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Phylogenetic Diversity of Cyanobacteria from Qatar Coastal Waters

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ABSTRACT

Cyanobacteria represent the major microorganism phyla, being diverse and widespread group inhabiting most of the earth's environments. The recent increase of occurrence of toxic cyanobacterial strains in the marine environment attracts attention of the scientific community and environmental managers. The deterministic factors leading to such events are under scrutiny and are closely linked to our understanding of the diversity and environmental response of these strains to environmental conditions. The extreme environment witnessed in the Arabian Gulf is likely to nurture the occurrence of such harmful events. In recent times advanced molecular methodologies for the detection and genetic characterization of cyanobacteria were developed based on DNA amplification techniques. We aim in this work to better understand the diversity of the cyanobacterial natural communities found in Qatar marine environment through a genotypic characterization (phylogenetic analysis) with the objective to *i*. assess the local diversity, and *ii*. provide consistent reference for future comparative analysis, biotechnological applications and monitoring. In this study, QUCCCM strains from Qatar coastal were used to amplify fragments of the 16S rRNA gene followed by phylogenetic analysis. This methodology showed to produce accurate identification of the considered strains and analyze their evolutionary relationship. 28 taxa were identified among them 21.4% belong to the genus Geitlerinem, 25% Chroococcidiopsis,10.7% Synechococcus, 10.7% Stanieria, 7.1% Euhalothece, 7.1% Geminocystis, 3.6% Leptolyngbya, 3.6% Oscillatoria, and 3.6% Dermocarpella. The biogeographic distribution of the strains and their potential toxicity is discussed.

Keywords: Arabian Gulf. Cyanobacteria. Genotypic characterization. Phylogenetic analysis. HAB. Phylogenetic diversity.

LIST OF ABBREVIATIONS

S in 16S rRNA	Svedberg units
Bp	Base pair
BLAST	Basic Local Alignment Search Tool
CTAB	Hexadecyl trimethyl-ammonium bromide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
F	Forward Primer
EDTA	Ethylenediamine tetra-acetic acid
HABs	Harmful algal blooms
ITS	Internal transcribed spacer
Kb	Kilo base
MEGA	Molecular Evolutionary Genetics Analysis
PCR	Polymerase Chain Reaction
R	Reverse Primer
Sp.	Species
rDNA	ribosomal DNA
RNA	Ribonucleic acid
rRNA	ribosomal Ribonucleic Acid
Rpm	Round per minutes
QUCCCM	Qatar University Culture Collection of Cyanobateria and Microalgae
UV	Ultraviolet

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INTRODUCTION

Cyanobacteria

Cyanobacteria are gram-negative photosynthetic prokaryotes with a long evolutionary history appeared approximately 2600– 3500 million years ago that have been analysed based on fossil records, biomarker analyses and phylogenetic relationships with other living form (Hedges et al, 2001). The various autotrophic blue-green algae are morphologically diverse and dominant group of organisms in the environments (Whitton and Potts 2000) occurring as filamentous, unicellular, planktonic or benthic and colonial (coccoid) forms (Burja et al, 2001). They are found in cold and hot, alkaline and acidic, marine, freshwater, brackish, terrestrial, and symbiotic environments, establishing competitive growth in almost any environment that has, at least temporarily, liquid water and sunlight (Gupta et al. 2013). This is due to the presence of Photosystem II (water-plastoquinone oxidoreductase); a protein complex located in the thylakoid membrane where the light-dependent reactions of oxygenic photosynthesis of cyanobacteria occurs (Wikipedia, 2014). In this process, electrons are extracted from water and thus are not limited to environments with other scarcer reduced electron donors, as are other non-oxygenic photosynthetic prokaryotes. Indeed, some cyanobacteria species evolved as specialised cells, with the ability of nitrogen fixation (heterocytes), survival in stressed conditions (akinetes) and dispersal and multiplication (hormogonia) (Gupta et al. 2013). Therefore identifying these relevant cyanobacteria strains that grow in the extreme environment of Qatar would help in keeping records of their diversity as well as finding the strains that have several positive biotechnological applications and showing significant role in the biogeochemical cycle, and the production of bioactive compounds, or negative effects such as harmful algal blooms (HABs) where environmental mitigation and/or adaptation measures are needed.

Cyanobacteria genetic diversity and physiological properties are recognised as key features enabling cyanobacteria to successfully grow in the diverse range of environments (Badger and Price, 2003).

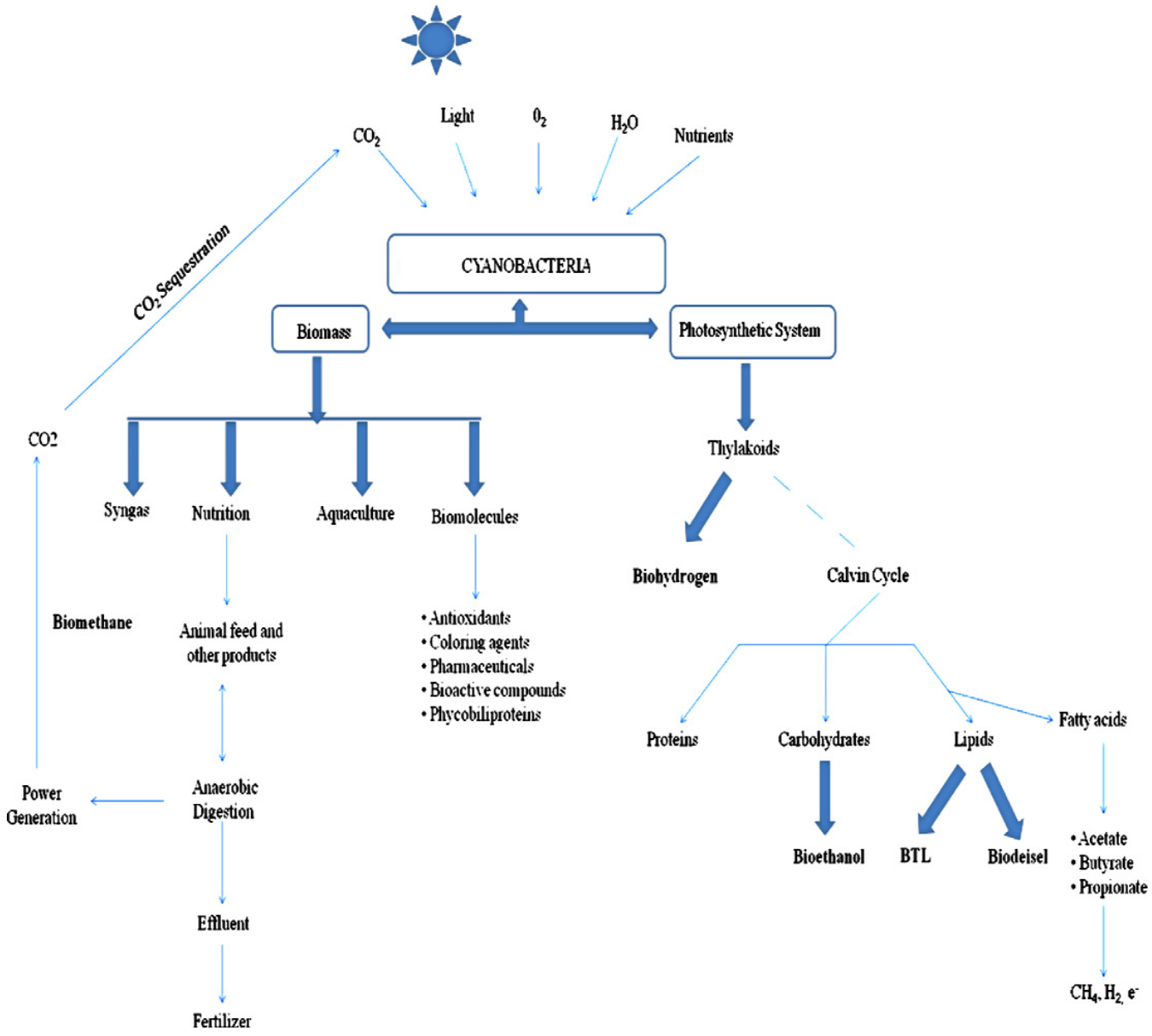

Figure 1. A schematic diagram of the potential use of cyanobacteria strains is shown with the production of a multitude of high-value co-products (Parmar et al., 2011).

Beneficial applications

Several cyanobacteria strains are already or potentially used for the production of beneficial co-products in different sectors (Figure 1).

Besides having tremendous ecological significance as food supplements/nutraceuticals, carbon sequestering and bio remediating agents (Prasanna et al., 2008), Cyanobacteria are known to produce wide group of bioactive compounds (secondary metabolites) synthesized via a nonribosomal pathway by a family of multi-enzymatic complexes called nonribosomal peptide synthetases (NRPS) and polyketide synthases (PKS) (Carmichael and Liu, 2006). They have diverse biological activities together with antiviral, antibacterial, antifungal, antimalarial, antitumoral and anti-inflammatory properties, having therapeutic, industrial and agricultural significance that is an emerging area of interest (Gupta et al., 2013).

Recently group of researchers investigated and illustrated the enormous capacity of marine cyanobacteria to integrate NRPS and PKS biosynthetic pathways, to have significant potential for development as chemotherapeutic agents. For instance, investigation of a Fijian collection of *Lyngbya majuscula* was shown in 2002 to produce a highly unusual neurotoxic dimeric lipopeptide, named somocystinamide A that have apoptosis-inducing on cancer cells (Joshawna et al., 2010).

Because of their simple growth needs, they are the ideal model organisms for deeper understanding of several metabolic processes and for the production of recombinant compounds for medicinal and commercial value (Gupta et al., 2013).

In recent years, cyanobacteria have gained interest for producing third generation biofuels by both biomass and H_2 production (Gupta et al., 2013). Advances in genetic manipulation of crucial metabolic pathways will form an attractive platform for production of numerous high-value compounds (Rosenberg et al., 2008). The development of a number of transgenic strains boosting recombinant protein expression, engineered photosynthesis and enhanced metabolism encourage the prospects of modified cyanobacteria for biofuel generation (Parmar et al., 2011).

Negative effects

Cyanotoxins are also products of the secondary metabolism of cyanobacteria similarly synthesized via a nonribosomal pathway, but they have toxic properties due to their mode of action, such as hepatotoxins (microcystins, nodularins and cylindrospermopsin), neurotoxins (anatoxin-a, saxitoxins) and dermal toxins (lyngbyatoxin, aplysiatoxin) (Carmichael and Liu, 2006) effects to general inhibition of protein synthesis. Chemical structure fall into three broad groups: cyclic peptides, alkaloids and lipopolysaccharides (Kaarina et al, 1999). The occurence of these toxins (Table 1) in natural environments might have negative impacts on aquatic ecosystems and their users through the production of dense blooms in eutrophic systems leading to a degradation of water quality by the release of off-flavors, toxins, water discoloration and accumulation of surface scums (Carmichael, 1992).

Table 1. List of known cyanobacteria and the toxins they produce (cyanosite, 2014)

Genus	Toxins produced
Anabaena	Anatoxins, Microcystins, Saxitoxins
Anabaenopsis	Microcystins
Aphanizomenon	Saxitoxins, Cylindrospermopsins
Cylindrospermopsis	Cylindrospermopsins, Saxitoxins
Hapalosiphon	Microcystins
Lyngbya	Aplysiatoxins, Lyngbyatoxin a
Microcystis	Microcystins
Nodularia	Nodularin
Nostoc	Microcystins
Phormidium (*Oscillatoria*)	Anatoxin
Planktothrix (*Oscillatoria*)	Anatoxins, Aplysiatoxins, Microcystins, Saxitoxins
Schizothrix	Aplysiatoxins
Umezakia	Cylindrospermopsin

Phylogeny

Initially, detection, identification and enumeration of cyanobacteria were conducted with microscopic techniques, which are based on morphological criteria that ultimately require the presence of an experienced observer. Conversely, newer and faster tools are necessary and in this sense, molecular methodologies have been developed. They have the advantages, in comparison with traditional methodologies, of being highly specific, sensitive and more rapid. Polymerase Chain Reaction (PCR) is a molecular tool lately applied to cyanobacteria investigations that along with gene sequencing allow the identification of cyanobacteria species from environmental samples, with the advantage of removing the time needed for laboratory growth of the cyanobacteria cultures (Moreira et al., 2013). Other studies have relied on molecular approaches to understand the diversity of microorganisms and their metabolites in natural environments (Tringe and Rubin, 2005).

Such molecular applications were used in bioremediation studies to identify the strains among mixed population. Sanchez et al. (2006) tested if cyanobacteria have a role in oil biodegradation by microbial mats that degrade petroleum components in polluted environments. Researchers used an illuminated continuous packed-column reactor filled with perlite soaked with crude oil. In the reactor different microorganisms grew attached to the column filling at the expense of petroleum under microaerophilic conditions. They investigated the diversity of the biofilm with molecular tools using 16S rRNA bacterial and archaeal primers and 18S rRNA eukaryotic primers for amplification. No cyanobacteria amplification was observed. On the other hand, they detected the presence of bacteria belonging to different groups, (Sanchez et al., 2006).

