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Research Article

Isolation And Identification of Cyanobacteria from Selected Caves of Bulabog Putian National Park Dingle, Iloilo, Western Visayas, Philippines

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ABSTRACT

Cyanobacteria are a photosynthetic, gram-negative, and diverse group of prokaryotic organisms inhabiting a wide range of habitats, including caves. This study was aimed to isolate and identify cyanobacteria in selected caves of Bulabog Putian National Park, Dingle, Iloilo, Western Visayas, Philippines. Six strains of cyanobacteria were identified based on morphology and 16S rRNA gene sequence analysis. The sequence homology search by BLAST program revealed that the closest relative of the six strains were *Leptolyngbya sp*. (94.62% similarity), *Calothrix sp.* (97.07% similarity), *Chroococcidiopsis cubana* (99.21% similarity), *Onodrimia javanensis* (96.27% similarity), *Chroakolemma pellucida* (94.74% similarity), and *Albertania skiophila* (98.40% similarity), belonging to Order Chroococcidiopsales, Nostocales, and Synechococcales. The phylogenetic tree revealed the taxonomic positions of the six cyanobacterial strains. Based on the results, six newly identified strains of cyanobacteria have been discovered from the caves of Bulabog Putian National Park which is the only finding reported. Further study, such as the polyphasic approach and other taxonomic characterizations, is needed to describe these new novel taxa of cyanobacteria.

Keywords: Cave, Cyanobacteria, 16S rRNA

Introduction

Underexplored environments are important in providing fresh venues for discovering novel chemicals with biotechnological value. One of the environments is the subterranean ecosystem. Caves are environments that vary greatly from those found on the surface and are of great scientific interest due to having diverse microorganisms that have long evolved in stable conditions like in limestone cave of the Western Loess Plateau of China [1], and Bozana cave in Serbia [2, 3]. They are considered extreme environments due to scarcity in nutrients and oxygen level compared to the surface, and the microorganisms living here have adapted to cave habitat conditions and are generally unique [4]. Among the microorganisms usually found in caves, Cyanobacteria are receiving a tremendous deal of inte-rest due to its ability to produce novel com-pounds with antibacterial activity against gram positive and gram-negative bacteria [5], fungi [6], and cancer cells [7]. They are the most diverse and ubiquitous group of bacteria, thriving in both terrestrial and aquatic ecosystems and can adapt to different habitats such as [8], rock surface [9], marine water [10], and in extreme environments like hot springs [11], Arctic oceans [12], desert soil [13, 14], and caves [15]. Caves are considered to be a unique environment for cyanobacteria to grow since they have a meager amount of weather unpredictability and exposure

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to sunlight [16]. In addition, most cyanobacteria are photoautotroph present in cave entrances directly or indirectly hit by sunlight [17]. Some are heterotroph, making them survive a prolonged dark environment [18]. It is note-worthy that cyanobacteria can adapt to the cave environment by interacting with minerals and some of the processes that reshape the caves' structure, such as the formation of speleothems like stalactites and stalagmites [19]. Furthermore, these organisms and cave habitats are rela-tively under-studied and insufficiently investigated [20]. Having to survive in a harsh environment, cyanobacteria produce a variety of secondary metabolites that enable them to survive in different environments [21].

