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The First Report on the Molecular Identification of Fresh Water Microalgae from Waterfalls of Paracelis, Mountain Province, Philippines

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Abstract

Microalgae belong to diverse groups of complex unicellular photosynthetic microorganisms typically found in the freshwater and marine environment. Towards the isolation and an attempt to report the first molecular identification of freshwater microalgae from the waterfalls of Paracelis, Mountain Province, Philippines, two microalgae were successfully isolated independently, purified, and cultivated for DNA extraction, polymerase chain reaction, and sequencing using gene 16s rRNA and rbcL markers. Based on the 16s rRNA sequence, one of the isolates has a 92.05% sequence similarity to *Oscillatoriales cyanobacterium* (KJ611413). On the other hand, one isolate has 98.27% sequence similarity with *Chlorella pyrenoidosa* (AB240145) using the rbcL. The morphological features of two microalgae were described and the phylogenetic trees were constructed using the gene marker.

Keywords: 16s rRNA; *Chlorella pyrenoidosa;* microalgae; *Oscillatoriales cyanobacterium;* phylogeny; rbcL.

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1. Introduction

Microalgae are complex unicellular and photosynthetic microorganisms. Representing a large variety of species and can flourish under a wide range of environmental conditions, including freshwater, brackish water, seawater, and even wastewater [1]. They played a significant role in the ecological balance between several types of macrophytes and their respective aquatic environment [2]. Microalgae are incredibly diverse, and their species are estimated to about 200,000–800,000. Microalgae also called phytoplankton by biologists, are very small plant-like organisms between 1-50 µm in diameter without roots or leaves [3] or up to 100 µm or higher [4, 5]. Considered as one of the most important main primary producers, algae are responsible for about half of the oxygen gas (O_2) production, and most of the dimethyl sulfide released into the atmosphere [6, 7].The utilization of micro-algae for the production of feed, food, food additives, pharmaceuticals, and fine chemicals is growing in importance. The cultivation of algae as a research tool is expanding rapidly as modern research becomes interested in such diverse fields as physiology, molecular biology, waste disposal, and medicine [8]. Moreover, the microalgae have been used in wastewater bio-treatments [9], as food for humans and animals [10], as feed-in aquacultures [11, 12], for the production of pigments [13] and in agriculture [14]. In the Philippines, most of the studies on the identification of microalgae has been done based on their morphology or classical approach. Classic morphological taxonomic classification of microalga based upon the description of cell and colony structures can be subjective leading to some errors like the double classification of the same organism. Isolation of important microalgae from nature and its identification using only its morphological characteristics remains a big challenge among researchers. The identification of microalgae requires a microscope with high magnification, identification of some species even impossible [15]. A morphological species notion is often used for eukaryotic microorganisms, but molecular data have also indicated patterns of local adaptation and endemism among some algae where morphological differences are difficult to determine [16]. Noteworthy, morphology and DNA-based approach molecular data were proven to be the most effective approach for species discrimination producing consistent species identification [15]. However, microalgae were found to be very diverse even in their molecular makeup. Identification of useful markers to reveal its identity is one of the key challenges for its identification. Therefore, the use of different universal gene markers has been an option and suggested use for the identification of several taxa of algae [17, 18, 19].The analysis of DNA sequences has been widely used for the identification of both blue-green using the 16S ribosomal ribonucleic acid (rRNA) genes and eukaryotic algae using the 18S rRNA also known as rDNA and chloroplast DNA (cpDNA) [20]. However, even 18S rRNA has been reported to be an effective method for identification with high accuracy [21], sometimes amplification of the 18S rDNA gene gives variable size bands giving inaccurate results [22]. On the other hand, the ribose and bisphosphate carboxylase (rbcL) region sequence of the chloroplast gene among photosynthetic organisms was also found useful in phylogeny study [23]. In this study, two gene markers (rbcL and 16S rRNA) were used to identify the freshwater microalgae collected from Paracelis, Mountain Province, Philippines. The identity of the microalgae was initially identified using its morphological features and structure.

2. Materials and methods

2.1. Sample collection

Water samples were collected at multiple sites in each waterfall and from the top and bottom of the water at each location to capture the dominant microalgae species in each site. Samples of about 100 ml each were collected in 500 ml sterile plastic bottles. The air and water temperature were recorded during the collection. The water pH was also determined. Samples were maintained in cold conditions and brought to the laboratory.

2.2. Isolation and cultivation of the microalgae

To obtain monoclonal microalgal species, the samples were diluted using the serial dilution technique. The 100 ml of the microalgae were obtained from the original and were serially diluted up to 10-6 dilutions. From every dilution, 100 µl samples were transferred on 500 ml media containing 0.1 g L(-1) urea and 1.0 g L(-1) of NPK (Nitrogen: Phosphorous: Potassium) fertilizers [24] and were incubated at 25°C with approximately 12-12 h light-dark periods. The culture was continuously aerated with air pumps with air stones and was added with the media after 7 days. The microalgae in each dilution bottle were then monitored and examined daily using the biological microscope (Solaris, BEL Engineering) with a built-in camera with a 1000X magnification to observe mono species culture for isolation and cultivation. One hundred ml of monoclonal isolates were then transferred to another bottle for cultivation and incubated as mentioned above. Cultivation was done for 6 weeks obtaining monoclonal suspension by centrifugation.

