Marine cyanobacteria from Mauritian waters

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Abstract

Cyanobacteria are phototrophic gram negative prokaryotes which include more than 150 genera and 200 species. Tropical marine ecosystems harbor a specific cyanobacterial flora while those forming the benthic community are known for their profilic source of natural products as well as their toxin production. Since no recent research had been entertained in determining the diversity of benthic cyanobacteria in Mauritius, this study aimed at identifying the benthic mat-forming cyanobacteria which inhabit the Mauritian lagoons. Samples were collected around the coastal areas of the Island during the summer season from Blue Bay, Albion, Balaclava and Grand River South East. Based on phenotypic and genotypic characters, 11 cyanobacterial strains were successfully identified to genus level, out of 12 samples collected. DNA extraction was performed using a Chelex-100 method which resulted in amplifications products when PCR was carried out. These cyanobacteria belong to the filamentous group of *Lyngbya*, *Leptolynbya*, *Oscillatoria* and *Anabaena* genera and the non-filamentous group of *Merismopedia* genera. Phylogenetic inferences based on 16s rRNA sequences revealed that the cyanobacterial strains evolved from a common ancestor, forming monophyletic clades. The identification of cyanobacterial strains up to the species level was not successful using both morphological and molecular characterization, suggesting the need for other important information such as ultrastructural morphology, ecophysiological characters or whole genome sequencing to ascertain their identity.

Introduction

Cyanobacteria also known as *blue-green algae*, *cyanoprokaryotes* and *prokaryotes*, are an ancient lineage of prokaryotes which are believed to have existed over 3.5 billion years ago. They played a vital role in the evolution of life on earth as both a major player in the creation of an aerobic atmosphere and as plastids precursors in higher plants.[1] They are by definition, oxygenic photoautotrophs that are able to harness sunlight energy using chlorophyll a and various accessory pigments to survive in a much diversified environment.

These microorganisms have very useful properties such as carbon sequestration which can be achieved by using microalgae as biocatalyst to convert carbon dioxide to photosynthetic metabolic products. Cyanobacteria are also important producers of secondary metabolites, including biologically active compounds, many of which are yet to be explored.[2] For instance, benthic marine filamentous cyanobacteria *lyngbya majuscula* have been found to produce a large number of bioactive compounds.[3,4] Biologically active peptides present in cyanobacteria have similar structural features as that of metabolites present in ascidians and sponges of coral reef ecosystems.[2]

Besides all the benefits that cyanobacteria can provide to mankind, one notorious drawback is cyanotoxin. The cyanotoxins, secondary metabolites produced by many strains, may enter animal and human body causing damage to cells, organs and tissues. Cyanotoxins can be classified as hepatotoxins, neurotoxins, cytotoxins and dermatotoxins.[5,6] Some cyanobacterial mats have been found to act as poison for scleractinian corals, killing live coral tissue.[7] In the past few years, benthic marine cyanobacteria in the tropical and subtropical regions have been studied quite extensively as these prokaryotes are important producers secondary metabolites which are not only structurally diverse but also highly bioactive.[8-10] Regardless of their toxicity, the pharmaceutical importance of these bioactives has been highly documented.

Mauritius has a rich marine biodiversity and bio-prospecting of our marine living resources can contribute to new avenues of pharmaceutical products which can give a new dimension to our economy. Benthic cyanobacterial mats which are very common in our lagoons, especially within the intertidal zone and coral reef ecosystem, are potential sources of bioactive compounds. Moreover, with the advent of global warming, occurrence of cyanobacterial bloom and related toxin production may become more frequent and has to be closely monitored for prevention of intoxication. Thus it is of upmost importance to identify the cyanobacterial mats communities in our lagoons.

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Due to the high economic importance of cyanobacteria in biotechnological applications, it is an imperative to define the dominant taxa of cyanobacteria in the coastal areas around Mauritius so as to enable further research in cyanobacteria and its numerous potential. In this study, cyanobacterial mats, mainly benthic, were studied and their taxonomic identification was made using morphology and molecular tools.