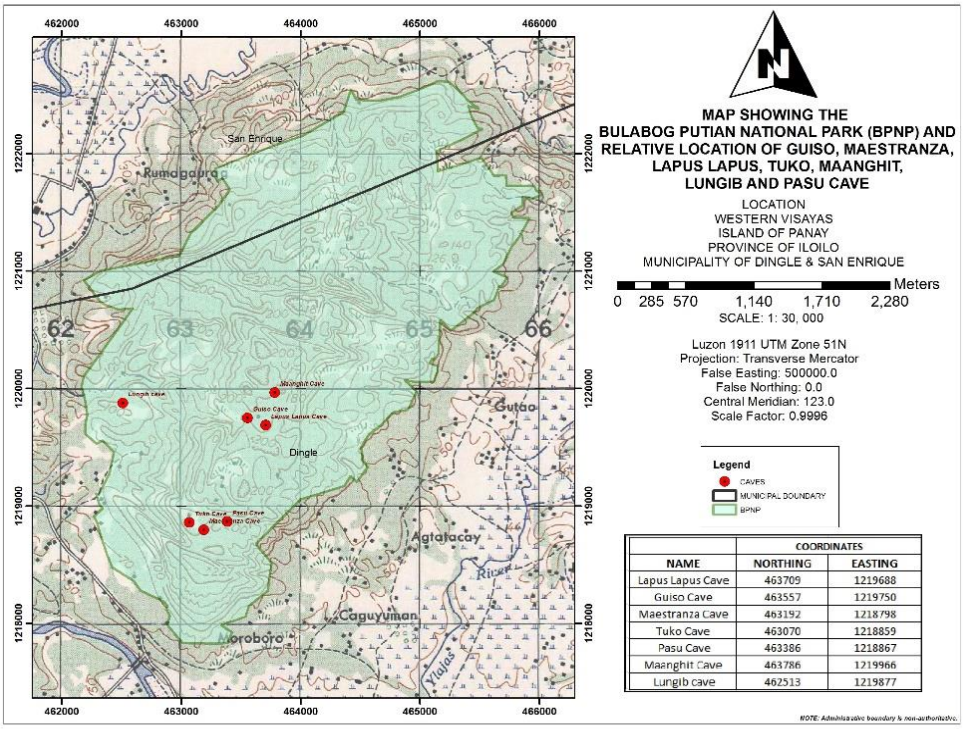
Cyanobacteria or blue-green algae have been primarily studied based on their morphology as prokaryotic organisms [8, 22, 23]. Cyanobacterial species were recognized baased on phenotypic criteria such as cell morphology, sheath characteristics or cell ultrastructure [11]. Morphological analyses of species continue to be extremely important in Cyanobacterial diversity studies, serving as the foundation for preliminary investi-gation and avoiding difficulties in identification. Investigating different morphologies of the cyano-bacterial genus can add more knowledge about the morphological variations among the groups. This in turn, can be used as a basis for taxon identifica-tion and separation. Recently, cyanobacteria were identified and categorized using different morphological characteristics like cell dimensions, shape, color, type of branching, sheath characteristics, and cell contents [24, 25, 26]. These criteria were based on the literature published by Komarek and Anagnostids 1998 and 2005, and Komarek 2013 [27, 28, 29]. For example, morphometric characters such as the length and width of the vegetative cell, akinete, and length and width of trichomes most variable traits were found to account for 99 percent of the overall variance among the four groups which were *Anabaena, Aphanizomenon, Trichormus,* and *Nostoc* [30]. Moreover, genus *Anabaena* has been identified, with uniform trichomes, absence of sheath, and presence of free or floccose or soft mucilaginous thallus and heterocysts, intercalary, and presence of spores near the heterocyst or between the heterocysts [31]. Cyanobacterial isolates at different stages of growth can be examined morphologically and described using the keys provided by Desikachary (1959) [31], Rippka *et al.* (1979) [32], and Bergey's Manual of Systematic Bacteriology (2001) [33]. Desert soil cyanobacteria have been investigated morphologically using various morphometric traits throughout their life cycle [14]. However, some morphological characters may display phenotypic commonality with struc-tures of other species or may change due to long culture periods [32], resulting in ambiguity in the essential identification. Classification of cyanobacteria can be resolved only by utilizing other essential criteria such as genetic, ecology, physiology, and morphology. In recent years, species have been identified or characterized utilizing morphological, molecular, and ecological data [34, 35].

