed (green pigment intensification or increase in turbidity), the cultures were purified using dilution to extinction technique in deep well plates until pure axenic sultures were acquired (Rippka, 1988). Solid cultures were established by spread plating an aliquot of the sample on the respective solid medium followed by incubation at 30 °C till growth was observed. Pure cultures were obtained by repeated streaking on fresh medium.

2.2. Morphological and molecular identification

Colony morphology of pure strains growing on plates was recorded using an inverted light microscope (Nikon Eclipse T1-SM) while the cell morphology, cells arrangement, and presence of filaments with or without heterocysts as well as pigmentation was documented using light microscope at 400X magnification under oil immersion. Further characterization and assignment was done as described by Van De Meene *et al*. (2006) Cells for total genomic DNA extraction were harvested by centrifuging 1 ml of the liquid culture at 14,000 × g for 5 min. The cell pellets was re-suspended in100 μl solution A lysis buffer (50 mM Tris pH 8.5, 50 mM EDTA pH 8.0 and 25% sucrose solution). 5 μl of 20 mg / l lysozyme was added and the resulting mixture incubated at 37 °C for 15 min so as lysis of the cell wall to occur. 400 μl of DNA extraction buffer (10 mM Tris pH 8.5, 5 mM EDTA pH 8.0 and 1% SDS) was added into the tube and inverted severally. 10 μl of Proteinase K (20 mg / l) was added and the mixture incubated at 65 °C for 15 min. Extraction of nucleic acids was using the freeze-thaw method followed by phenol: chloroform: isoamyl alcohol (25:24:1 v / v) separation. The resultant mixture was centrifuged at 13200 rpm for 5 min at 4 ºC. The nucleic acids were recovered from the aqueous phase using (pH 5.2) sodium acetate: isopropanol precipitation method. Pellets containing the nucleic acids were dried, suspended in sterile Polymerase chain reaction (PCR) water and stored at –20 °C until use. The quality and purity of the extracted DNA was checked by running an aliquot (2 μl) on a 1% agarose gel electrophoresis and thereafter visualized under UV light using a transilluminator. DNA was not extracted from the solid cultures but they were directly used in PCR amplification (colony gradient PCR) (Uniwersytet *et al*., 2007).

Total DNA from each isolate was used as a template for 16S rRNA gene amplification. This process was performed using PCR machine (Sure cycler 8800 Agilent Technologies) in a total reaction volume of 25 μl containing the following reagents: 14.0 μl PCR water, 5.0 μl polymerase buffer, 1.5 μl of primer pair (5'-AGAGTTTGATCCTGGCTCAG-3'and 5'-AAGGAGGTGATCCAGCC-3'), 0.6 μl of dNTPs, 0.9 μl of DMSO 0.6 μl of $MgCl_2$, 0.3 μl Taq polymerase and 0.5 μl of the DNA. The control experiment contained all the above with no nucleic acid template. The PCR machine was set follows: initial denaturation for 5 min at 95ºC followed by 35 cycles of denaturing at 94 ºC for 1.0 min, primer annealing at 54 ºC for 1.0 min, 1.0 min for chain

extension at 72 ºC and a final extension step at 72 ºC for 5 min. The quality and size of the amolicons was checked by running an aliquot (3 µl) on a 1% agarose gel electrophoresis in 1× TBE and thereafter visualized under UV light using a transilluminator after staining with fluorescent dye. PCR amplicons were sent for sequencing at Inqaba Biotech, South Africa. Sequences were aligned using Chromas lite alignment editor software and their closely related database sequences retrieved using (BLAST) algorithm search program of NCBI (Altschul *et al*., 1995). Sequence alignments and construction of phylogenetic tree was done using MEGA 7 software (Kumar *et al*., 2016).

3. Results

3.1. Sampling and physiochemical data of the sites

Physiochemical data shows that the different sites differ in the water chemistry and this may have an influence on the cyanobacterial diversity. Notable is the concentration of Sodium ions which influences the salinity and the Bicarbonate ions which are responsible for the alkalinity (Table 1). Haloalkaliphilic species of cyanobacteria such as *Microcystis, Synechocystis, Anabaena* and *Spirulina* have been reported from Lake Magadi by Wagacha *et al*. (2014). Table 1 summarizes the most important environmental parameters of the lake in the different sampling periods (July, August and September).