Materials and Methods

Sample collection and morphological identification

Benthic cyanobacterial mats were sampled in four different regions across the island – Balaclava, Grand River South East, Albion and Blue Bay. Samples were divided into 2 aliquots. One aliquot was stored in the freezer compartment (-20°C) for later DNA analysis and a second aliquot was preserved into 15% Lugol's iodine solution and kept in the dark for microscopic analysis. The morphological study of the cells and filaments was carried out using a light microscope (Leica) equipped with a digital camera. A number of morphological charac-

ters for each cyanobacterial strain were selected and scored using a data matrix. A dendogram was generated to study the phenetics of the cyanobacteria strains collected, based on morphological traits.

DNA extraction and quantification

Cyanobacterial mats (approximate surface area of 1cm^2 each) stored at -20°C were transferred to 1.5 ml microcentrifuge tubes and 1 ml of sterile water was added. The samples were centrifuged at 13,000 rpm for 5 minutes. The pellets were each grinded with a mortar and pestle using liquid nitrogen.[11] The procedure used for DNA extraction was a modified protocol from Mohlenhoff *et al*. (2001).[12] 200 µl of 5 % chelex-100 (Sigma- Aldrich) were added to each microcentrifuge tubes containing cyanobacteria following by 4 µL of proteinase K (20 mg/ml). After incubation and centrifugation, 2 µl RNAse (10 mg/ml) was added to each tube and incubated at 37°C for 30 minutes. To assess the quantity and quality of DNA extracted, absorbance values at 260 nm, 280 nm and 230 nm were recorded for each sample.

Polymerase chain reaction

A set of primers, CYA 106F and an CYA 781R [equimolar quantity of CYA 781R (a) and CYA 781 R (b)], were used to amplify part of the 16S rRNA genes. The products were run on 1.5% agarose gel and were sequenced. The consensus sequences were then fed into the NCBI BLAST online system.

Molecular data analysis

Consensus sequences of 5 cyanobacterial strains and the reference sequences retrieved were then aligned using MAFFT software (online version) and ultimately phylogenetic analysis was carried out using the pairwise distance method (Maximum Parsimonious tree) using the MEGA6 software.

Results

Morphological identification and analysis of benthic cyanobacteria

From Grand River South East, microalgae *Symploca* sp. was found on a hard substrate in the warm shallow lagoon of a coral reef ecosystem. *Anabaena* sp. was spotted in bloom, floating in the seawater across the lagoon forming blue-green colonies with heterocysts. *Lyngbya* sp. was spotted in bloom, forming hair-like filaments which were reddish brown in colour. The non-heterocystous cyanobacteria *Leptolyngbya* sp. was collected on hard subsbrate (dead coral) in the shallow lagoons. Dark purple cyanobacterial mat of *Moorea producens* was collected from a hard substrate in the coral reef ecosystem of shallow lagoons. From Balaclava, *Lyngbya* sp was collected from the shallow lagoons on a live diseased coral. The only non-filamentous species collected in the study was *Merismopedia* sp. and it was collected on benthic sediments in the shallow lagoons. From Blue-Bay, microscopic analysis showed very thin filaments which suggest that species may have belonged to the genus *Leptolynbya*. Dark green cyanobacterial colonies of *Anabaena* sp. were collected on a dead coral in the same shallow lagoon.

From Albion, *Lyngbya* sp. was collected on hard substrate and formed dense dark brown turfs. *Leptolyngbya* sp. was also collected as a gelatinous mat, brown in colour. The samples that have been collected and identified are as per Table 1, with details on the substrate they were collected, size of trichome, and general shape of end cells. Figure 1 is a photographic illustration of some of the microalgaes collected.

Based on morphological traits, Figure 2 shows a dendrogram which was generated for the 12 cyanobacterial strains collected. The dendogram showed that the cyanobacteria have clustered into three main clades. The upper clade constituted of 2 subgroups Synenococcales (*Leptolyngbya* sp.1, sp.2, sp.3) and Nostococcales (*Anabaena* sp.1, sp.2). Surprisingly, *Symploca* sp. (Sample G1) was clustered into the upper clade rather than in the middle clade, the Oscillatoriales. The middle clade composed of the class Oscillatoriales and the lower clade comprised of the class Chrooccocales (*Merismopedia* sp.). The unidentified sample showed closed similarity with *Symploca* sp.