DNA sequences are used in the reconstruction of evolutionary relationships among organisms and have led to new genetic based systematic classifications by applying molecular

techniques to amplify some portions of the genome in order to characterize and deduce phylogenetic relationships of cyanobacteria. Phylogenetic analysis has increased considerably in the recent years helping in confirming or revising traditional taxonomical studies (Premanandh et al., 2006).

To gather relationships that span the diversity of known life, it is necessary to look at genes conserved through the billions of years of evolutionary divergence. These genes consist of hypervariable regions, where sequences have diverged over evolutionary time as well as strongly conserved regions often flank these hypervariable regions. Primers are designed to bind to conserved regions and amplify variable regions (Długosz and Wiśniewski, 2006). Sequences from tens of thousands of clinical and environmental isolates are available over the Internet through the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) that also provide search algorithms to compare new sequences to their database.

In these phylogenetic studies 16S rDNA sequences was used. The 16S rRNA (Small subunit of ribosome) gene is the most conserved (least variable) DNA in all cells. It has a universal distribution in prokaryotes and its functional consistency in both the variable and conserved regions and rather high information content represent important characteristics needed for a good phylogenetic marker gene. Sequences of 16S rRNA gene are relatively easy to align, and a large database has accumulated (currently over 6000 cyanobacterial sequences), allows comparison of the newly obtained 16S rRNA gene sequences (Gupta et al., 2013).

QUCCCM

In 2010, Qatar University initiated an algal biofuel project, led by Prof. Dr. Malcolm Potts, the project started off by isolating native cyanobacteria (Figure 2) and microalgae from the desert and marine environments of Qatar which led to the establishment of the Qatar University Culture Collection of Cyanobacteria and Microalgae (QUCCCM).

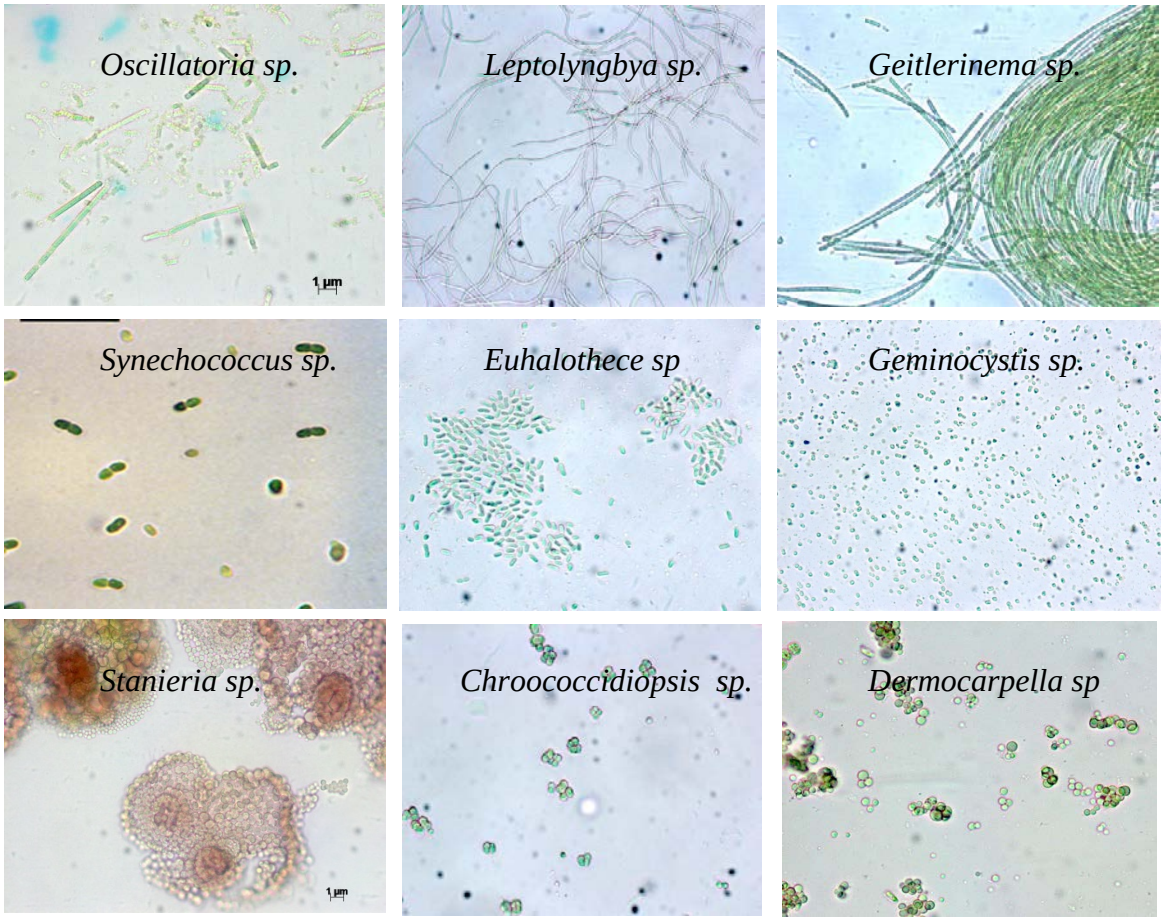

Figure 2 Microscopic images of some QUCCCM strains (Biofuel Lab)

Aim and Objectives:

Our general aim in this research was to investigate local cyanobacteria strains from Qatar; we used a polyphasic approach in the analyses of cyanobacterial systematics for the first time in the identification of the phylogenetic relationship among cyanobacteria strains isolated from Qatar marine environment. The specific objectives were:

1) To identify local isolates of cyanobacteria by molecular methods; within the Qatar University Culture Collection of Cyanobacteria and Microalgae (QUCCCM)
2) To assess phylogenetic relationships among the studied species

This investigation advance our understanding of this key community and enhance our ability in using these organisms to provide viable solutions to local and regional challenges such as food/feed production, energy and environmental health preservation.

Literature Review

Cyanobacteria relevance as key community in the marine environment and potential uses (Qatar, the Gulf and worldwide)

Al-Thani and Potts (2012) emphasized connections between the cyanobacteria and global warming. They expounded that huge populations of ancient cyanobacteria and other microalgae are credited with the formation of Earth's oil deposits, as well as, marine cyanobacteria contribute significantly to the fixation of atmospheric carbon through their photosynthesis. Consequently, spills from the commercial trafficking of oil often accumulate in coastal regions where cyanobacterial mats are established and mitigate the effects of oil pollution. They also stated that cyanobacteria are a viable source of biofuel, environmental friendly oil.

In other works Neil et al (2012) stated that cyanobacteria are the most ancient phytoplankton on the planet responsible for the formation of HABs in freshwater, estuarine, and marine ecosystems. Recent research suggests that eutrophication and climate change are two processes that may promote the proliferation and expansion of cyanobacterial harmful algal blooms that are known to produce a wide variety of toxins that generally refer to compounds that cause animal and human poisonings or health risks (Neil et al., 2012).

Notably, HABs are complex events (Heisler et al., 2008) affected by multiple factors occurring simultaneously cause their formation. Consequently on 18 February 2003, a bloom in the southern Arabian Gulf was reported that was a consequence of various hydroclimatic variations. The bloom surrounds (Figure 3) the peninsula of Qatar, and stretches from Bahrain in the west, along the shores of the United Arab Emirates and Oman to the Strait of Hormuz (Al-Thani and Potts, 2012).

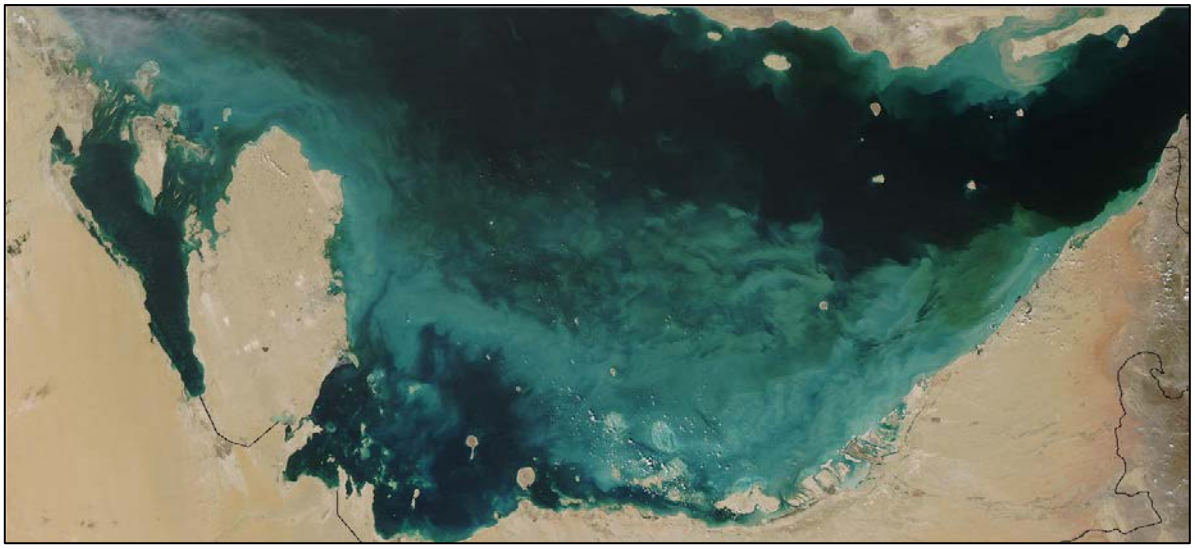

Figure 3 In the southern Arabian Gulf, the bloom coloured the waters turquoise-blue in this true-colour Aqua Moderate Resolution Imaging Spectroradiometer (MODIS) image from NASA, 2003.

Recent enhancement of remote sensing capabilities allowed a better detection and monitoring of harmful cyanobacterial blooms in the world ocean (Shen et al., 2012) that lead to better documentation of these events as well as to increase scientific and public awareness.

Cyanobacteria identification and systematics (taxonomic and molecular)

In an adjacent ocean, the Pacific, analysis of cyanobacterial diversity has been conducted (Nitin & Adhikary, 2014). The authors studied the strains that colonise archaeologically important stone monuments and building facades of India where cyanobacterial biofilms lead to weathering the substratum. They obtained 16S rRNA gene sequences of cyanobacteria species isolated from stone temples and monuments as well as on building facades from several locations of India and determined their molecular phylogeny comparing the gene sequences of species from identical surfaces of other regions of the globe. They found that the species principally belonging to the genera *Hassallia, Tolypothrix, Scytonema, Lyngbya* and *Calothrix*, which appeared soon after wetting of the biofilms. Several other species of genera A*ulosira, Nostoc, Camptylonema, Dichothrix, Chlorogloeopsis* and *Westiellopsis* occurred as associated organisms as they appeared upon prolonged culture of the biofilms (Nitin & Adhikary, 2014).

The first polyphasic study of cyanobacteria from Brazilian mangrove ecosystems using morphological, genetic and biological approaches had been conducted. Fifty cyanobacterial strains were examined where unicellular, homocytous and heterocytous morphotypes were recovered, representing five orders, seven families and eight genera (*Synechococcus, Cyanobium, Cyanobacterium, Chlorogloea, Leptolyngbya, Phormidium, Nostoc and Microchaete*). All of these novel mangrove strains had their 16S rRNA gene sequenced and BLAST analysis revealed sequence identities ranging from 92.5 to 99.7% when they were compared with other strains available in GenBank. The results showed a high variability of the 16S rRNA gene sequences among the genotypes that was not associated with the morphologies observed (Caroline et al, 2014).

Lake Naivasha in Kenya was studied for the abundance of cyanobacteria in comparison to the entire phytoplankton community between 2001 and 2013 (Lothar Krienitz et al, 2013). Characterization of the uncultured field clones of the dominant cyanobacteria in field samples was done for the first time. Molecular phylogenetic investigations were carried out It has been observed microscopic dominance of cyanobacterial taxa in Lake Naivasha in 2010 and 2011 is supported by evidence from molecular markers (16S rRNA gene, 16S–23S ITS sequences and cpcBA-IGS region).

Among other studied aquatic systems, temperate estuaries of North and Centre of Portugal, were characterised for cyanobacterial diversity and their secondary-metabolite profiles. Forty four benthic forms of cyanobacteria was isolated and correlated to morphological (e.g. cell shape, cell size, presence/absence of sheaths) and chemical characteristics to establish species relationships among the taxa examined and comparing to the phylogeny constructed from the 16S rRNA gene sequences (Viviana et al, 2012). (i) *Chroococcales (Cyanobium, Synechocystis and Synechococcus), (ii) Oscillatoriales (Leptolyngbya, Microcoleus, Phormidium and Romeria) and (iii) Nostocales (Nostoc and Nodularia)* were found.