Table 1: Summary of physiochemical parameters

Parameter	S3 July	S4 July	S3 Aug	S4 Aug	S2 Aug	S2 Sep
pH	10.5	10.5	11.3	11.1	10.7	10.7
Temperature	27	27	34.6	33.6	35.2	37.2
TDS (g/L)	135.4	134.6	139.2	139.8	148.2	145
P	77.2	63.8	108	107	73.7	117
K	2,430	1,960	3,300	3,270	2,860	4,280
Mg	8.20	6.31	<0.02	0.30	<0.02	2.63
Na	120,000	100,000	121,000	118,000	96,500	143,000
Ca	16.4	9.23	0.20	1.13	0.96	0.34
NO_3	<0.01	<0.01	0.20	<0.01	<0.01	5.98
NO_3N	<0.01	<0.01	0.045	<0.01	<0.01	1.35
HCO_3	229,000	196,000	242,000	233,000	181,000	213,000
$CaCO_3$	74.6	48.9	0.58	4.05	2.48	11.6

3.2. Isolation and purification of cyanobacteria

We obtained 11 axenic cyanobacterial strains (eight from liquid culture, and three from solid culture) which were morphologically and molecularly characterized. Isolates recovered from the liquid medium (BG11) were affiliated to the orders *Chroococcales*, *Oscillatoriales*, *Pleurocapsales* and *Nostocales* while solid medium BG11 supported taxa affiliated to the orders *Chroococcales* and *Pleurocapsales.* The isolates C3, C5, C9 and C10 were observed to be motile. It was noted that growth was better on liquid media as compared to solid medium probably because cyanobacteria are inhibited by agar (Urmeneta *et al.*, 2003). The isolates affiliated to orders *Nostocales* and *Spirulina* did not grow on the solid media even after supplementing it with vitamin B12. The obtained liquid and solid cultures are shown in Figures 1-2.

Figure 1: Axenic cyanobacterial cultures growing in liquid media (from left to right C1, C2, C4 and C5 respectively)

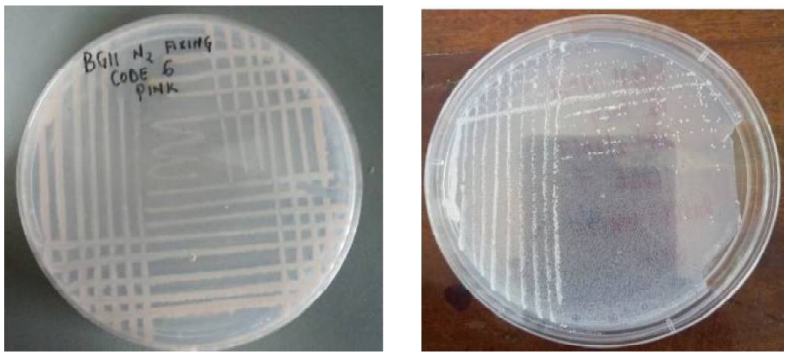

Figure 2: Axenic cyanobacterial cultures growing in solid media (from left to right Isolate C16 and C13 respectively)

3.3. Morphological characterization of pure cultures

To visualize the cell morphology, cells arrangement, and presence of filaments with or without heterocysts under the microscope for solid cultures, methyl blue stain was used (Figure 3a). No staining was done for the

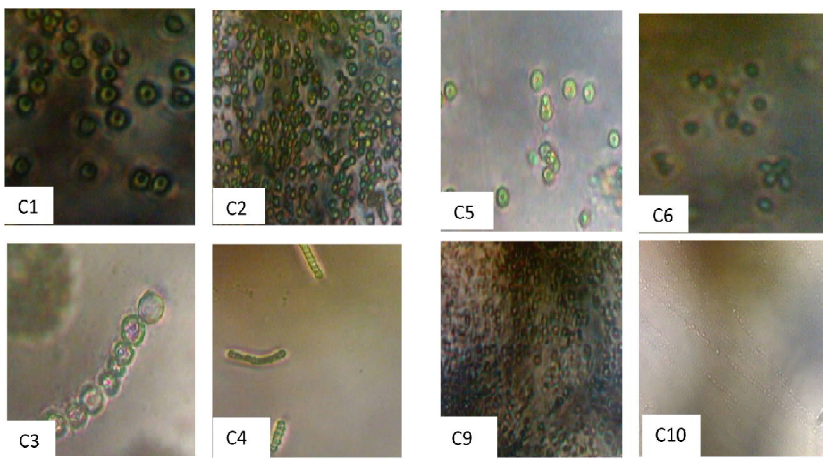

Figure 3a: Microscopic images of liquid cultures C1, C2, C3, C4, C5, C6, C9, C10 at a magnification of x400