DNA and phylogenetic analysis

DNA purity of the samples was revealed by the ratio A260/A280 (Table 2). Out of 12 extracted DNA samples, 11 had values of A260/A280<1.8 and one sample (Unidentified-BB1) had a corresponding ratio of more than 1.8. These values showed that DNA was highly contaminated with proteins, polysaccharides and RNA despite proteinase K treatment and RNAse treatment during the DNA extraction procedure.

The low value of ratios A260/A230 of 11 samples of extracted DNA indicates high salt contaminants in the samples and it is also the case for sample BB1 which has a very high corresponding ratio. PCR amplification products of 6 samples were

Table 1. Morphological analysis of collected cyanobacteria.

Location	Sample ID	Species	Habitat (Substrate type)	Thallus	Sheath	Trichome size (µm)	Constrictions at cross walls	Shape of end cells	Heterocysts	Size (µm)	
										Vegetative cell (Length/ width)	Heterocyst Length/ width)
Grand River South East	G1	*Symploca* sp.	Hard	Filamentous	Present	6.67	Absent	Rounded	Absent	6.67/6.67	-
	G2	*Anabaena* sp.	Hard and soft	Filamentous	Absent	6.36	Present	Rounded	Present	6.36/4.55	5.90/6.82
	G3	*Lyngbya* sp.	Hard and soft	Filamentous	Present	17.8	Absent	Flat	Absent	17.6/2.20	-
	G4	*Leptolyngbya* sp.	Hard	Filamentous	Present	1.8	Present	Rounded	Absent	-	-
	G8	*Leptolyngbya* sp.	Hard	Filamentous	Present	<10	Present	Rounded	Absent	-	-
	G9	*Moorea producens*	Hard	Filamentous	Absent	13.3	Absent	Rounded	Absent	13.2/2.10	-
Balaclava	BL1	*Lyngbya* sp.	Hard	Filamentous	Present	20	Absent	Rounded	Absent	-	-
	BL2	*Merismopedia* sp.	Soft	Non-filamentous	Present	-	Absent	Rounded	Absent	6.20/4.40	-
Blue Bay	BB1	Unidentified	Hard	Filamentous	Absent	<10	-	Rounded	Absent	-	-
	BB2	*Anabaena* sp.	Hard	Filamentous	Absent	5.5	Present	Rounded	Present	3.30/5.50	5.60/5.50
Albion	A1	*Lyngbya* sp.	Hard	Filamentous	Present	22	Absent	Rounded	Absent	22/1.10	-
	A2	*Leptolyngbya* sp.	Hard	Filamentous	Present	2.2	Present	Rounded	Absent	2.20/3.30	-

obtained namely G1, G2, G3, G4, G8 and G9, each having approximately 700 bp.

Phylogenetic analysis was performed using MEGA 6 software. A Maximum Parsimonious tree based on 16SRNA sequences was generated using the 6 consensus sequences of the cyanobacterial strains and 7 reference sequences obtained from NCBI (Figure 3).

An estimate of evolutionary divergence (Table 3) between sequences was also generated using the Maximum Composite Likelihood model.[13] Evolutionary analyses were conducted in MEGA 6.[14]

Table 3 shows the genetic distance between *Symploca* sp. (G1) and *Symploca atlantica* CCY9617 (GQ 402026.1) was found to be as little as 0.06. Corresponding genetic distance between *Moorea prudecens* (G9) and *Oscillatoria sancta* PCC 7515 (NR 114511.1) was also very low (0.05). Genetic distance between *Leptolyngbya* sp. 1 (G4) and *Leptolyngya* PCC 7375 (AB039011.1) was 0.11. *Leptolyngbya* sp. 1 (G4) and *Leptolyngbya* sp. 2 (G8) has a genetic distance of 0.22 which indicates close relatedness. The unidentified sample (G3) shared a genetic distance of 0.42 with *Leptolyngbya* sp. 1 (G4) which also inferred close relatedness. Both *Anabaena* sp. (G2) and *Gloebacter violaceus* (AJ007273.1) shared large genetic distances (>1) with other cyanobacteria.