For decades, cyanobacteria were identified based on their 16S rRNA [13, 14, 36], *nifH,* and *hetR* [37] gene sequences in addition to morphological, biochemical, and ecological analyzes. However, genetic relationships sometimes conflict with morphological classification [38]. Some morphological species and strains are not identifiable, resulting in morphological and gene-tic evaluation difficulties. This is due to the lack of a culture and long-term laboratory cultivation [30]. Thus, cyanobacterial isolates should be thoroughly studied by several morphological characters and molecular genetic markers such as the 16S *rRNA* gene. The caves in Bulabog Putian National Park feature their unique geological formations such as stalactites and stalagmites. The caves have not yet been explored by researchers in microbiology and biotechnology. Thus, this study aimed to isolate and identify Cyanobacteria in selected limestone caves of Bulabog Putian National Park, Dingle, Iloilo, Western Visayas, Philippines.

Material and Methods

Description of sampling sites

Bulabog Putian National Park is a protected wildlife and natural park located in the northern part of Iloilo. This is situated in the municipality of Dingle and San Enrique in Panay Island, Western Visayas, Philippines, with coordinates of 11º0.03'N 122º 37'E (Figure 1), about 37 km away from Iloilo City. Permit to collect rock samples from the caves was acquired for this park is under the management of the Protected Area Management Board (PAMB), Department of Environment and Natural Resources (DENR) Region VI, covering an area of 854.33 hectares along a 40 km trail in the rainforest. The park was known for its unusual geological formations such as stalagmites and stalactites, including Moroboro and Talinab springs, which serve as supplemental water sources. It is the only limestone mountain formation in Iloilo, containing thirty-three caves with different bats and other animals such as tailless scorpion, gecko, and insects. The park is well known for



NAME	COORDINATES	
	NORTHING	EASTING
Lapus Lapus Cave	463709	1219688
Guiso Cave	463557	1219750
Maestranza Cave	463192	1218798
Tuko Cave	463070	1218859
Pasu Cave	463386	1218867
Maanghit Cave	463786	1219966
Lungib cave	462513	1219877

Figure 1. Collection sites of Cyanobacteria in selected caves of Bulabog Putian National Park in Dingle, Iloilo in Panay Island, Western Visayas, Philippines (image: DENR Region 6)

its forest caves with a wide diversity of flora and fauna, and researchers in microbiology and biotechnology have not yet explored the caves.

Samples collection

The collection of samples was done from May 15, 2019, to May 17, 2019. Seven caves were identified and named by the protected area management board (PAMB) of the Department of Environment and Natural Resources (DENR) Region VI was randomly selected as sampling sites. These were accessible among the caves in Bulabog Putian National Park and are good sites for cyanobacterial diversity studies due to their unique features, such as stalagmites, stalactites, lakes, and natural springs. The sampling was made in two distinguish sites in each limestone cave. One site was the physical entrance with sunlight and well-oxygenated. Thus, this was called the light zone; the other site was the farther chambers illuminated only by weak natural daylight or artifi-cial light, known as the dark zone [39]. Samples were collected using a sterile scalpel and placed in sterile plastic tubes from the walls and ceilings of limestone caves with evidence of biological colonization [40]. A small quantity was scraped from the rock substrate and placed in wide-mouthed, 2 to 4 cm diameter screw-cap tubes about 10 to 20 mL capacity, containing a few drops of sterile water to keep the interior moist [41]. Twenty-four samples were collected in each cave, twelve from the light zone and the other twelve from the dark zone. Six subsamples were scraped from the walls about 1 to 3 feet above the cave's floor and the other six were scraped from the ceiling about 4 to 5 feet above the cave's floor under sterile conditions. Environmental variables such as light intensity (lux), temperature (°C), and relative humidity (RH %) were measured for each limestone cave. The samples were stored in a cooler without adding water for 1 hour until further treatment in the laboratory.

Isolation and culture enrichment

The materials scraped from the rock substrat of the cave with few drops of sterile water were transferred to sterile 25 mL test tubes containing 5 ml F medium. After two weeks of incubation at 28°C under a continuous light with illumination at 3000 lux, it was transferred into another sterile 25 mL test tube with 10 mL F medium.