In Tunisia, a study was conducted on the genetic characterization of several cyanobacterial strains isolated from seven Tunisian water bodies used for drinking, irrigation and fishing (Afef et al., 2011). An evaluation of cyanobacteria toxicological potential was performed using molecular biology approaches and 16S rRNA, 16S–23S rRNA ITS, and rpoC1 sequences were used for the identification of the isolates and conducting a phylogenetic analysis. Sequences for the Tunisian isolates were highly similar to each other, however, the presence of potentially toxic cyanobacteria, *Cylindrospermopsis raciborskii* and *Microcystis aeruginosa*, was confirmed as well as revealed that the majority of *Microcystis* strains had the six characteristic segments of the microcystin synthetase mcy cluster (mcyA, -B, -C, -D, -E and -G) indicating, therefore, their potential to produce microcystin (Afef et al., 2011).

In a similar investigation, Konstantinos et al, (2011) aimed to monitor the occurring cyanobacteria in two eutrophic, shallow Mediterranean lakes which had high cyanobacterial diversity in blooms, by comparing the diversity by morphological observation and phylogenetic analysis by Neighbour-joining tree after PCR amplification of the 16S rRNA gene with cyanobacterial-specific primers. This study gives an example for successful cyanobacterial bloom analysis as they successfully sequenced 118 clones which were grouped in 23 Cyanobacteria and 11 chloroplast-like phylotypes. Phylogenetic analysis revealed all common bloom-forming Cyanobacteria that belonged to the genera *Microcystis, Anabaena, Aphanizomenon, Cylindrospermopsis-Raphidiopsis group, Limnothrix* and *Planktothrix*, comprising most of the diversity previously recognized by morphological observations in cyanobacterial morphospecies in these lakes (Konstantinos et al, 2011).

The genus *Planktothrix* was believed to be an important water-bloom forming cyanobacterial group that has been found in dense water blooms around China, therefore Shen et al, (2010) conducted the study of genetic diversity and phylogeny of *Planktothrix* strains isolated from

seventeen Chinese water bodies, based on 16S rRNA, rbcLX and rpoC1 genes. They found that the strains morphologically classified into two groups as *Planktothrix agardhii* and *Planktothrix mougeotii.* However, phylogenetic analyses based on the three gene regions, revealed that *Planktothrix* strains analyzed in this study were mainly divided into three clades, corresponding to *P. agardhii, P. pseudagardhii,* and *P. mougeotii* (Shen et al, 2010).

A study on thirteen strains of *Microcystis wesenbergii*, isolated from blooms in different Chinese waters, investigated for the genetic diversity within this *Microcystis species* (Wenhua et al, 2010). They found that there was a highly variable region (cpcBA-IGS) between *M. wesenbergi*i strains and other species of *Microcystis*. This encouraged proceeding with the design and development of a rapid molecular method to detect and distinguish *M. wesenbergii* in nature. Occurrence of the wesenbergii-cluster, using cpcBA-IGS-based phylogeny of *Microcystis* in this study, supported by high bootstrap values and posterior probability, showed a high divergence of cpcBA-IGS sequences between *M. wesenbergii* and other species of *Microcystis*. This demonstrated that the cpcBA-IGS region could be used to characterize *M. wesenbergii* and distinguish *M. wesenbergii* from other morphospecies of Microcystis (Wenhua et al, 2010).

The diversity of *Cylindrospermopsis raciborskii*, known for its ability to produce potent toxins, was studied which was increasingly spreading in temperate freshwater habitats worldwide. Isolates from European and African strains from Lake Zierkersee and Lake Victoria respectively, represent the invasive population of *C. raciborskii*. The researchers genetically characterised with respect to ITS1, PC-IGS, nifH and rpoC1 (RNA polymerase) genes and compared to corresponding sequences of *C. raciborskii* available in the GenBank and complemented with morphological analyses of the strains (Sigrid et al, 2008). Their findings showed a low degree of *C. raciborskii* diversity in comparison with other groups of

cyanobacteria that might be due to selection mechanisms, physiological tolerance, climatic change or radiation after the last ice age. Most likely, a combination of coexisting mechanisms enables *C. raciborskii* to proliferate into temperate areas, thus underline the complexity of the phylogeography of this species.

A Molecular characterization was conducted for 10 cyanobacteria cultures collected at the National Facility for Marine Cyanobacteria (NFMC) from the across different geographical locations within the Indian subcontinent which were characterized based on their morphological features (Jagadeesan et al, 2006). It was concluded that the molecular analysis of *cpcBA*-IGS and the 16S-23S ITS region supports the polyphyletic nature of *Phormidium* and *Leptolyngbya* species. Despite the low sample numbers, more studies comprising 16S rRNA gene regions may provide a better understanding of the systematics of order *Oscillatoriales* and resolve the relationships between *Phormidium, Leptolyngbya,* and other *Oscillatoriales* (Jagadeesan et al, 2006).

The largest oil spill in human history occurred in 1991 during the Gulf War (Kuwait/Iraq) led to the pollution of over 770 km of coastline from southern Kuwait to Abu Ali Island (Saudi Arabia) with oil and tar, affecting most of the local plant and animal communities. Consequently, cyanobacteria colonized most of the oil polluted shores. The focus of the study conducted by Hans-Jorg (2003) was on the development and distribution of cyanobacteria mats, and the abundance and diversity of benthic fauna along transect lines at eight different study sites. Additionally the total coastline was surveyed on ground and by helicopter flights. The data have been collected during repeated visits to the study area in 1994, 1995, 1996, 1999, 2000, 2001 and 2002.

Composition of the mats was analysed using light microscopy. Where laminated cyanobacterial mat communities were found environmental conditions in the studied ecosystems changed completely from former diverse crab colonies to laminated

cyanobacterial mat communities following the deterministic environmental conditions and communities succession schematically presented in Figure 4.

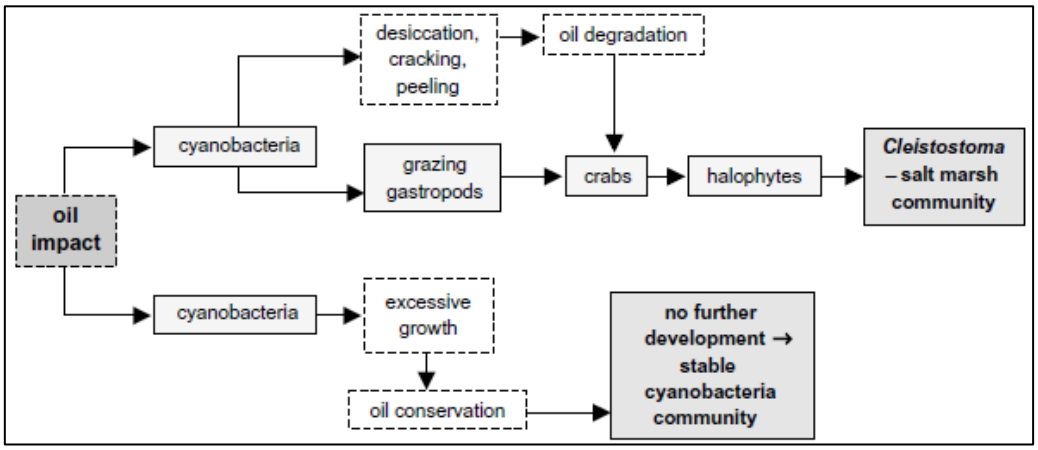

Figure 4: Simplified successions at oiled crab Cleistostoma – salt marshes in the Kuwait – Saudi Arabia coasts following the 1991 Gulf war related pollution (Hans-Jorg, 2003)

They found that the oil spill did not damage the cyanobacteria mats sites (the total area 1.55 km2), but promoted their extensive development to a high degree with a good possibility of survival even in habitats where they did not occur before (such as *Cleistostoma* crab colonies). Regeneration of the original ecosystem is impossible as long as the laminated cyanobacterial mats seal the soil surface and thus prevent oil degradation (Hans-Jorg, 2003).

In a related study (Al-Thukair et al., 2007) considered cyanobacterial mats, along the coast of the Eastern Province of Saudi Arabia, were affected by severe oil pollution following 1991 oil spill. Their investigation of composition of cyanobacteria and diatoms was carried out using light microscopy, and Denaturant Gradient Gel Electrophoresis (DGGE) technique. Light microscopy identification revealed dominant cyanobacteria to be affiliated with genera *Phormidium*, *Microcoleus*, and *Schizothrix*, and to a lesser extent with *Oscillatoria*, *Halothece*, and *various diatom species*. The analysis of DGGE of PCR-amplified 16S rRNA fragments showed that the diversity of cyanobacteria decreased as proceed from the lower to the upper intertidal zone. Accordingly, the tidal regime, salinity, elevated ambient air

temperature, and desiccation periods had a great influence on the distribution of cyanobacterial community in the oil polluted intertidal zone of Abu Ali Island.

Lack of knowledge of the Qatari cyanobacteria species and their phylogeny

Investigations about cyanobacteria communities in Qatari environment are limited. Recent interests about these communities promoted the study of cyanobacteria found in Qatar environment but they were mostly limited to desert and extreme aquatic ecosystems such as coastal hypersaline lagoons.

Hypolithic microbial communities are found underneath rocks and encountered in the southern desert of Qatar where quartz rocks are ubiquitous and are a substrate for hypoliths colonized by green hypolithic microbial communities dominated by colonizing cyanobacteria (Al-Thani, 2014). Using light and electron microscopic analyses, the cyanobacterial community structure of hypoliths was shown to be dominated by cyanobacteria affiliated to the genera *Chroococcidiopsis, Aphanothece, Pleurocapsa, Oscillatoria, Lyngbya, Leptolyngbya, Phormidium* and *Scytonema*). More investigation by molecular techniques was suggested to understand their taxonomy, physiology and ecology (Al-Thani, 2014).

Sabkha is a transliteration of the Arabic word for a salt flat area that is widely distributed throughout the Qatar Peninsula. Mahasneh et al. (2006) investigated the biological characteristics of cyanobacteria communities within this area. Extensive field and laboratory studies were conducted from October 2001 to June 2003. Among the findings, it has been found that the upper-intertidal zone is composed of cyanobacterial mats, including species of *Calothrix, Anabaena, Phormidium, Microcoleus, Oscillatoria, Lyngbya, and Scytonema*. Inland sabkhas – dry, vegetation-free flats in undrained desert basins – are covered with salt crust containing cyanobacteria of the genera *Synechococcus, Anacystis, Microcoleus, Oscillatoria*, and *Gloeocapsa* (Mahasneh et al., 2006).

With population growth and development in Qatar, concerns have been raised as regard to the occurence of HABs, which are commonly associated with toxin producing species. Natural and aquacultured fish deaths were observed nearby Kuwait Bay (1999). United Arab Emirates and the Gulf of Oman (2008–2009) were associated with cyanobacterial blooms. A recent study by Antonietta et al. (2013) on phytoplankton species diversity, biomass (chlorophyll a concentration) and primary productivity was conducted in the coastal waters surrounding Qatar (Arabian Gulf) at 13 stations in February 2010, July 2010, February 2011 and May 2011. 125 species were identified; the majority of phytoplankton species were diatoms (82), with 41 identified dinoflagellates and 2 cyanobacteria. In contrast, both biomass and productivity were low. It has been mentioned that this did not reflect an absence of other species in Qatar during this study but rather the difficulty in identifying many groups of phytoplankton, particularly the smaller and cell wall-less species (Antonietta et al., 2013).

It can be concluded from the above literature review that molecular identification along with phylogeny have become an important tool for studying cyanobacteria evolution in any particular area or investigating the occurring bloom event associated to cyanobacteria species. The primary cause of evolution is the mutational change of genes in DNA sequence caused by nucleotide substitution, insertions/ deletions, recombination, gene conversion, and so forth may spread through the population by genetic drift and/or natural selection (see, e.g., Nei 1987; Hartl and Clark 1997). Establishing a valid phylogenetic tree for the cyanobacteria species found in Qatar coastal waters will help in identifying the lineage of these species, their evolutionary relationship and support the development of monitoring and mitigation programs to minimize environmental and human health issues beside the potential use of identified strains in beneficial applications.

Methodology

The 28 strains used in this study were collected along the coastline of Qatar and in offshore location as shown in Figure 5. Sampling was conducted from 2010 to 2013. Isolation and purification of the strains were done in the Biofuel Laboratory and Qatar University Culture Collection of Cyanobacteria and Microalgae (QUCCCM) was stablished.

Figure 5 QUCCCM sampling sites around Qatar coast (green) and offshore (bown). Samples were collected since 2010 to 2013

In the following diagram (Figure 6) we report the sequential methodologies used during this research from train isolation to the final phylogenetic tree.