The Maximum Parsimonious tree generated (Figure 3) using the 16SRNA sequences showed the presence of two main clades, the lower clade comprising of the Nostococcales (*Anabaena* sp.) and the upper clade comprising of the Oscillatoriales from which the organisms evolved. Boostrap values are indicated at the nodes of the tree. Highest boostrap value obtained was 96%, indicating that *Oscillatoria sancta* PCC 7515 (NR 114511.1) and *Moorea prudecens* (G9) have close genetic relationships (they share a common ancestor). On the other hand, there is only 33% support that *Moorea bouillonii* PAL08-16 (GU182894.1) and *Symploca* sp. (G1) share a common ancestor.

Discussion

Sampling and morphological characterization

Benthic cyanobacteria occurred as scattered mat-forming colonies across the seafloor and on corals. The morphology of mat-forming cyanobacteria is dependent on the environmental conditions that prevail as well as the dominant species.[15] Samples stored in 15% Lugol's iodine solution were

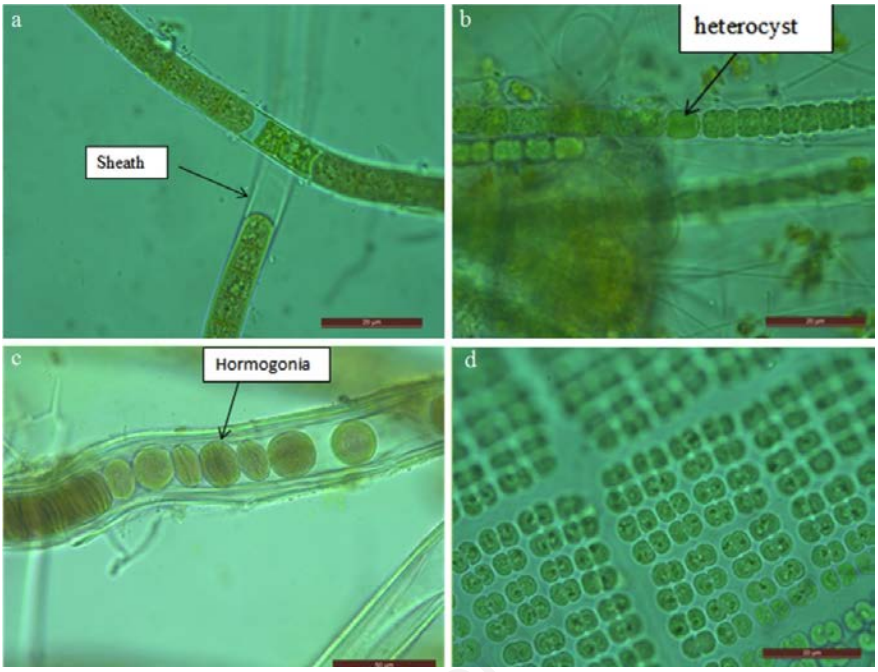

Figure 1. Photomicrograph of filaments of *Symploca* sp. with empty sheaths (live sample; 100× magnification) (a); Photomicrograph of *Anabaena* sp. (live) with heterocyst in the centre (b); photomicrograph of *Lyngbya* sp. (live) showing discrete cells coming out of filament (c) and photomicrograph of *Merismopedi*a sp. with distinct arrangement of colonies in quadrangular flat singular plane and oblong cells in regular perpendicular rows (d).

Table 2. DNA purity and quantity (ng/µl) determined using spectrophotometer.