After three weeks of incubation at 28°C, cyanobacterial strains were isolated by the agar plate spreading technique. One hundred microliters of cyanobacterial suspension was placed at the middle of agar-solidified F medium. Using L-shaped glass tubing, the suspension was spread out all

throughout the solid agar F medium. The plates were incubated at 28℃ under continuous white light at 3000 lux.

The streaking method was carried out using the F agar medium [24]. The plates were incubated at 28℃ under a continuous white light with illumination at 3000 lux. The agar plates were checked regularly to monitor the growth of cyanobacterial colonies. Each colony formed was examined under a light compound microscope to confirm the presence of cyanobacterial cells and was picked up and transferred onto new agar plates with the same medium to obtain unicyano-bacterial isolates. The cells were preserved on a 25 mL test tube containing F medium and isolates were maintained under photoautotrophic condi-tions, under continuous illumination at 3000 lux and incubated at 28℃.

Cyanobacterial culture purification

Purification of cyanobacteria was done using 200 mg/mL nystatin, 2.19 mL co-amoxiclav, and UV radiation, which was employed according to the method described by Garcia-Pichel *et al.* (1992) [42]. The isolates grown in the test tubes were exposed to UV rays for 30 minutes. The tube that showed healthy cyanobacterial growth was selected to further propagate the cultures in larger volumes [23]. The cyanobacterial strain was cultured in a 500 mL flask containing 150 mL of F medium without shaking. The incubation temperature was 28 ± 2℃ and it was illuminated at 3000 lux with a white continuous light and regime of 16 hrs light/8 hrs dark. Axenic cyanobacterial strains were maintained in the laboratory until further use.

Morphological identification of cyanobacteria

Diacritical morphological traits used in botanical species descriptions were utilized for morphological identification. For coccoid cyanobacteria, qualitative morphological characters such as the color of culture (plate or tube), color and shape of cells, presence or absence of sheath, motility, arrangement of cells or form, and quantitative morphological characters such as diameter, breadth, and length of cells were analyzed. Morphological characters such as color of culture, size and shape of vegetative cells, heterocysts, akinetes, and hormogonia, length and width of vegetative cells, color of sheath, and presence or absence of heterocysts and akinetes, were taken into analysis for filamentous cyanobacteria. Microphotograph and observations were performed using a light compound microscope equipped with digital camera Touptek wifi 1080P, and were taken in 100× objective with oil immersion. The morphometric measurements of the cyanobacteria microphotographs were carried out using image analysis software ImageJ [40]. Microphotographs were taken before morpho-metric measurements in the exponential growth phase [34], and calibration was done using software ImageJ based on the stage micrometer with 100× magnification (OIO) in the microscope. The taxonomic classification was based on morphological characteristics pre-sented in the standard classical literature [31, 32, 44, 45, 46] and in more recent taxonomic revisions [30, 47, 48, 49, 50].

DNA extraction, PCR amplification, and sequencing

Following the manufacturer's manual, the cyanobacterial DNA was extracted using PurelinkTM Genomic Plant DNA purification kit (Invitrogen/Thermo Fisher). Cyanobacterial cells were pelleted by centrifugation and was grinded using micro pestle before genomic extraction. DNA products were electrophoresed in a 1% agarose gel and checked with ethidium bromide under ultraviolet light. The resultant DNA was stored at -20℃ until further use.

The extracted cyanobacterial DNA was sent to Macrogen Inc., Seoul, South Korea for polymerase chain reaction (PCR) for the 16S *rRNA* gene using cyanobacteria-specific primers CYA359F 5'GGGGAAT (C/T)TTCCGCAATGGG-3' and CYA781R 5'GACTAC (A/T)GGGG-TATCTAATCCC(A/T)TT-3' [48] and for sequencing. The resulting sequences were used to confirm the identification of the cyanobacteria and for phylogenetic analysis. The consensus forward and reverse 16S rRNA gene sequences obtained in the study were assembled using DNABaser software. The resulting contiguous se-quence was compared with sequence information available in the National Center for Biotechnology Information (NCBI) database using BLASTn. (http://www.ncbi.nlm.nih.gov/BLAST).