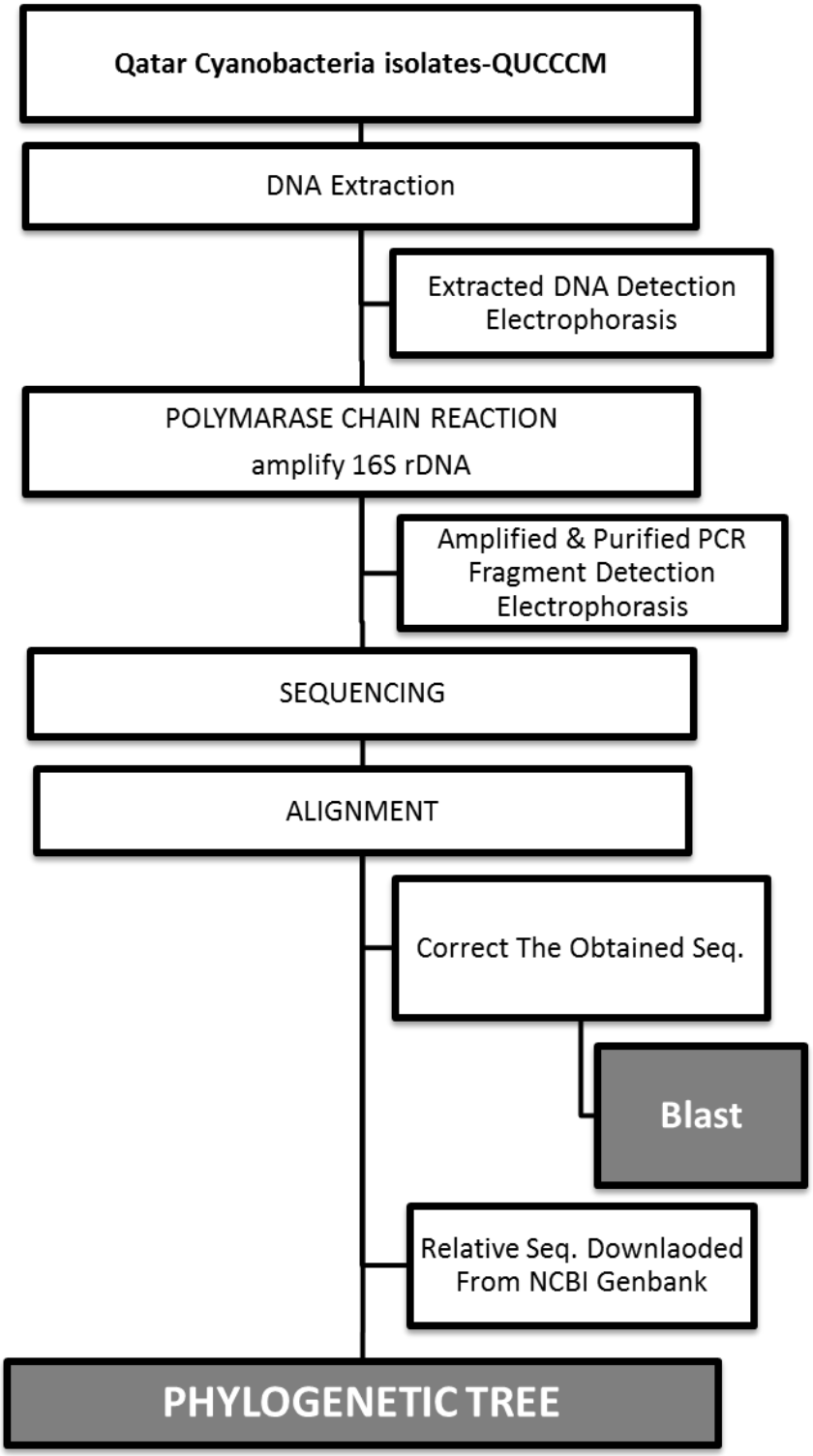

Figure 6 Step by step approach for the production of the phylogenetic tree. Final outputs are indicated with grey background

DNA extraction

Genomic DNA was isolated from the cyanobacteria cells cultured on solid media by using several isolation protocols: CTAB (Doyle JJ, 1987), Sigma - GenElute™ Plant Genomic DNA Miniprep Kit , and Qiagen-DNeasy plant mini kit. Among them the better quality of DNA was selected and used for further processes. For a detailed description of the electrophoresis protocol refer to the Annex I.A.

Agarose Gel Electrophoresis

To analyze the quality and quantity of each extracted DNA sample, gel electrophoresis technique was performed. For a detailed description of the electrophoresis protocol refer to the Annex I.B.

Polymerase Chain Reaction

To amplify 16S rRNA gene, specific primers and corresponding temperature settings in the PCR machine were used. In Figure 7 the general PCR reaction is reported.

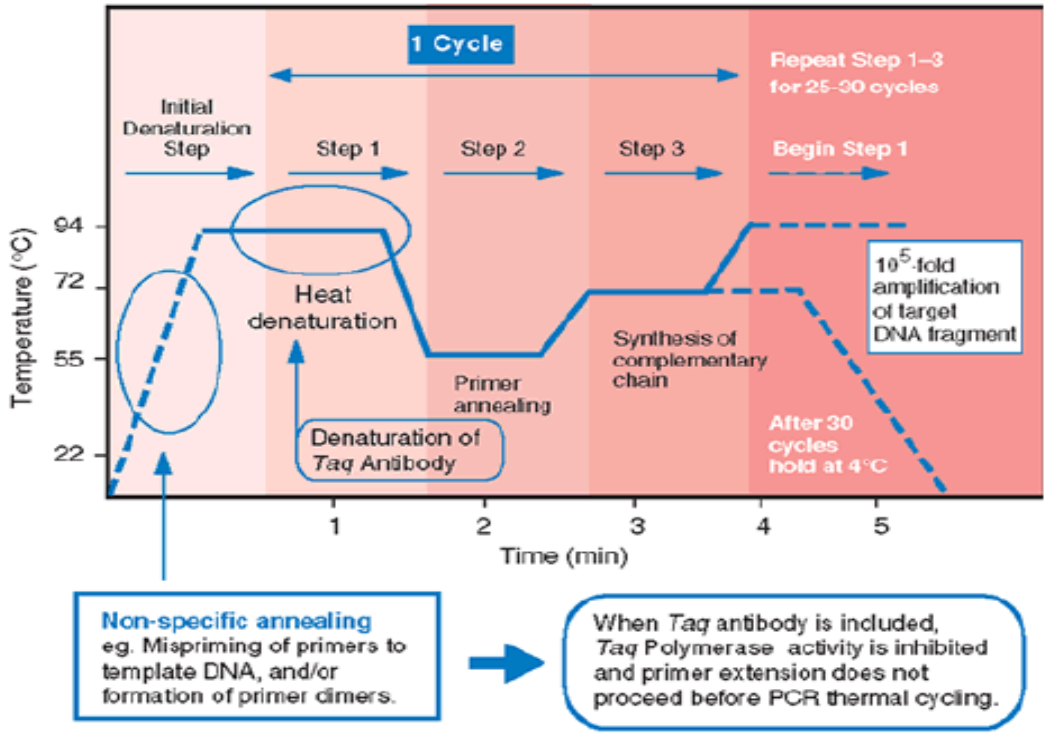

Figure 7 PCR reaction showing the temperature (Jennifer-hernandez, 2010)

Table 2 PCR reaction mix for 16S rRNA gene amplification

Buffer 5x	10 µL
dNTP (10µM)	1 µL
27F1 (20pMol) '5-AGAGTTTGATCCTGGCTCAG-3	1 µL
1516R (20pMol) 5'-ATCCAGCCACACCTTCCGG-3'	1 µL
GoTaq Polymarase	0.25 µL
H2O (filtered and autoclaved)	33.75 µL (depends on the DNA volume)
DNA	2 µL (depends on the DNA quality)
Total PCR volume	**50 µL**

- PCR cycles:

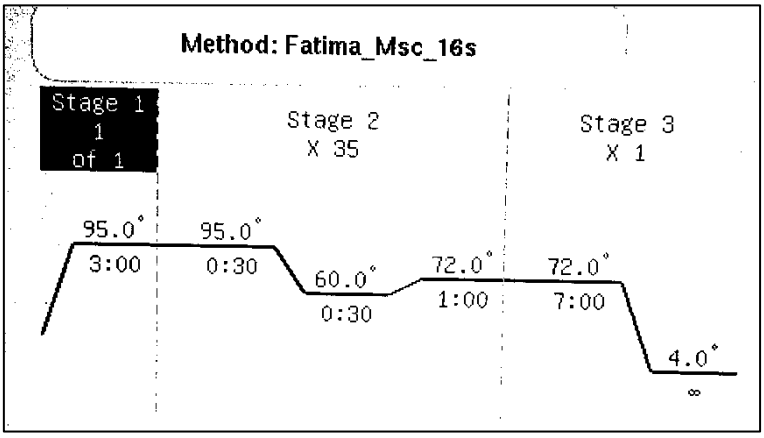

Figure 8 16S rDNA amplification PCR

Agarose Gel Electrophoresis

To analyze the quality and quantity of the amplified fragment, some gel electrophoresis technique was done. For a detailed description of the electrophoresis protocol refer to the Annex II

Purification

To utilize the required amplified PCR fragment with specific size, Gel purification technique with purifications kits ere used. For a detailed description of the electrophoresis protocol refer to the Annex III.

PCR-Sequencing

Each PCR product were divided into 4 tubes that each tube following 16s rRNA sequencing primers (Table 3) and BigDye sequencing kit was used (Table 4) and placed for slandered BigDye PCR reaction (figure 10):

Table 3 Sequencing 16S rDNA Primers

27F1(3.2pMol)	5'-AGAGTTTGATCCTGGCTCAG
1332R(3.2pMol)	5'-TGACCTGCGATTACTAGCGA
773F(3.2pMol)	5'-GTAGTCCTAGCCGTAAACGAT
334R(3.2pMol)	5'-GCTGCTCATCCTCTCAGACCA

Sequencer PCR reaction

Table 4 Sequencing Reaction mix

BigDye Terminator sequencing buffer	2 µL
Sequencing RR	4 µL
Primer (3.2pMol)	1 µL
H_2O (filtered and autoclaved)	11 µL
DNA	2 µL (depends on DNA Concen ng/ µL)
Total volume	**20µL**

1.4.1.1 Sequencing PCR Cycles:

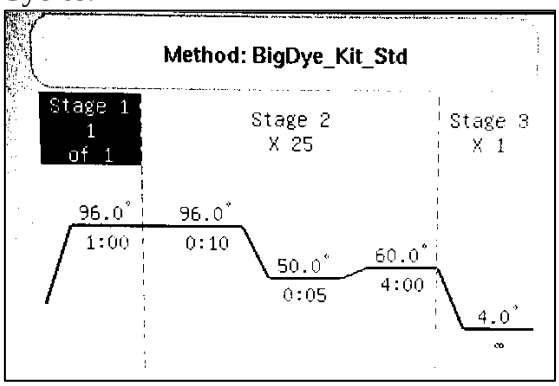

Figure 9 Sequencing PCR

Corresponding sequencing, PCR purified by BigDye purification kit and laoded in 3500 series genetic analyser (figer 11) and ran the bigdye_sequencer_standard_assay_pop7.1

Alignment

The obtained raw sequences were manually corrected by Molecular Evolutionary Genetics Analysis (MEGA 6) by CLUSTAL W alignmen.

Corrected sequences were blasted in NCBI and downloaded sequence data for reference cyanobacteria available in the NCBI database in comparison to DNA sequences obtained in this study.

DNA sequences, both examined in this study and obtained from GenBank, were aligned using CLUSTAL W.

Phylogenetic tree

The phylogenetic tree was constructed from the multiple-aligned data using the neighbor-joining (NJ) algorithmic Kimura's two-parameter as implemented within the Molecular Evolutionary Genetics Analysis (MEGA) version.6 program package.

Theory behind the software: Kimura 2-parameter distance

This model distinguishes between two types of substitutions: transitions, where a purine is replaced by another purine (A<-->G) or a pyrimidine is replaced by another pyrimidine (C<-->T), and transversions, where a purine is replaced by a pyrimidine or vice versa (A or G <--> C or T). The model assumes that the rate of transitions is different from the rate of transversions.

Kimura's two parameter model (1980) corrects for multiple hits, taking into account transitional and transversional substitution rates, while assuming that the four nucleotide frequencies are the same and that rates of substitution do not vary among sites.

Table 5 The Kimura 2-parameter model

	A	T	C	G
A	-	β	β	α
T	β	-	α	β
C	β	α	-	β
G	α	β	B	-

Table 6 MEGA provides facilities for computing the following quantities

Quantity	**Description**
d: Transitions + Transversions	Number of nucleotide substitutions per site.
s: Transitions only	Number of transitional substitutions per site.
v: Transversions only	Number of transversional substitutions per site.
$R = s/v$	Transition/transversions ratio.
L: No of valid common sites	Number of sites compared.

Formulas for computing these quantities are as follows:

Distances ; where *P* and *Q* are the frequencies of sites with transitional and transversional differences respectively

$$d = -\tfrac{1}{2}\log_e(w_1) - \tfrac{1}{4}\log_e(w_2)$$

$$s = -\tfrac{1}{2}\log_e(w_1) + \tfrac{1}{4}\log_e(w_2)$$

$$v = -\tfrac{1}{2}\log_e(w_2)$$

$$R = s/v$$

$$w_1 = 1 - 2P - Q$$
$$w_2 = 1 - 2Q$$

Variances ; Where

$$\mathrm{Var}(d) = \left[c_1^2 P + c_3^2 Q - (c_1 P + c_3 Q)^2\right]/L$$

$$\mathrm{Var}(s) = \left[c_1^2 P + c_4^2 Q - (c_1 P + c_4 Q)^2\right]/L$$

$$\mathrm{Var}(v) = \left[c_2^2 Q(1-Q)\right]/L$$

$$\mathrm{Var}(R) = \left[c_5^2 P + c_6^2 Q - (c_5 P + c_6 Q)^2\right]/L$$

$$c_1 = 1/w_1,$$
$$c_2 = 1/w_2,$$
$$c_3 = \tfrac{1}{2}(c_1 + c_2),$$
$$c_4 = \tfrac{1}{2}(c_1 - c_2),$$
$$c_5 = c_1/v,$$
$$c_6 = (c_4 - c_2 R)/v$$

A rooted phylogenetic tree (Figure 12) summarizes hypothesized evolutionary relationships among species or other biological units such as lineages within species (Vellend et al, 2011).