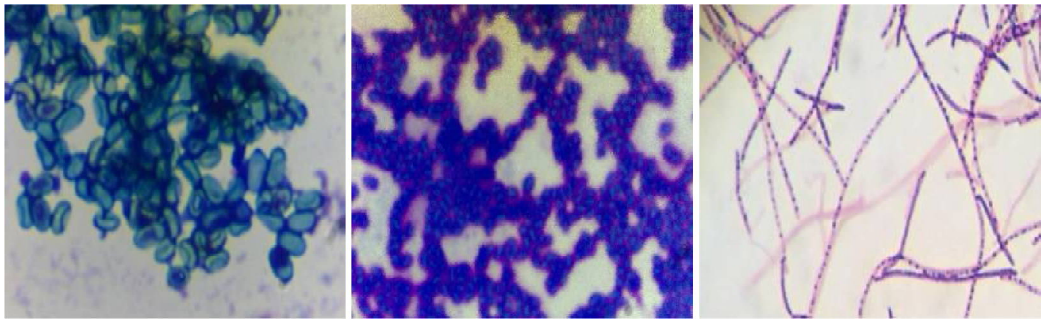

Figure 3b: Microscopic images of the isolates C13, C14, C16 grown on solid medium as seen under a compound microscope at x400

liquid cultures as they were clearly visible at x400 magnification (Figure 3b). The cell morphology was the typical cyanobacterial morphologies such as those observed for *Chroococcales, Oscillatoriales, Pleurocapsales* and *Nostocales*. Motility was observed in the liquid cultures of C3, C5, C9 and C10 while cells occurred solitary or in pairs after division. Cell content had no separation on centro- and chromatoplasma (Vos *et al*., 2011). Cultures of C1, C2, C3, C5, C6, C9, C10 and C16 could presumably fix their own nitrogen since nitrates were not provided in their growth medium. However, isolates C4, C13 and C14 only grew when nitrates were introduced into their growth media. The isolates C10 and C16 had visible specialized cells (heterocysts).

3.4. Molecular characterization

BLAST analysis of the sequences against the NCBI database showed that all the isolates belonged to the phylum cyanobacteria distributed in the following orders: *Chroococcales* (3 isolates), *Oscillatoriales* (2 isolates), *Pleurocapsales* (5 isolates) and *Nostocales* (1 isolate). The percentage similarity values ranged between 86%-99% similarity. Isolates C3 and C10 had percentage similarity of 99.45% to *Spirulina* and *Nodularia* species respectively. It was found out that C10 was closely related to *Anabaenopsis abijatae* while C3 was closely related to *Spirulina* major. Isolates C1, C2, C4, C5, C13 and C16 formed a cluster of their own and therefore were not affiliated to any known cyanobacterial groups. Their percentage similarity fell below 90% hence they could represent novel cyanobacterial lineages. The phylogenetic relationship of the recovered isolates is summarized in Figure 4.

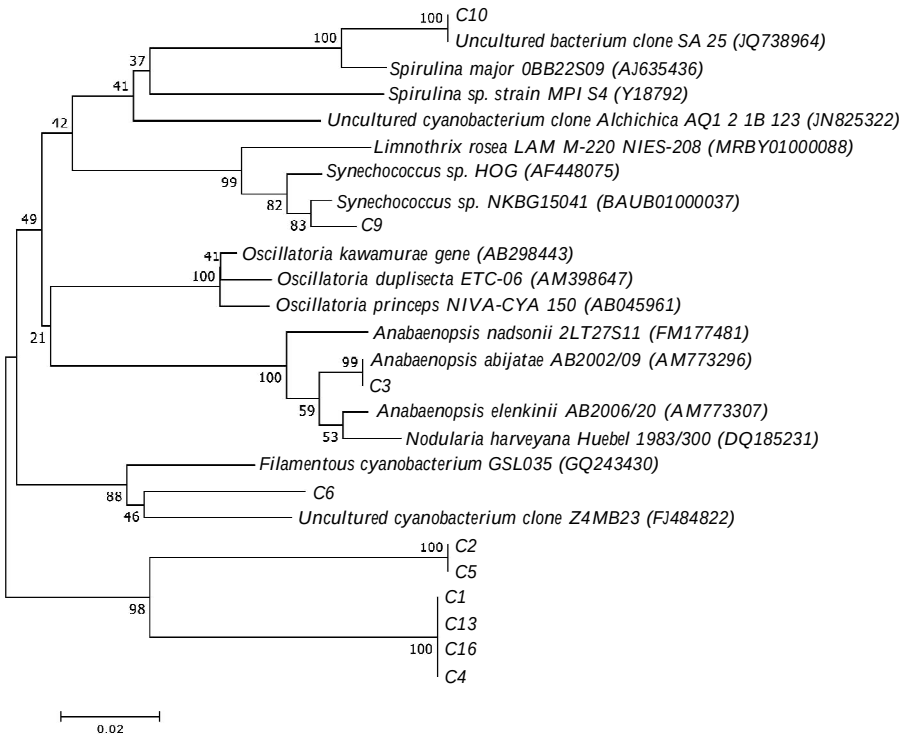

Figure 4: Unrooted phylogenetic tree showing the relatedness of the cultured cyanobacterial isolates to closest relatives