Results and Discussions

Cyanobacteria are ubiquitous, photosynthetic, and diverse groups of prokaryotic organisms. They adapt and survive to different environmental conditions including subterranean ecosystems. Caves have environments that differ significantly from those found on the surface and are considered as extreme environments due to its scarcity in nutrients and oxygen level compared to that at the surface. Despite of its habitat conditions, caves contained diverse taxa of cyanobacteria [1].

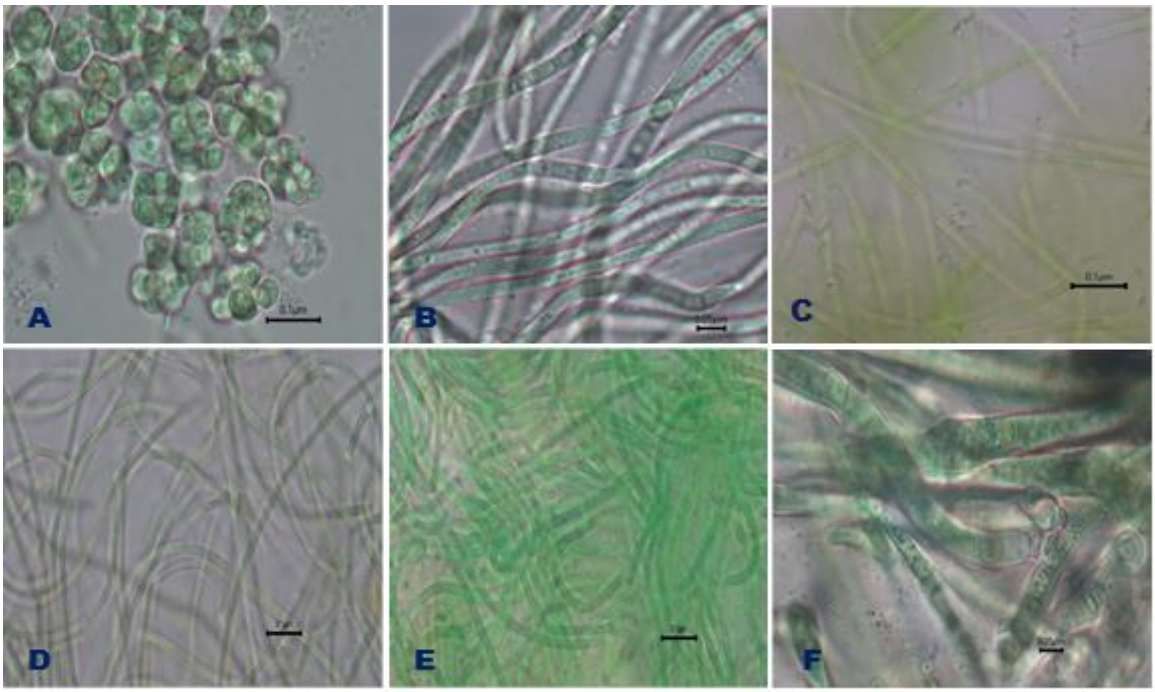


Figure 2. Microphotographs of Cyanobacterial Strains Isolated from Bulabog Putian National Park. A) *Chroococcidiopsis cubana* B) *Leptolyngbya sp.* C) *Onodrimia javanensis* D) *Albertania skiophila* E) *Chroakolemma pellucida* F) *Calothrix sp.*

Cyanobacteria can be grouped morphologically into unicellular, spherical shapes that form singly, in pairs or colony, and fila-mentous that are unbranched, false-branched or branched form [44, 45]. Based on the above mentioned morphological characterization and according to Geitler 1932 [46]; Desikachary 1959 [31]; Rippka et al. 1979 [32]; Castenholz 1989 [47]; Komarek and Anagnostids 1989 [46], and in more recent taxonomic revisions [30, 49 – 55] the present study isolated and morphologically identified six strains of cyanobacteria belonging to genera *Chroococcidiopsis, Leptolyngbya, Albertania, Chroakolemma, Onodrimia, and Calothrix, a*nd confirmed their identification based on 16S rRNA gene sequences.