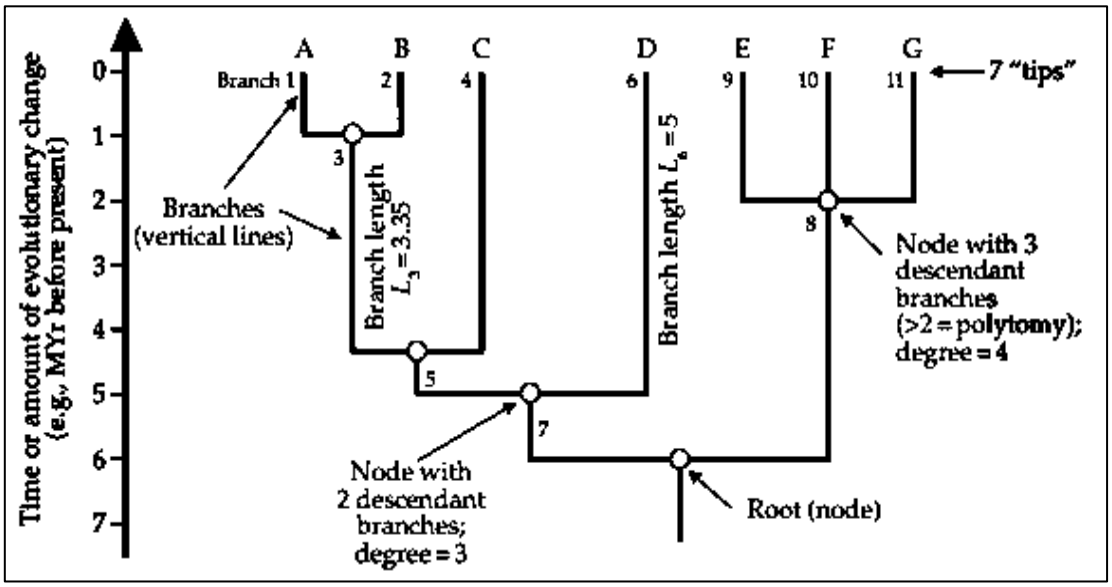

Figure 10 The components of a phylogenetic tree (Vellend et al, 2011).

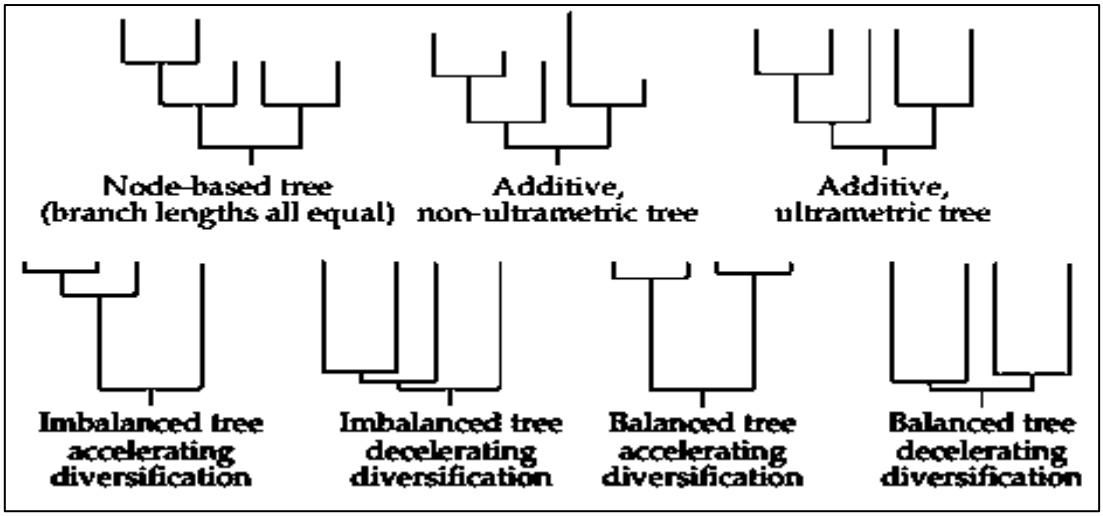

Figure 11 The different representations and shapes of trees illustrating their properties (Vellend et al, 2011)

Phylogenetic Diversity

Based on the obtained evolutionary relationship, a phylogenetic diversity index is calculated as a measure to quantify how much evolutionary history is represented by a group of species (Richard, 2014).

Simply, it informs about the relative quantities or proportions of individuals belonging to the different species. For example, a site containing a thousand of species may not seem particularly diverse if the majority of the species share the same phylogeny.

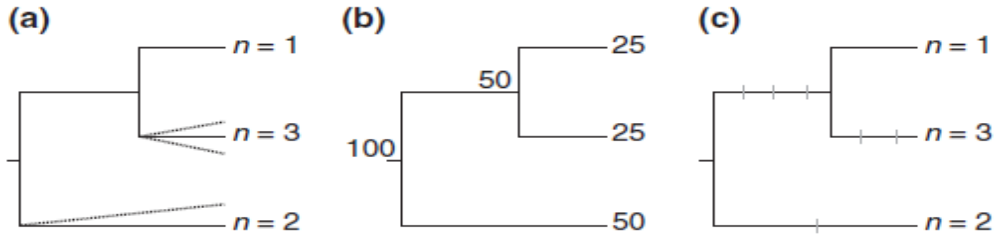

Figure 12 Graphical representation of the three abundance weighted phylogenetic diversity metrics. (a) phylogenetic-abundance evenness, scales terminal branches in proportion to species abundance. (b) Imbalance of abundances at the clade creates an expected abundance based on splitting abundances among higher clades. (c) Abundance weighted evolutionary distinctiveness partitions branch lengths among descendent individuals within a community (Marc et al, 2010).

Table 7 Phylogenetic Diversity index

Metric	Presence-absence (PA) version (Faith, 1992)	Equation
Phylogenetic diversity (PD)	Sum of all branch lengths in the portion of a phylogenetic tree connecting the focal set of species (PD, PDn)	$B \times \frac{\sum_i^B L_i N_i}{\sum_i^B N_i}$ Where; *B*, number of branches in tree; L_i , length of branch *i* N_i Number of species that share branch *i* ;

Results and Discussion

In total 28 marine samples of QUCCCM of cyanobacteria were selected for this study and DNA was extracted. The genotyping result of the 16S rRNA gene with the DNA sequencing method is presented in table below (Table 10). Obtained 16S rDNA sequences were further analyzed by constructing phylogenetic tree (figure 19). six of samples were *Geitlerinema sp.* and six *Chroococcidopsis sp.* followed by three *Synechococcus sp*., three *Stanieria sp,*. Two *Euhalothece sp.*, two *Geminocystis sp.*, a *Leptolyngbya sp., a Dermocarpella sp.*, and a *Oscillatoria sp.* Two samples were fluctuating in relation with the given GenBank references. Calculated the phylogenetic diversity index for QUCCCM identified strains.

Gel of Extracted DNA

Genomic DNA for each strain was extracted by three different protocols, among them better quantity and quality DNA was selected according to its appearance on ethidium bromide stained agarose gel (Figure 15) e.g. strain QUCCCM 110 & 111 (Table 8) were better extracted by CTAB, very weak band was extracted by QIAgen but no extraction done by sigma.

Figure 13 Ethidium bromide-stained gel shows differences in extracted genomic DNA by different extraction protocols

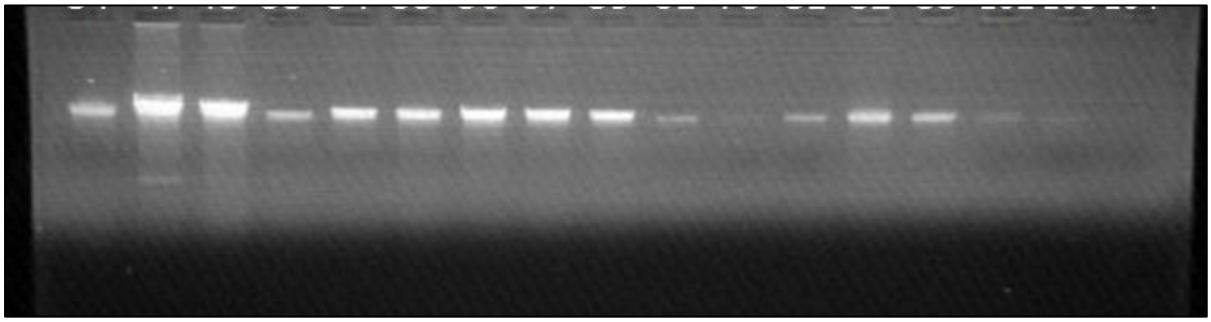

Figure 14 genomic DNA was detected in the photo on an ethidium bromide-stained gel

The selection of good quantity and high quality genomic DNA for each strain from the different extraction protocol is summarized in following (Table 9):

Table 8 isolated genomic DNA from different strains by different extraction protocols.

Genomic DNA extraction Protocol	Strains
CTAB protocol	QUCCCM 61
	QUCCCM 102
	QUCCCM 104
	QUCCCM 106
	QUCCCM 108
	QUCCCM 110
	QUCCCM 111
	QUCCCM 117
Sigma - GenElute™ Plant Genomic DNA Miniprep Kit	QUCCCM 112
	QUCCCM 115
Qiagen-DNeasy plant mini kit	QUCCCM 12
	QUCCCM 20
	QUCCCM 25
	QUCCCM 29
	QUCCCM 34
	QUCCCM 47
	QUCCCM 48
	QUCCCM 53
	QUCCCM 54
	QUCCCM 55
	QUCCCM 56
	QUCCCM 57
	QUCCCM 59
	QUCCCM 78
	QUCCCM 81
	QUCCCM 82
	QUCCCM 83
	QUCCCM 113

Polymarase Chain Reaction:

Each amplified and purified 16S rDNA fragment for each QUCCCM strains was detected on ethedium bromide stained agarose gels to detect its presence then to check its quantity and quality as follows (figure 16):

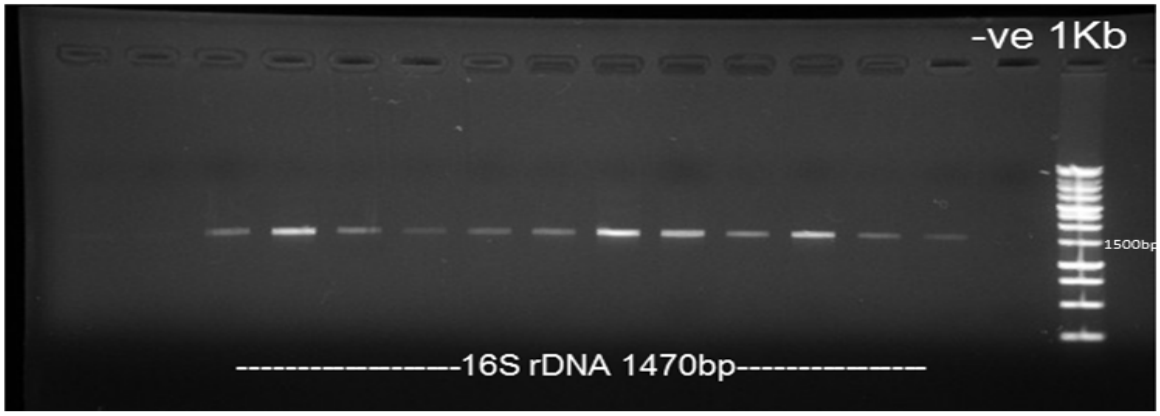

Figure 15 Amplified 16S rDNA was detected (~1.5kb) in the photo on an ethidium bromide-stained gel after the PCR-amplifilication. The last line shows DNA marker (Promega, 1kb).

Sequences

Four sequencing primers were used to cover the 1470bp long 16S rDNA fragment and around 500bp were very well sequenced by the forward primers and around 700b (figure 17) by the reverse primers which was long enough to cover 1.2kb in the following alignment step.