2.3. Morphological characterization

Morphological characterization of the monoclonal isolates was done microscopically using a light microscope as previously described. Cell shape, size, and color features were observed for characterization [20].

2.4. Genomic DNA Extraction

DNA extraction was done after 4 weeks of monoclonal cultivation. DNA extraction was done following the protocol of Murray and Thompson [25] using the cetyltrimethylammonium bromide (CTAB) method with minor modification. Using the four 50 ml tubes with 40 ml of monoclonal isolates were centrifuged at 6000 rpm for 30 min. Pelleted monoclonal isolates were freeze-dried with liquid nitrogen and powdered with mortar and pestle. One-hundred milligrams of samples were transferred in a 1.5 ml tube and added with 750 µl of pre-warmed (65°C) 2x CTAB and 50 µl 20% sodium dodecyl sulfate (SDS). The mixture was mixed using a vortex and incubated in a digital water bath for 45 min at 70°C and mixed with vortex every 5 min. The 500 µl of chloroform was added and the sample was thoroughly mixed and centrifuged at 10,000 rpm for 10 min. The upper phase was transferred into a new 1.5 mL tube and 400 µL of ice-cold isopropanol was added and incubated overnight. After incubation, the tubes were spun with a centrifuge at 10,000 rpm for 10 min and added with ice-cold isopropanol and mixed gently and was spun again at 10,000 rpm for 3 min and 70% ethanol was added to wash the DNA. Finally, alcohol was drain dried by inverting on a clean paper towel to get rid of the excess liquid. Afterwards, the pellet was dissolved in 20 µl of TE buffer.

2.5. Polymerase Chain Reaction

The conserved region of 16S rRNA and rbcL regions were amplified using Thermal Cycler Machine (Applied

Biosystems 2720) with thermal cycling conditions of initial denaturation at 95°C for 3 min; 35 cycles of 95°C for 30 sec; 55°C for 30 sec; and 72°C for 1 min; and final extension of 1 min at 72°C. The primers used in this study were shown in Table 1. The 50 ml reactions were carried out in 0.2 ml PCR tubes containing 16.9 ml of distilled water, 2.5 ml of 10X Buffer A, 1 ml of dNTP's, 10 mM each of forward and reverse primer, 1ml of DNA template, and 0.1 ml of KAPA Taq DNA polymerase (Sigma-Aldrich). PCR products were run for 30 minutes at 100V on 1% agarose gel containing 1 ml of gel red. The PCR product was analyzed under the gel photo documentation system (Labnet GDS-1302 Enduro Imaging System). The PCR product was sent to First Base Laboratory in Malaysia for sequencing. For identification, sequence analysis using the Basic Local Alignment Search Tool (BLAST) was performed to determine the maximum identity score of the target sequence with the existing sequences in the NCBI database [26].

Table 1: The sequences of 16S rRNA and rbcL primers used in the amplification of monoclonal isolates.

Primer name	Sequence	Reference
16S rRNA	Forward (5'- CCAGCAGCCGCGGTAATACG-3') Reverse (5'-ATCGG (C/ T) TACCTTGTTACGACTTC-3'	[27]
rbcL	Forward (5'-ATGTCACCACAAACAGAGACTAAAGC-3') Reverse (5'-TCGCATGTACCTGCAGTAGC-3')	[28]

2.6. Phylogenetic Analysis

The phylogenetic analysis was performed with the related sequences obtained from the GenBank. The phylogenetic tree was constructed using the default parameter of MEGA X [29].

3. Results

3.1. Collection site and Morphological characterization of the isolated microalgae

Two freshwater microalgae were isolated from the two waterfalls from Paracelis, Mountain Province, the Philippines (Figure 1) using the serial dilution method. The environmental parameters such as air temperature, water temperature, and water pH were collected. The coordinates of the collection sites were presented in Table 2.

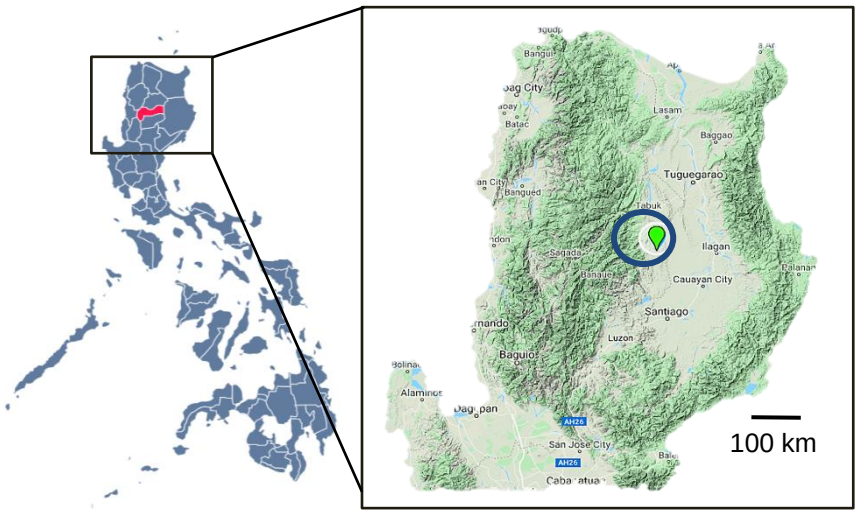

Figure 1: Map showing the collection site in Paracelis, Mountain Province.