The morphometric characteristics of the cyanobacteria strains are given in Table 1. Based on the available literature, CyaC9 was identified as a member of the genus *Chroococcidiopsis* classified as coccoid or unicellular cyanobacteria. This strain form colony, 0.08 µm width and 0.10 µm length with rounded cube cells having 0.03 µm diameter, and dull green to bright green in color. The cells or colony are surrounded by thin, firm, colorless, and layered sheaths that allow them to stick to other filamentous cyanobacteria (Figure 2) *Chroococcidiopsis* usually formed an irregular colony due to impulsive multiple fission, having a different mode of division of enlarged cells. Isolates CyaC38, CyaC24, CyaC19, and CyaC16 encompass strains that are filamentous cyanobac-teria without heterocyst, belonging to genera *Leptolyngbya*, *Albertania*, *Chroakolemma*, *Onodrimia* respectively. The trichomes are somewhat similar among the strains with width of 0.04 µm and slightly attenuated at the ends (Figure 2). The vegetative cells are shorter than wide (Table 1), blue-green to olive green or bright green in color, and end cells are rounded (Figure 2). The filaments are slightly constricted at the cross walls and are distinct.

CyaC5 was a member of the genus *Calothrix*, the only filamentous cyanobacteria with heterocyst isolated from selected Bulabog Putian National Park and its characteristics were summarized in Table 1. This isolate has both heterocyst which is intercalary and apical, 0.05 µm width and 0.02 µm length, oval and pale green in color, and akinete, 0.04 µm width and 0.02 µm length. The filaments are unbranched, short and slightly wide ned towards the end (Figure 2). The isolated cyanobacteria from selected caves of Bulabog Putian National Park were hard to distinguish based on its morphology because cyanobacteria can change their morphology depending on the kind of environment [56]. This is the morphological or pheno-

Table 1. Morphological characteristics of cyanobacteria isolated from selected caves of Bulabog Putian National Park, Dingle, Iloilo, Western Visayas, Philippines. Measurements are given as mean standard deviation for n= 100 for coccoid cyanobacteria and n=30 for filamentous cyanobacteria

Isolate Name	Strain	Dimensions			Cell Descriptions				Baeocytes	Heterocyst	Akinetes
		Diameter	Breadth	Length	Shape	Color	Arrangement	Sheath			
1. CyaC9	*Chroococcidiopsis cubana*	Cell: 0.03± 0.01	Colony: 0.08±0.01	Colony: 0.10±0.02	Rounded cubes to spherical	Dull green to bright green	Colony	+	+		
2. CyaC38	*Leptolyngbya sp.*	TR: 0.02± 0.006	VC: 0.02±0.002	VC: 0.01±0.002	VC: elongate	VC: Blue green					
3. CyaC24	*Albertania skiophila*	TR: 0.01± 0.002	VC: 0.01±0.003	VC: 0.02±0.003	VC: cylindrical	VC: Pale green					
4. CyaC19	*Chroakolemma pellucida*	TR: 0.01± 0.002	VC: 0.01±0.003	VC: 0.02±0.003	VC: cylindrical	VC: Bright green					
5. CyaC16	*Onodrimia javanensis*		VC: 0.01±0.003	VC: 0.02±0.003	VC: cylindrical	VC: Yellow green					
6. CyaC5	*Calothrix sp.*		VC: 0.06±0.009 He: 0.05±0.014 Ak: 0.04±0.008	VC: 0.03±0.008 He: 0.03±0.011 Ak: 0.02±0.007	VC: Elongate He: Oval Ak: rounded	VC: Pale green				+	+