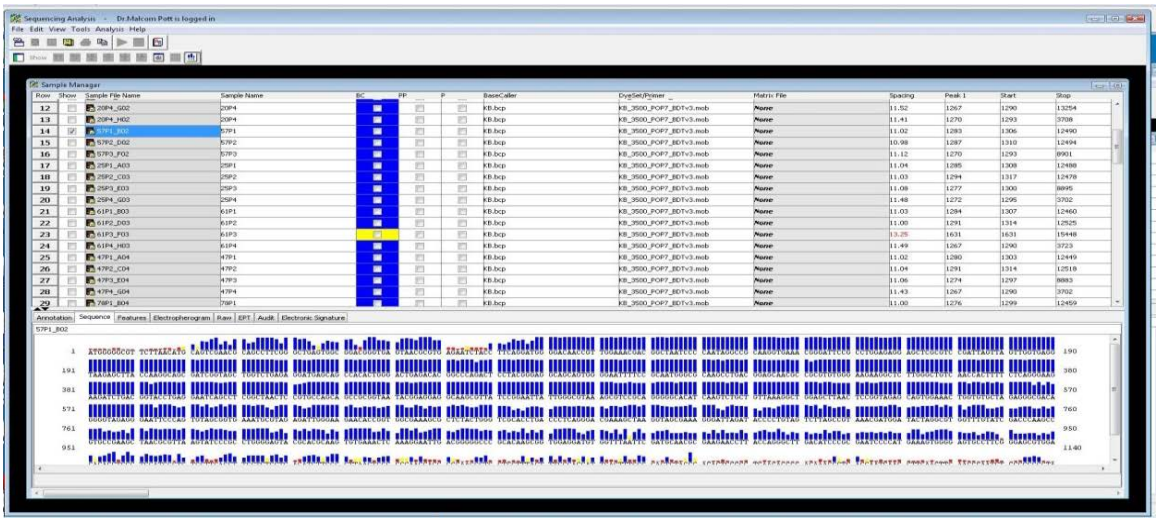

Figure 16 sequences obtained from the 3500 Series Genetic analyser system

Alignment

Thru MEGA 6. Software, 28 QUCCCM sequenced fragments were corrected manually and aligned to many referenced cyanobacteria sequences downloaded from GenBank (figure 18) then saved the alignment as mega format

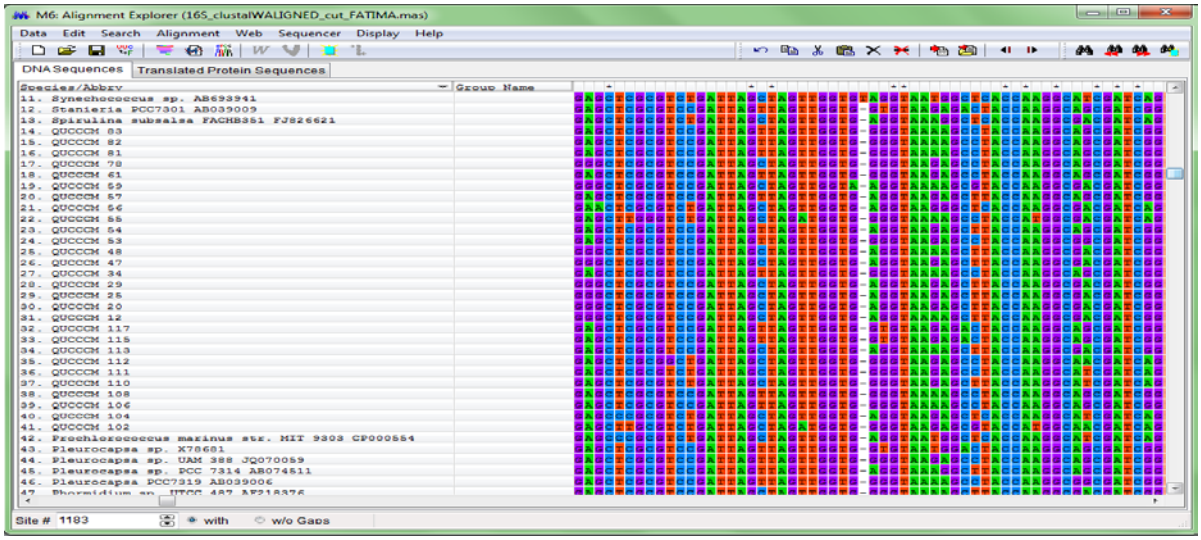

Figure 17 show the alignment of both the QUCCCM corrected 16S rDNA sequences and GenBank refrence sequences.

Phylogenetic tree

The evolutionary history was inferred using the Neighbour-Joining method (Saitou et al, 1987). The optimal tree with the sum of branch length = 2.13915325 is shown. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein J.1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree (figure 19). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura K., Nei M., and Kumar S. 2004) and are in the units of the number of base substitutions per site. The analysis involved 87 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 984 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura K., Stecher G., Peterson D., Filipski A., and Kumar S. 2013).

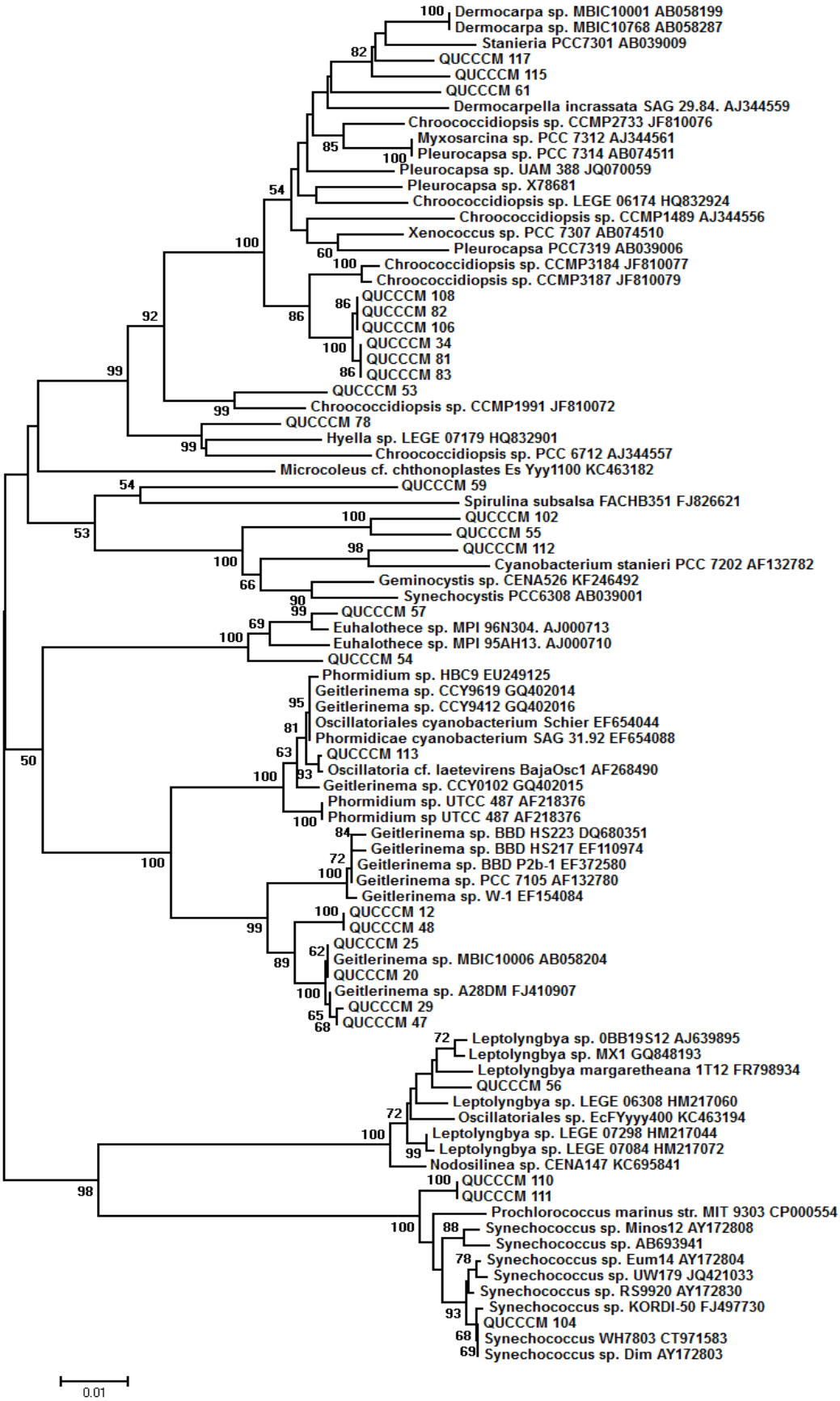

Figure 18 Evolutionary relationships of taxa of 16S rRNA gene for local isolated cyanobacteria from Qatar.

Table 9 The table represents the phylogenetically Identified QUCCCM strains in relation to genus and addition of order from NCBI Taxonomy Browser, 2009. Frequencies among the genus and the orders was calculated.

Order	Frequency among orders	Genus	Strain	Frequency among genus
Oscillatoriales	*32.2%*	*Geitlerinema sp.*	QUCCCM 12	**21.4%**
			QUCCCM 20	
			QUCCCM 25	
			QUCCCM 29	
			QUCCCM 47	
			QUCCCM 48	
		Leptolyngbya sp.	QUCCCM 56	**3.6%**
		Oscillatoria sp.	QUCCCM 113	**3.6%**
		(54% in bootstrapping) *Spirulina sp.*	QUCCCM 59*	-
Pleurocapsales	*42.8%*	*Chroococcidopsis sp.*	QUCCCM 53	**25%**
			QUCCCM 78	
			QUCCCM 81	
			QUCCCM 82	
			QUCCCM 83	
			QUCCCM 106	
			QUCCCM 108	
		Dermocarpella sp.	QUCCCM 61	**3.6%**
		Stanieria sp.	QUCCCM 112	**10.7%**
			QUCCCM 115	
			QUCCCM 117	
		Chroococcidiopsis / Pleurocapsa sp.	QUCCCM 34	-
Chroococcales	*25%*	*Euhalothece sp.*	QUCCCM 54	**7.1%**
			QUCCCM 57	
		Geminocystis sp.	QUCCCM 55	**7.1%**
			QUCCCM 102	
		Synechococcus sp.	QUCCCM 104	**10.7%**
			QUCCCM 110	
			QUCCCM 111	

- *Confidence identification low ….hypothetic unreported new species*

Oscillatoriale are filamentous in morphology resulted by Hormogonia, the distinct reproductive segments, repeated cell divisions occurring in a single plane at right angles to the main axis of the filament (Figure 20). The multicellular structure consisting of a chain of identical cells is called a trichome that may be straight or coiled. Size and shape of the cells are greatly variable among the filamentous cyanobacteria (Luuc et al., 1999).

Figure 19 Morphological structure of the species in order *Oscillatoriales* (Luuc et al., 1999).

Pleurocapsales reproduce by the formation of small, spherical cells known as spores. This mode of reproduction distinguishes them from all other cyanobacteria. develop on plates as small, compact colonies, which are often tough and adhere to the agar surface. members of the Pleurocapsales are multicellular (figure 21) and do not display polarity (John, 1978).

Figure 20 Morphological structure of the species in order *Pleurocapsales* (Luuc et al., 1999).

The unicellular blue-green algae assigned to a single order *Chroococcales,* are coccoid or rod-shaped organisms which multiply by binary fission, and they may form loose colonies in which the constituent cells are held together (figure 22) by a common slime layer or by sheaths. In structural terms, they appear to be equivalents of unicellular true bacteria (Stanier, 1971).

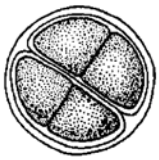

Figure 21 Morphological structure of the species in order *Chroococcales* (Luuc et al., 1999).

Relevance of the identified strains to Qatar Environment

- ***The Phylogenetic Diversity;***

Biodiversity represent the variability of all living organisms on the planet, (Wilson, 1988). It refers to species richness and evenness, and also the phenotypic and genetic variation that species hold as well as all of the spatial and temporal variation inherent to communities and ecosystems (Richard, 2014). A quantitative measure of phylogenetic diversity was defined as the minimum total length of all the phylogenetic branches required to span a given set of taxa on the phylogenetic tree (figure 23). Larger PD values can be expected to correspond to greater expected feature diversity (Faith, 2010).