Species	Sample	A280	A260	A230	A260/A280	A260/230	DNA conc. (ng/ µl)
Symploca sp.	G1	0.024	0.033	0.153	1.38	0.22	165
Anabaena sp.1	G2	0.069	0.093	0.248	1.35	0.38	465
Lyngbya sp.1	G3	0.038	0.055	0.231	1.45	0.24	275
Leptolyngbya sp.1	G4	0.105	0.103	0.25	0.98	0.41	515
Leptolyngbya sp.2	G8	0.06	0.042	0.187	0.70	0.22	210
Moorea prudecens	G9	0.055	0.082	0.257	1.49	0.32	410
Lyngbya sp.3	A1	0.07	0.073	0.165	1.04	0.44	365
Leptolyngbya sp.3	A2	0.067	0.026	0.178	0.39	0.15	130
Unidentified	BB1	0.052	1.004	0.185	19.31	5.43	5020
Anabaena sp.2	BB2	0.029	0.082	0.393	2.83	0.21	410
Lyngbya sp.2	BL1	-	-	-	-	-	-
Merismopedia sp.	BL2	-	-	-	-	-	-

Table 3. Pairwise distances between sequences.

	1	2	3	4	5	6	7	8	9	10	11	12
1. Leptolyngbya PCC7375 |AB039011.1| 1556 bp												
2. Moorea bouillonii PAL08-16 |GU182894.1| 1556 bp	0.14											
3. Symploca atlantica CCY9617 |GQ402026.1| 1556 bp	0.12	0.06										
4. Symploca sp G1 1556 bp	0.15	0.08	0.06									
5. Microcoleus vaginatus CNP 3-KK2 |EF654064.1| 1556 bp	0.15	0.12	0.11	0.12								
6. Oscillatoria sancta strain PCC 7515 |NR 114511.1| 1556 bp	0.18	0.11	0.11	0.11	0.12							
7. Lyngbya aestuarii strain PCC 7419 |NR 114680.1| 1556 bp	0.16	0.11	0.11	0.13	0.12	0.10						
8. Moorea producens G9 1556 bp	0.20	0.13	0.13	0.12	0.15	0.05	0.13					
9. Leptolyngbya sp1 G4 1556 bp	0.11	0.13	0.12	0.14	0.14	0.17	0.16	0.19				
10. Leptolyngbya sp2 G8 1556 bp	0.29	0.32	0.30	0.33	0.31	0.33	0.32	0.36	0.22			
11. Unidentified sp G3 1556 bp	0.44	0.44	0.47	0.44	0.45	0.44	0.49	0.46	0.42	0.56		
12. Ananbaena sp G2 1556 bp	1.44	1.39	1.34	1.33	1.42	1.41	1.48	1.39	1.48	1.19	1.39	
13. Gloeobacter violaceus |AJ007273.1| 1556 bp	1.08	1.04	1.08	1.10	1.06	1.03	1.08	1.06	1.07	1.26	1.15	1.26

all found to be degraded, probably due to photodegradation of the preservative while samples of cyanobacteria preserved in 4% formaldehyde resulted in successful microscopic analysis in a study conducted by Charpy *et al*. (2010).[16]

The cyanobacteria were identified to the genus level based on morphological traits such as thallus type, colour, trichome size, presence of sheath, presence of heterocysts, size of vegetative cells and substrate type. The genera which were dominant among the samples were those of *Lyngbya*, *Leptolyngbya*, *Anabaena*, *Symploca*, *Moorea* and *Merismopedia.* Due to the morphological plasticity of some strains, species level identification was difficult and would require expert knowledge as well as other information about ultrastructure.