TR (trichome), **VC** (vegetative cell), **He** (Heterocyst), **Ak** (Akinete)

typic plasticity that makes the cyanobacterial isolates difficult to identify morphologically. The identification of cyanobacteria isolated from selected caves of Bulabog Putian National Park using molecular marker such as 16S rRNA gene was needed to confirm the identification based on morphological characters. For several years 16S rRNA sequence gene has been the pillar in the taxonomy of prokaryotes, and can measure relationships among all cyanobacterial species. The six isolated cyanobacterial strains were sub-jected to molecular analysis based on 16S rRNA gene sequence. The resulting sequences were ana-lyzed using BLAST search from NCBI website. According to DNA sequence analysis, CyaC38 was found to be similar to *Leptolyngbya sp.* (94.62% similarity), strain CyaC5 was closely related to *Calothrix sp.* (97.07% similarity), CyaC9 was *Chroococcidiopsis cubana* (99.21% similarity), CyaC16 was *Onodrimia javanensis* (96.27% similarity), CyaC19 was *Chroakolemma pellucida* (94.74% similarity), and CyaC24 was *Albertania skiophila* (98.40% similarity).

The BLASTn result analysis based on 16S

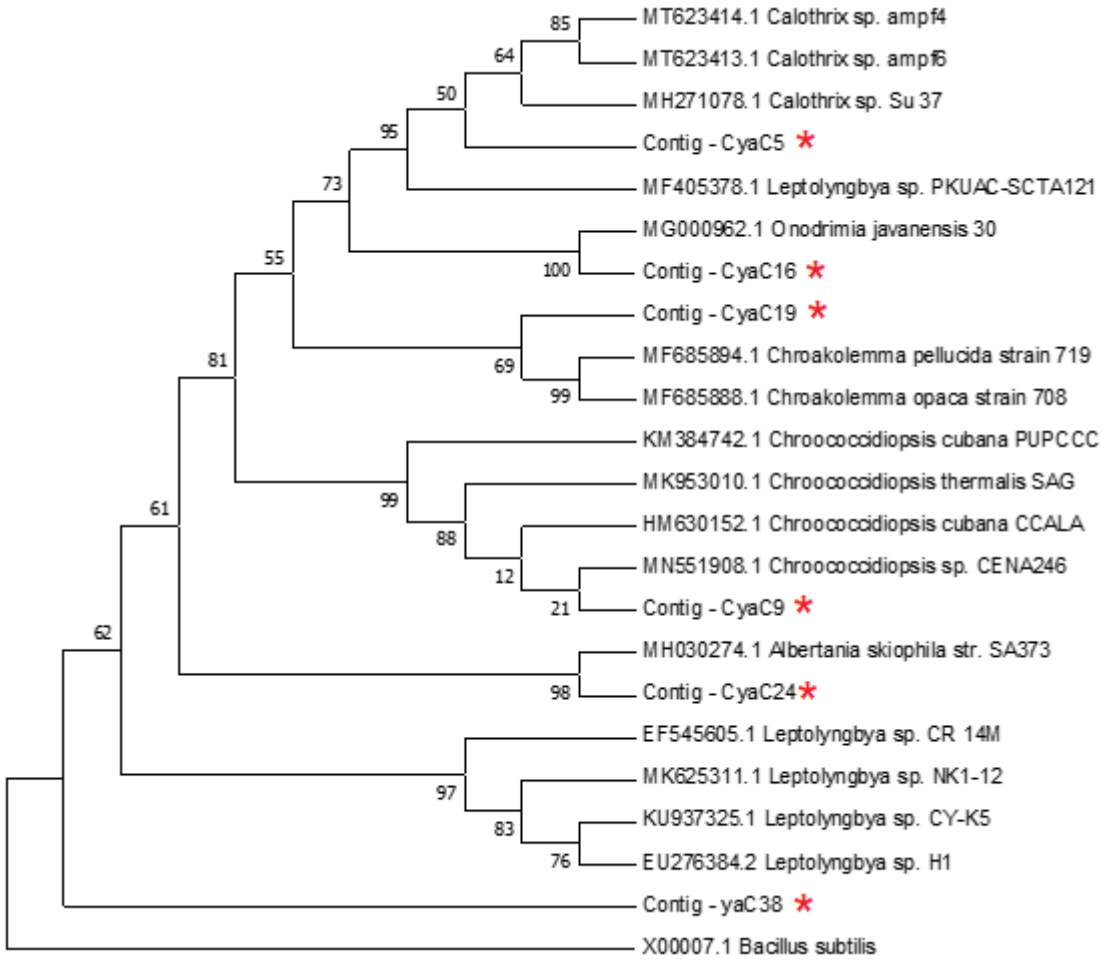


Figure 3. Phylogenetic position of the six cyanobacterial strains isolated from selected cave of Bulabog Putian National Park based on 16S rRNA gene sequences data. Phylogenetic tree was constructed using Maximum Likelihood method, 1000 bootstrap replications with Bacillus subtilis as outgroup. Cyanobacterial strains of the present study is mark by a red star (*)

Table 2. BLAST search results of cyanobacteria strains used in this study based on 16S rRNA gene sequence analysis

Strain	Sequence Similarities	Accession No.	Identity (% Homology)
1. CyaC38	*Leptolyngbya sp*. HI	EU276384	94.62%
2. CyaC5	*Calothrix sp.ampf4*	MT623414	97.07%
3. CyaC9	*Chroococcidiopsis cubana* SAG 39.79	MK484708	99.21%
4. CyaC16	*Onodrimia javanensis 30*	MG000962	96.27%
5. CyaC19	*Chroakolemma pellucida strain* 719	MF685887	94.18%
6. CyaC24	*Albertania skiophila str. SA 373*	MH030274	98.40%