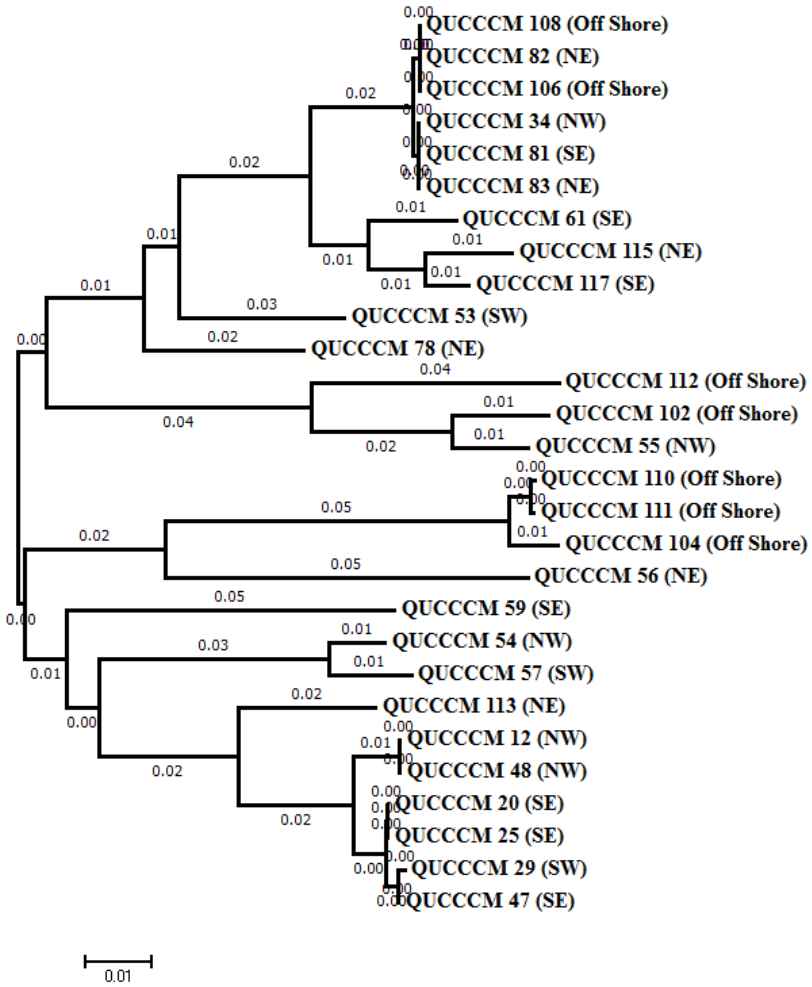

Figure 22. Constructed phylogenetic tree of QUCCCM strains for the purpose of phylogenetic diversity index calculation. Coastal sampling sites are mentioned in the tree; SE-south east, SW- south west, NE- north east and NW- north west.

The phylogenetic diversity (PD) of our set of strains was calculated (PD=0.14), this relatively low value (based on Faith and Baker, 2007) despite the high number of cyanobacteria may be explained by the extreme conditions occurring in the Arabian Gulf and in the coastal environment of Qatar specifically. These environmental conditions limited the number of order and genus susceptible to adapt such extreme conditions. This phylogenetic diversity index appears to be very helpful to assess the genotypic diversity among our local strains and guide future monitoring of the cyanobacterial community in Qatari marine environment.

- ***The occurrence of the identified species in Qatar extreme environments (extremophile): extreme temperature and salinities conditions***

By means of what mentioned above in the introduction, some cyanobacteria species evolved as specialised cells adapted to live in habitats where various physicochemical parameters reach extreme values, therefore they are among the extremophiles which are microorganisms with the ability to thrive in extreme environments such as high salinity and high temperature due to the unique enzymes used by these organisms, called extremozymes, enable these organisms to function in such forbidding environments.(NOAA, 2014). Organisms require one or more extreme conditions in order to grow are called extremophilic, but organisms which can tolerate extreme values of one or more physicochemical parameters though growing optimally at normal conditions are called extremotolerant (Pabulo, 2013). This can be linked to the existence of Qatar local isolates as cyanobacteria to be the best adapted group to various extreme conditions is (Pabulo, 2013). Thermophiles, grow at very high temperatures, are *Synechococcussp, (*Kailash, 2010), Chroococcidiopsis sp. (Nobuhiro, 1997). Halophiles, grow in habitats where salt concentration is in excess of seawater, are *Oscillatoria sp.* (Thomrat et al, 2011), *Euhalothece sp.* (Marc et al, 2006), *Chroococcidiopsis* sp., *Spirulina subsalsa,* and *Leptolyngbya* sp. (Raeid et al, 2011).

- ***Potential toxin producers strains***

Within the identified 28 species we conducted a bibliographic analysis about their potential toxicity or toxin production. Eighteen strains are belonging to potentially toxic marine cyanobacteria. The potential produced toxins are shown in the below table 11.

Table 10 The QUCCCM strains and their potential produced toxins

Strain	Genus	Toxin	Reference
QUCCCM 12	*Geitlerinema sp.*	Mitsoamide: A cytotoxic linear lipopeptide	(Eric et al, 2007)
QUCCCM 20			
QUCCCM 25			
QUCCCM 29			
QUCCCM 47			
QUCCCM 48			
QUCCCM 56	*Leptolyngbya sp.*	Aplysiatoxins, Lyngbyatoxin	(cyanosite, 2014)
QUCCCM 113	*Oscillatoria sp.*	Anatoxins, Aplysiatoxins, Microcystins,Saxitoxins	
QUCCCM 53	*Chroococcidopsis sp.*	neurotoxic cyanobacterial toxins, β -N-methylamino-L-alanine (BMAA) 2,4 diaminobutyric acid (DAB)	(PAUL, 2009)
QUCCCM 78			
QUCCCM 81			
QUCCCM 82			
QUCCCM 83			
QUCCCM 106			
QUCCCM 108			
QUCCCM 104	*Synechococcus sp.*	microcystins	(Wayne et al, 2006)
QUCCCM 110			
QUCCCM 111			

- ***Toxic Effects***

The potential productions of the reported toxins in Table 11 by local cyanobacteria may cause severe health issues through food poisoning. As reported by Nagai et al. (1996) cyanobacteria species are causative agents of the human poisoning incidents in Hawaii in September of 1994 by producing the aplysiatoxins. This toxin is known to be a potent irritant and carcinogen for both animals and humans.

Hepatocytes from animals treated with microcystins appear to die by a process of programmed cell death or cell suicide called apoptosis. In Brazil, 1996, Caruaru Syndrome was reported. Microcystins and cylindrospermopsin were found in the water as well as the blood and livers of the patients. 131 patients experienced visual disturbances, nausea, vomiting, and muscle weakness following routine dialysis then developed acute liver failure and 52 eventually died (Azevedo et al, 2002).

In August 2002, a case of 3 died dogs was reported and showed results of ingesting anatoxin contaminated water that is known for acute neurological signs and often death. 3 dogs died after swimming in the Eel river in, California. Microscopic examination of water revealed the presence of filamentous algae as *Planktothrix* spp. and *Lyngbya* spp in the Eel river in California. Based on the rapid onset of severe neurological signs and suspected exposure to a neurotoxicant, in-depth toxicological analyses were performed that detected anatoxin and demonstrated the first documented case of anatoxin as poisoning in dogs dying of acute neurotoxicosis (Birgit et al, 2008)

- ***Beneficial biotechnology applications of the identified strains***

Paradoxically, the synthetic analogues of aplysiatoxin have been researched for anti-cancer effects where researchers demonstrated that a simple analogue of the tumor-promoting aplysiatoxin is an antineoplastic agent rather than a tumor promoter (Nakagawa et al. 2009).

There is no doubt that Microcystin-LR (MC-LR) belongs to the most potent toxins in aquatic environments, but that does not necessarily reflect the primary function of microcystins. Activation of nuclear factor erythroid 2-related factor 2 (Nrf2) by Microcystin-LR provides advantages for Liver Cancer Cell Growth that treatment of that cells with MC-LR resulted in significant increases in (Nrf2) that is key in mediating the protective antioxidant response against various environmental toxicants (Gan et al, 2010).

Diphtheria is an acute toxin-mediated disease caused by toxigenic strains of *C. diphtheriae.* Since long back it was reported that Diphtheria toxoid is a successful immunizing agent consist of (anatoxin-Ramon) to secure permanent immunity as quickly as possible (Max et al, 1927). The value of diphtheria toxoid (anatoxin-Ramon), the modified toxin of diphtheria, in active immunization of man was firmly established since Glenny's experiments with animals and Ramon's clinical demonstration of its efficiency (Weinfeld, 1929).

These findings suggest **the need for regular monitoring programs along the coasts of Qatar to timely identify the occurrence of harmful cyanobacteria** and to draw the mitigation and control actions that are needed. These frequency and geographic distribution of the monitoring program could be based on our sampling location corresponding to the potential toxins producers strains and biology.

Table 11 Beneficial traits of the identified strains

Strain	Genus	Beneficial Traits
QUCCCM 54	*Euhalothece sp.*	Produce water soluble compounds Mycosporine-like amino acids (MAAs) when grown at high light intensities. MAA show strong absorption in the UV-B region of the spectrum therefore it has been demonstrated in the protection against UV-induced damage (Marc et al, 2006).
QUCCCM 57		
QUCCCM 56	*Leptolyngbya*	Contain toxin-related gene that has antimicrobial effects against the Gram positive bacteria and Candida genera (Mohsen, 2013).
QUCCCM 104	*Synechococcus*	1. Their photosynthetic antenna (phycobilisomes) are adapted to the spectral quality of light in the ocean, as they can synthesize unique photosynthetic pigments Ability to carry out a large portion of carbon fixation in the oceans (Genoscope, 2014). 2. the production of biodegradable plastic, polyhydroxyalkanoate (PHA), by genetically engineered cyanobacteria (Hideo et al, 2011)
QUCCCM 110		
QUCCCM 111		
QUCCCM 113	*Oscillatoria sp*	1. Obtained methanol extract, showed antifungal activity on *S. cerevisae* and *S. cerevisae* (Katircioglu, 2005). 2. Obtained ethanol extract showed antibacterial activity on *Pseudomonas aeroginosa* (Katircioglu, 2005).

Conclusion

The obtained phylogenetic trees helped in branching the isolates by computing the similarities and the distance of differences among the isolates and compare them to the known (published) cyanobacteria. This allowed the accurate identification and detection of the relevant Qatar cyanobacteria diversity.

Several informative conclusions are drawn from this research:

- Qatar marine cyanobacteria populations showed a high species richeness. Indeed, **we identified 28 taxa** among them *Geitlerinema sp., Euhalothece sp.,* and *Geminocystis sp.* **have never been reported in Qatar waters**
- Six isolates (QUCCCM 12, 20, 25, 29, 47 & 48) belonged to the potentially toxic marine cyanobacteria *Geitlerinema sp.* potentially producer of a cytotoxic known for human lung tumor cells
- Six isolates (QUCCCM 53, 78, 81, 82, 83, 106 & 108) belonged to the marine cyanobacteria *Chroococcidiopsis sp.* and are known to produce a neurotoxic amino acid.
- QUCCCM 34 identification fluctuated between the marine cyanobacteria *Pleurocapsa sp.* and *Chroococcidiopsis sp.* This has been explained that genus *Chroococcidiopsis* has been classified within the order *Pleurocapsales*, based on a unique reproduction modus by baeocytes. (Antje Donne, 2013).
- Three isolates (e.g. QUCCCM 112, 115, 117) belonged to *Stanieria sp*. and partially to *Dermocarpa sp.* These two genus were initially assumed as the same (Stech, 1919). Their phylogenetic relationship reveals that they share a close ancestor (sister relationship)
- QUCCCM 55 and 102 belonged to marine cyanobacteria *Geminocystis sp.*

- Several isolates are potentially newly identified strains with low relationship to known cyanobacteria species (e.g. QUCCCM 59 showing only 54% in bootstrapping replication to the nearest strain i.e. *Spirulina*)
- QUCCCM 113 belong to *Oscillatoria sp.* that has been reported from Qatar previously by Mahasneh et al., 2006. Shoichiro et al reported 2002 that *Oscillatoria* commonly form water-blooms that reduce water quality and prevent the use of water-bodies for drinking and recreation.
- QUCCCM 56 belongs to a marine *Leptolyngbya* strain known to contain toxin-related gene that has more antimicrobial effects.
- 2 isolates (QUCCCM 54 & 57) belonged to the unicellular natronophilic cyanobacterium *Euhalothece sp.*
- Three isolates from offshore waters (e.g. QUCCCM 104, 110, 111) are distinct from the isolated coastal strain *Synechococcus sp.* that has been reported from Qatar previously by Mahasneh et al. (2006). It is known for its ability to carry out a large portion of carbon fixation in the oceans,
- Geographic fidelity among some of the QUCCCM genus are not high in the country. Several genus exhibit degility to specific environment condition (biogeographical distribution. Indeed, QUCCCM 110, 111, and 104 all belong to *Synechoccus* were sampled from the same offshore location (figure 23).

We were able to distinguish between populations in geographically separate regions along the Qatari coasts identifying where potentially toxic cyanobacteria may occur. A regular monitoring of the occurrence and potential toxicity of the cyanobacteria in the coastal zone is recommended to timely identify potential environmental and human health hazards

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Annex I.

A: Genomic DNA extraction

I.A.1 CTAB Protocol

1. Cells are resuspended in 500 µL of pre-warmed 65°C CTAB extraction buffer that is prepared as following:

   2% CTAB (hexadecyltrimethylammonium bromide)
   100 mM TrisHCl [pH=8]
   20 mM EDTA,
   1.4 M NaCl
   0.2% β-mercaptoethanol [added just before use]
2. With the addition of glass beads, lysed the cell in tissue lyser for 4min twice.
3. 2hr incubation at 65°C in between gently mixed by inverting.
4. Spin 5min at maximum speed 14000 rpm.
5. Carefully transferred the aqueous phase, above the white interface layer, to a clean microtube.
6. Added 500µL of phenol:chloroform:isoamylalcohol (25:24:1) solution - worked in the fume hood.
7. **Gently** mixed by inverting and Spin 5min at maximum speed 14000 rpm.
8. Carefully transferred the upper phase to a clean microtube with 500µL chilled absolute ethanol.
9. Incubated overnight in 20°C
10. Spin 15min at maximum speed 14000 rpm, discard the supernatant
11. Added 500µL ethanol 70% and mixed by inverting
12. Spin 15min at maximum speed 14000 rpm, discard the supernatant
13. Dry the pellet by leaving tube open @ room temperature.
14. Resuspend pellet in sterile 100µL TE (pH 8.0)mixed with1 µL RNase (DNase-free) and incubate for 30 min at 37°C.
15. Stored in -20°C.