The dendrogram (Figure 1) showed presence of two main clades distinguished by the thallus type of the cyanobacteria. *Merismopedia* sp. which has the largest rescaled distance on the dendogram, is found in a distinct group of non-filamentous (coccoid) cyanobacteria. On the other hand, the smallest rescaled distance is represented by the three sister clades of *Leptolyngbya* sp. (*Leptolyngbya* sp. 1 *Leptolyngbya* sp. 2 and *Leptolyngbya* sp. 2). *Leptolyngbya* sp. 2 and *Leptolyngbya* sp. 3 also form sister clades, however, it would have been expected that *Leptolyngbya* sp. 1 be positioned in the same sister clade but instead, *Moorea prudecens* has been positioned. As expected, *Anabaena* sp. 1 and *Anabaena* sp. 2 have been clustered together as they both form part of the heterocystous cyanobacteria forming trichomes with vegetative and heterocyst cells. Lastly, *Symploca* sp. and the unidentified sample (BB1) were clustered together. However, *Symploca* sp., belonging to the order Oscillatoriales, would have been expected to cluster with the *Lyngbya* and *Moorea* species, which also belong to the same order. This is probably due to the smaller trichome size and the presence of constrictions at the cross walls which account for its position in the dendogram.

DNA extraction

Chelex method has been reported to extract DNA from microorganisms which in turn resulted in successful PCR amplifications.[17] The protocol which was adopted from Moller *et al*. (1992) for the lysis of the cells using grinding in liquid nitrogen and Mohlenhoff *et al*. (2001) for the chelex-100 method.[11,12] Ultimately, the outcome was the extraction of DNA from 10 samples of which 6 samples successfully gave amplification products. As for the other samples, DNA was not present which may be due to several factors such as disintegration or loss of the DNA during manipulation. The main disadvantages of using mechanical lysis such as grinding in liquid nitrogen was reported to be the degradation of the DNA due to shearing stress and loss of samples during the process.[18] Alternatively, Morin *et al*. (2010) proposed the freeze-thawing technique for cell lysis which involved three alternating freezing in liquid nitrogen and thawing in a waterbath at 37°C.[18]

The quantity of DNA extracted from the samples was satisfactory, ranging from130 ng/µl to 5020 ng/µl (Table 2). However, absorbance ratios, A260/A280 ,which deviated largely from the recommended range of 1.8 to 2.0, showed a lot of protein contamination of the DNA, questioning the effectiveness of the RNAse treatment at the specified concentration and incubation time used. Absorbance ratios A260/A230 was also far from the recommended ratios which suggest presence of high salts in the DNA samples. Repeated washing of the cyanobacterial mats with sterile water prior to DNA extraction would have probably removed the excess salt in the samples.

Analysis of morphological and molecular data

The maximum parsimonious tree which was generated using the 16SRNA sequences showed the presence of two main clades (Figure 3). The upper clade showed

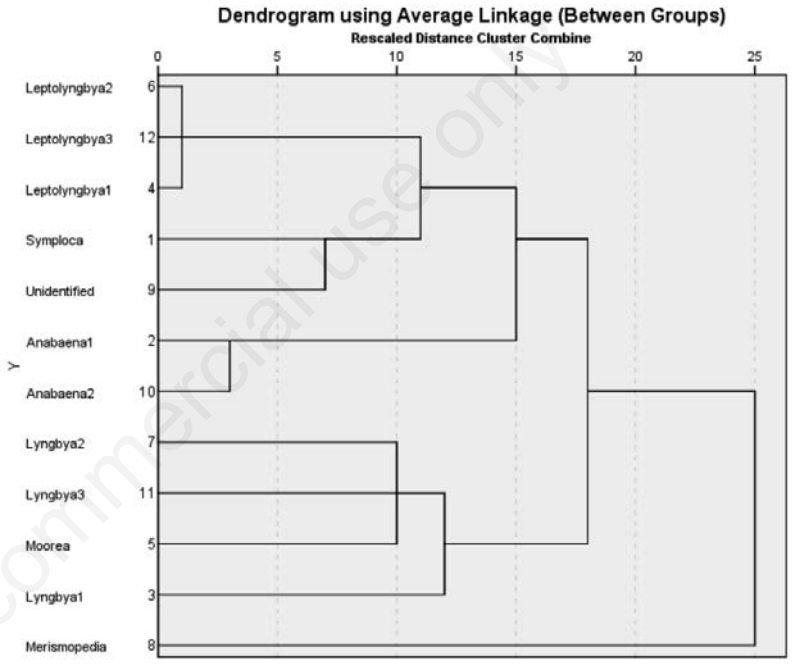

Figure 2. Dendogram showing the clustering of cyanobacteria based on morphological characters.