Lake Magadi as an alkaline saline ecosystem exhibit elevated alkalinity, pH and salt content (Kambura *et al.*, 2016). This study demonstrated that extreme environments such as Lake Magadi are inhabited by a diverse, polyphyletic array of culturable cyanobacteria which are underrepresented in currently available nucleotide databases. Harsh conditions in the lake were even increased during dry periods, which can be very stressful for most cyanobacterial species. Organisms able to tolerate elevated pH and excess of ions present in the water adjust their cellular content to be in osmotic equilibrium with the outside medium (Oren, 2007) and develop strategies to maintain the internal pH lower than the external environment, as particular cell walls, membranes and enzymes (Bell, 2012).

All the isolates were able to grow at pH above nine, temperatures of between 25-40 ºC in at least 21 days. With no added carbon source in the media, cyanobacteria still grew well since they are autotrophic. Recovery of cyanobacteria isolates from samples collected when highest values of alkalinity, salinity and temperature were recorded confirms that taxa in this environment are true extremophiles. Physiological adaptations favor alkaliphilic cyanobacteria as the main primary producer in the alkaline saline lakes as well (Grant and Sorokin, 2011). This phenomenal extremes were also recorded by Costa *et al*. (2016) while studying for cyanobacterial diversity from a similar environment. Their dominance may also be explained by the reduced interactions with other species (interspecific competitors and predators) that are rare or absent as the result of the strong selective pressure of the environment (Costa *et al*., 2016).

Haloalkaliphilic cyanobacteria had unique morphological characteristics such as heterocysts, which are involved in nitrogen fixation. The heterocysts were only visible in filamentous cyanobacteria C4, C10 and C16. Presence of taxa affiliated to *Chroococcales (27%), Oscillatoriales, Pleurocapsales and Nostocales was reported previously* by Wagacha *et al.* (2014), although their study relied on microscopical techniques. *Chroococcidiopsis* species are known for their ability to tolerate a diverse array of high conditions of salinity, extreme high temperatures or pH, high levels of radiation, conductivity and alkalinity (Magana-Arachchi and Wanigatunge, 2013). In this study thus taxon was represented by C2, C4, C5, C13 and C16. *Anabaenopsis elenkinii* (represented by isolate C10 at 99.45% similariy) preferentially thrives in alkaline and saline lakes, such as Lake Sonachi in Kenya and Lake Texcoco in Mexico (Ballot *et al.,* 2008). All isolates were able to tolerate high salt concentration of up to 10%. It has been reported that *Arthrospira* species is the most common cyanobacteria in the saline Rift valley lakes (Vonshak and Tomaselli, 2000; Ballot *et al*., 2004; Dadheech *et al*., 2009; Wagacha *et al*., 2014). This species has been extensively studied, particularly because of the significance as source of protein, vitamins and production of supplements (Ayachi *et al.,* 2007). However, in our study, *Arthrospira* species was only represented by isolate C10.

Molecular typing allows proper taxonomic assignment of an organisms as compared to traditional morphological methods (Taton *et al*., 2006; and Willame *et al*., 2006). This approach also permits the recognition of cryptic species and of different genotypes that display similar morphology within a population (Ernst *et al*., 2003). *Chroococcidiopsis* species for example had not been reported before in the saline Rift valley lakes. Therefore, Lake Magadi is still largely unexplored in terms of the microbial diversity. The uncultured diversity is an untapped resource for taxonomists as well as the biofuel and biotechnology industry.

Acknowledgment

The study was supported in part by the Alexander von Humboldt Equipment Grant, DAAD Material Resources Program, Equipment donation by Seeding Labs, USA, National Research Foundation, Kenya and The World Academy of Sciences. Edwin Ngetha was supported by the University of Embu postgraduate scholarship.

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Cite this article as: Ngetha Edwin, Julia Khayeli Akhwale and Romano Mwirichia (2019). Phylogenetic and morphological diversity of culturable cyanobacteria from Lake Magadi in Kenya. *African Journal of Biological Sciences* 1 (4), 24-31.