I.A.2 Sigma - GenElute™ Plant Genomic DNA Miniprep Kit

1. Mix loop full tissue cells in 350 µL of Lysis Solution [Part A] and 50 µL of Lysis Solution [Part B] to the tube; thoroughly mix by vortexing and inverting.
2. Lysed cells in tissue lyser with glass beads 4min x2.
3. A white precipitate will form upon the addition of Lysis Solution [Part B]. added 4 µl of (not supplied) RNase A stock solution (100 mg/ml) and incubated the mixture at 65 °C for 10 minutes with occasional inversion to dissolve the precipitate.
4. Precipitated Debris; Added 130 µL of Precipitation Solution to the mixture; mixed completely by inversion and place the sample on ice for 5 minutes. Centrifuged the sample at maximum speed for 5 minutes to pellet the cellular debris, proteins, and polysaccharides.
5. Filtered Debris; carefully pipetted the supernatant from step 3 onto a GenElute filtration column (blue insert with a 2 mL collection tube). Centrifuged at maximum speed for 1 minute. This removes any cellular debris not removed in step 3. Discarded the filtration column, but retain the collection tube.
6. Prepared for Binding; Added 700 µL of Binding Solution directly to the flowthrough liquid from step 4. Mixed thoroughly by inversion.
7. Prepared Binding Column; Inserted a GenElute Miniprep Binding Column (with a red o-ring) into a provided microcentrifuge tube. Added 500 µL of the Column Preparation Solution to each miniprep column and centrifuge at 14000 for 1 minute. Discard the flow-through liquid. Note: The Column Preparation Solution maximizes binding of DNA to the membrane resulting in more consistent yields.
8. Loaded Lysate; Carefully pipetted 700 µL of the mixture from step 5 onto the column prepared in step 6 and centrifuge at maximum speed for 1 minute. Discard the flow-through liquid; retain the collection tube. Return the column to the collection tube. Repeat the centrifugation as above and discard the flow-through liquid and collection tube.
9. First Column Wash, added ethanol to the Wash Solution Concentrate. Placed the binding column into a fresh 2 mL collection tube and applied 500 µL of the diluted Wash Solution to the column. Centrifuge at maximum speed for 1 minute. Discard the flow-through liquid, but retain the collection tube.
10. Second Column Wash Apply another 500 µL of diluted Wash Solution to the column and centrifuged at maximum speed for 3 minutes to dry the column.

11. Elute DNA; Transferred the binding column to a fresh 2 mL collection tube. Applied 50 µL of pre-warmed (65 °C) Elution Solution to the column and centrifuged at maximum speed for 1 minute. Repeat the elution with 50µL. The eluate contains pure genomic DNA. For long-term storage of DNA, –20 °C is recommended. Avoid freezing and thawing, which causes breaks in the DNA strand. Elution Solution will help stabilize the DNA at these temperatures.

I.A.3 Qiagen-DNeasy plant mini kit

1. Mixed loop full tissue cells into 400 µL of Buffer AP1 and vortex vigorously. No tissue clumps should be visible. Clumped tissue will not lyse properly and will therefore result in a lower yield of DNA.
2. Lysed cells in tissue lyser with glass beads 4min x2.
3. Added 4 µl of RNase A stock solution (100 mg/ml) and Incubate the mixture for 10 min at 65°C. Mix 2–3 times during incubation by inverting tube.
4. Added 130 µl of Buffer AP2 to the lysate, mix, and incubate for 5 min on ice.
5. Centrifuged the lysate for 5 min at full speed.
6. Applied the lysate to the QIAshredder spin column (lilac) sitting in a 2 ml collection tube and centrifuge for 2 min at maximum speed.
7. Transferred flow-through fraction 450 µL a new tube (not supplied) without disturbing the cell-debris pellet.
8. Added 675 µL volumes of Buffer AP3/E with added ethanol to the cleared lysate and mix by pipetting.
9. Applied 650 µl of the mixture from step 8, including any precipitate which may have formed, to the DNeasy mini spin column sitting in a 2 ml collection tube (supplied).
10. Centrifuged for 1 min at 8000 rpm and discard flow-through.*Reuse the collection tube in step 9.
11. Repeated step 9 with remaining sample. Discard flow-through* and collection tube.
12. Placed DNeasy column in a new 2 ml collection tube (supplied), add 500 µl Buffer AW to the DNeasy column and centrifuged for 1 min at 8000 rpm. Discard flow-through and reuse the collection tube in step 13.
13. Added 500 µl Buffer AW to the DNeasy column and centrifuge for 2 min at maximum speed to dry the membrane.
14. Spin again to ensure that no residual ethanol will be carried over during elution. Discard flow-through and collection tube.

15. Transferred the DNeasy column to a 1.5 ml or 2 ml microcentrifuge tube (not supplied)
16. Pipetted 50 µl of preheated (65°C) Buffer AE directly onto the DNeasy membrane.
17. Incubated for 5 min at room temperature and then centrifuge for 1 min at 8000 rpm to elute.
18. Repeated elution once more with 50µL and then stored in -20°C.

B. Electrophoresis to Detect Genomic DNA

1. 1g Agarose powder was measured and added 100 ml TAE Buffer to the bottle.
2. It was melted in a microwave until the solution becomes clear, heated the solution for several short intervals - do not let the solution boil for long periods as it may boil out of the bottle).
3. Solution was cooled to 50-55°C, swirling the bottle occasionally to cool evenly.
4. Placed the combs in the gel casting tray.
5. Mixed with 5µL ethedium bromide and poured the melted agarose solution into the casting tray and let cool until it is solid (it should appear milky white).
6. Carefully pulled out the combs.
7. Placed the gel in the electrophoresis chamber.
8. Added enough TAE 0.5x Buffer so that the buffer covers the gel.
9. Loaded 6 µl of (5µL DNA with 1µL 6X Sample Loading Dye) in the well
10. Recorded the order each sample loaded on the gel.
11. *Ran the gel* 100 watts, 30mins
12. *Took a pic in UV*

Annex II

Electrophoresis for PCR amplification detection:

1. Measured 1g Agarose powder and added 100 ml TAE Buffer to the bottle.
2. Melted the agarose in a microwave until the solution becomes clear, heated the solution for several short intervals - do not let the solution boil for long periods as it may boil out of the bottle).
3. Let the solution cool to about 50-55°C, swirling the bottle occasionally to cool evenly.
4. Placed the combs in the gel casting tray.
5. Mixed with 5µL ethedium bromide and poured the melted agarose solution into the casting tray and let cool until it is solid (it should appear milky white).
6. Carefully pulled out the combs.
7. Placed the gel in the electrophoresis chamber.
8. Added enough TAE 0.5x Buffer so that the buffer covers the gel.
9. Pipetted 6 µl of the DNA ladder standard (100bp or 1kb Promega) into one well on the gel.
10. Loaded 10µl of amplified PCR (green) in the well
11. Recorded the order each sample will be loaded on the gel.
12. *Ran the gel* 100 watts, 30mins

Annex III

Purification of amplified fragment:

III. A. Agarose Gel Electrophoresis Protocol

1. Measured 1g Agarose powder and added 100 ml TAE Buffer to the bottle.
2. Melted the agarose in a microwave until the solution becomes clear, heated the solution for several short intervals - do not let the solution boil for long periods as it may boil out of the bottle).
3. Let the solution cool to about 50-55°C, swirling the bottle occasionally to cool evenly.
4. Placed the combs in the gel casting tray.
5. Mixed with 5µL ethedium bromide and poured the melted agarose solution into the casting tray and let cool until it is solid (it should appear milky white).
6. Carefully pulled out the combs.
7. Placed the gel in the electrophoresis chamber.
8. Added enough TAE 0.5x Buffer so that the buffer covers the gel.
9. Loaded complete volume of amplified PCR (green) in 2 wells and leave space of 1 well then loaded other samples; to avoid contamination.
10. Recorded the order each sample loaded on the gel.
11. *Ran the gel* 50 watts, 60mins
12. Excised the DNA fragment from the agarose gel with a clean, sharp scalpel.

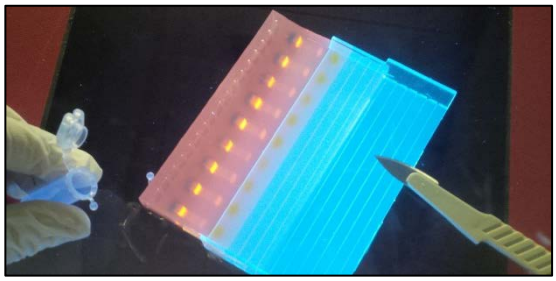

Figure 23 Purification (annex III)

III.B. Gel Purification kits

III.B.1 Promega - Wizard® SV Gel and PCR Clean-Up System (50)

1. Added 200µL Membrane Binding Solution
2. Vortex the mixture and incubated at 65°C for 10 minutes until the gel slice is completely dissolved.

3. Centrifuged the tube briefly at room temperature to ensure the contents are at the bottom of the tube.
4. Placed SV Minicolumn in a Collection Tube for each dissolved gel slice or PCR amplification.
5. Transferred the dissolved gel mixture to the SV Minicolumn assembly and incubated for 1 minute at room temperature.
6. Centrifuged the SV Minicolumn assembly in a microcentrifuge 14,000rpm for 1 minute. discard the liquid in the Collection Tube.
7. Returned the SV Minicolumn to the Collection Tube.
8. Washed the column by adding 700µl of Membrane Wash Solution, previously diluted with 95% ethanol (see Section 4.A), to the SV Minicolumn.
9. Centrifuged the SV Minicolumn assembly for 1 minute 14,000rpm. Emptied the Collection Tube as before and place the SV Minicolumn back in the Collection Tube. Repeated the wash with 500µl of Membrane Wash Solution and centrifuged the SV Minicolumn assembly for 5 minutes.
10. Emptied the Collection Tube and recentrifuged the column assembly for 1 minute with the microcentrifuge lid open (or off) to allow evaporation of any residual ethanol.
11. Carefully transferred the SV Minicolumn to a clean 1.5ml microcentrifuge tube.
12. Applied 50µl of Nuclease-Free Water directly to the center of the column without touching the membrane with the pipette tip. Incubate at room temperature for 1 minute.
13. Centrifuge for 1 minute 14,000rpm.
14. Discard the SV Minicolumn and store the microcentrifuge tube containing the eluted DNA at 4°C or –20°C.

III.B.2 Qiagen - QIAquick Gel Extraction Kit (50) Protocol

1. Added 200µL Buffer QG .
2. Incubated at 65°C until the gel slice has completely dissolved, mix by vortexing the tube every 2–3 min during the incubation.
3. Added 50µL isopropanol
4. Placed a QIAquick spin column in a provided 2 ml collection tube.
5. To bind DNA, applied the sample to the QIAquick column, and centrifuged for 1 min.
6. Discarded flow-through and placed QIAquick column back in the same collection tube.

7. To wash, added 750 µl of Buffer PE to QIAquick column. The DNA will be used for salt sensitive application, direct sequencing, let the column stand 2–5 min after addition of Buffer PE,before centrifuging.
8. centrifuged for 1 min.
9. Discarded the flow-through and centrifuged the QIAquick column for an additional 1 min 13,000 rpm.
10. Placed QIAquick column into a clean 1.5 ml microcentrifuge tube.
11. To elute DNA, added 50 µl of Buffer EB (10 mM Tris·Cl, pH 8.5) or H2O to the center of the QIAquick membrane and centrifuged the column for 1 min at maximum speed.

II.C. Agarose Gel Electrophoresis Protocol

To analyze the quality and quantity of the purified PCR samples, some gel electrophoresis technique was done.

1. Measured 1g Agarose powder and added 100 ml TAE Buffer to the bottle.
2. Melted the agarose in a microwave until the solution becomes clear, heated the solution for several short intervals - do not let the solution boil for long periods as it may boil out of the bottle).
3. Let the solution cool to about 50-55°C, swirling the bottle occasionally to cool evenly.
4. Placed the combs in the gel casting tray.
5. Mixed with 5µL ethedium bromide and poured the melted agarose solution into the casting tray and let cool until it is solid (it should appear milky white).
6. Carefully pulled out the combs.
7. Placed the gel in the electrophoresis chamber.
8. Added enough TAE 0.5x Buffer so that the buffer covers the gel.
9. Pipetted 6 µl of the DNA ladder standard (100bp or 1kb Promega) into one well on the gel.
10. Loaded 10µl of amplified PCR (green) in the well
11. Recorded the order each sample will be loaded on the gel.
12. *Ran the gel* 100 watts, 30mins