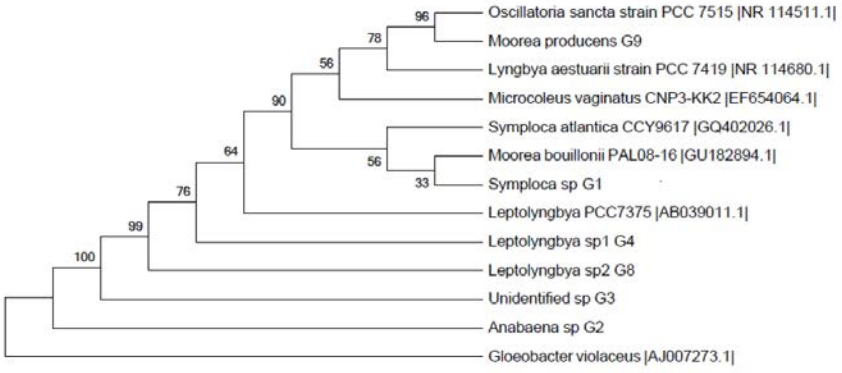

Figure 3. Maximum Parsimonious tree based on 16SRNA sequences of cyanobacteria, generated using MEGA 6 software.

100% support that the cyanobacteria belonging to the order Oscillatoriales, that is the representatives of the genus *Lyngbya*, *Oscillatoria*, *Moorea*, *Microcoleus*, *Symploca*, *Leptolyngbya*, all evolved from a common ancestor. The unidentified sample G3 was also grouped together in the same clade with 100% support, which inferred its close relatedness to the Oscillatoriales. The tree revealed an important anomaly; the cyanobacteria identified as *Moorea prudecens* (G9) form a sister clade, with high support, with the strain *Oscillatoria sancta* PCC 7515 sharing a genetic distance of only 0.05 with each other suggesting that the sample G9 was actually misidentified and should rather belong to the genus *Oscillatoria*. The dendogram generated from morphological characters confirmed the position of sample G9 to the order Oscillatoriales but its genus could not be determined.

Another anomaly detected in the tree was the position of *Moorea bouillonii* PAL08-16 and *Symploca sp*. (G1) as sister clades, with a support value of 33%. The genetic distance between the two strains, *Symploca sp*. (G1) and *Moorea bouillonii* PAL08-16, was only 0.08 whereas the genetic distance between *Symploca sp*. (G1) and *Symploca atlantica* CCY9617 was as little as 0.06. This suggested that a misidentification must have been made regarding the reference strain *Moorea bouillonii* PAL08-16 in the NCBI database. The lower clade showed with 100% support the position of *Anabaena sp*. (G2) to a distinct group which belong to the order Nostococcales. The outgroup, *Gloeobacter violaceus* (accession number AJ 007273.1) was used as an outgroup to root the maximum parsimonious tree.

Conclusions

Benthic marine cyanobacteria are known for their ability to produce structurally diverse prolific secondary metabolites having both biomedical and toxicogenic properties.[19] The identification of the mat-forming cyanobacteria was made possible by morphological traits, microscopic analysis, as well as DNA sequence data. Molecular analysis of six cyanobacterial strains were enabled from DNA extracted from a chelex-100 method followed by amplification of specific 16SRNA gene segments and sequencing of these amplicons by Sanger-method. The molecular data was more reliable in identification and classification of cyanobacteria since the morphological plasticity of this phenotypically diverse group of photosynthetic microorganism may lead to misidentification. The dominant taxa which were identified here belong to the genera *Lyngbya*, *Leptolyngbya*, *Oscillatoria*, *Anabaena* and *Merismopedia*. To be able to better differentiate between cyanobacterial strains, it is better to employ techniques such as the use of electron microscopy to determine the ultrastructures, such as thylakoids, which may help in taxonomic identification of the strains up to species level and the use of 16S-23s RNA Internal Transcribes Spacer (ITS) in complementary with 16SRNA molecular marker is a better option.

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