rRNA supports the morphological identification of the cyanobacteria isolates from Bulabog Putian National Park. Nevertheless, three cyanobacteria strains *Leptolyngbya* sp., *Chroakolemma pellucida*, and *Onodrimia javanensis* have the low percentage similarity of their 16S rRNA sequence data (> 97%) to its closest related taxon, showing that they were genetically different from its closest related taxon, thus can be a new strain of the same species. However, BLASTn result having less than 97% similarity to existing known species in the NCBI database would be only indicative of a new strain to that species, not prospective new species of itself [57]. To determine an isolate as new taxa, several analyses should be taken into account other than morphology and 16S rRNA

gene analysis. The low percentage similarity of its 16S rRNA is congruent to the results of the previous studies on cyanobacteria identification based on 16S rRNA gene with percentage similarity ranging between 92.5% and 99.7% [38, 58, 59]. Based on morpho-logical and molecular analysis, the cyanobac-teria isolated from selected caves of Bulabog Putian National Park belong to Order Chroococcidiopsales (*Chroococcidiopsis*), Nostocales (*Calothrix*), and Synechococcales (*Leptolyngbya*, *Albertania*, *Onodrimia*, *Chroakolemma)*. Figure 3 showed the phylogenetic position of the six strains among the closely related cyanobacteria. The phylogenetic tree revealed and confirmed that the cyanobacterial strains from selected caves of Bulabog Putian National Park, Dingle, Iloilo, Western Visayas, Philippines were distinct to their closest related taxon.

Conclusion

This study isolated and identified six cyanobacterial strains attained from selected caves of Bulabog Putian National Park, Dingle, Iloilo, Western Visayas, Philippines. The strains were successfully identified based on morphology and 16S rRNA gene sequence data analysis. The closest related taxa to the six strains were *Leptolyngbya* sp., *Calothrix* sp., *Chroococcidiopsis cubana*, *Onodrimia javanensis*, *Chroakolemma pellucida*, and *Albertania skiophila*, as determined by 16S rRNA gene sequence analysis using the BLAST search program.

Six strains of cyanobacteria have been identified belonging to the Orders Chroococcidiopsales (*Chroococcidiopsis*), Nostocales (*Calothrix*), and Synechococcales (*Leptolyngbya*, *Albertania*, *Onodrimia*, *Chroakolemma*) which normally present in extreme environments such as the caves in Bulabog Putian National Park. Addi-tional research, such as a polyphasic approach and other taxonomic characterization, is required to fully describe these novel cyanobacteria taxa.

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