Table 2: Environmental parameters and coordinates of the collection sites.

Sites	Air Temperature (Ave.)	Water Temperature (Ave.)	Water pH (Ave.)	Coordinates
Canabo Falls	23.9°C	21.5°C	7.42	17.163951,121.480663
Malinsawang Falls	27.1°C	25.4°C	7.85	17.110915,121.531282

The sample CS2 was collected from Canabo Falls, while the sample MF1 was from Malinsawang Falls. The CS2 isolate was unicellular and was spherical with 2-7 µm in diameter and is without flagella, somewhat forming small ovoid colonies, the chloroplast parietal with a single pyrenoid and forming free-floating colonies of 4 or more cells and some are solitary (Figure 2A). On the other hand, the sample MF1 has green, un-branched long single trichomes filamentous chain of cells that are straight to wavy and threadlike shape. It has a coin-like cell about 2-10 µm in length. The apical cell was rounded, conical in shape and the trichome end was shortly attenuated (Figure 2B).

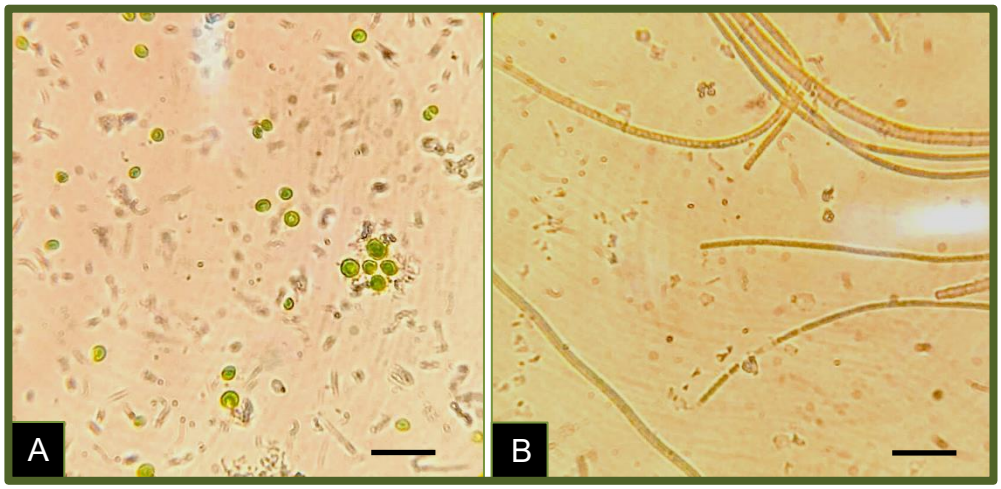

Figure 2: Morphology of the freshwater microalgae. A. CS2 isolated from Canabo Falls. B. MF1 isolated from Malinsawang Falls. Scale bars = 10 µm.

3.2. Molecular identification and phylogenetic analysis of isolated microalgae

The isolates were subjected to molecular identification using two gene markers, the 16S rRNA and rbcL gene marker. The number of microalgae subjected for molecular identification were depend on the number of species that have been isolated and purified after collection using serial dilution. Another limitation of this study was the gene markers used for molecular identification. However, we can be able to successfully identify the isolated microalgae using the available gene marker. The CS2 sample was successfully amplified using the rbcL while it failed to amplified the MF1. However, in an attempt to amplify the rbcL region of both isolates, the 16S rRNA primer successfully amplified the region from the MF1 but failed to amplify the region from CS2. BLAST analysis showed that the 16S rRNA region of CS2 has 98.50% similarity to *Chlorella pyrenoidosa* with GenBank accession no. AB240145. On the other hand, the rbcL region of MF1 has 92.05% similarity to *Oscillatoriales cyanobacterium* with GenBank accession number KJ611406. The phylogenetic tree was constructed using the Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model [30]. It was found out that the CS2 belonged to the order *Chlorellales*, family *Clorellaceae*, and genus *Chlorella* (Figure 3). The fourteen strains were separated by two clades. The CS2 was found to closely related with *Chlorella pyrenoidosa* (AB240145) the other 9 strains in the group were *Jaagichlorella luteoviridis* (MK295209), *Pseudochlorella pringsheimii* (MK295216), *Chlorella vulgaris* (MK295221), *Chlorella* sp. (KF975597), *Auxenochlorella protothecoides* (MK95295211), *Auxenochlorella pyrenoidosa* (MK842153), *Heterochlorella luteoviridis* (KM514870), and *Chlorella sorokiniana* (KT777992). The other three strains grouped in another clade were *Dictyosphaerium ehrenbergianum* (KM514898), *Micractinium singularis* (MN894287).

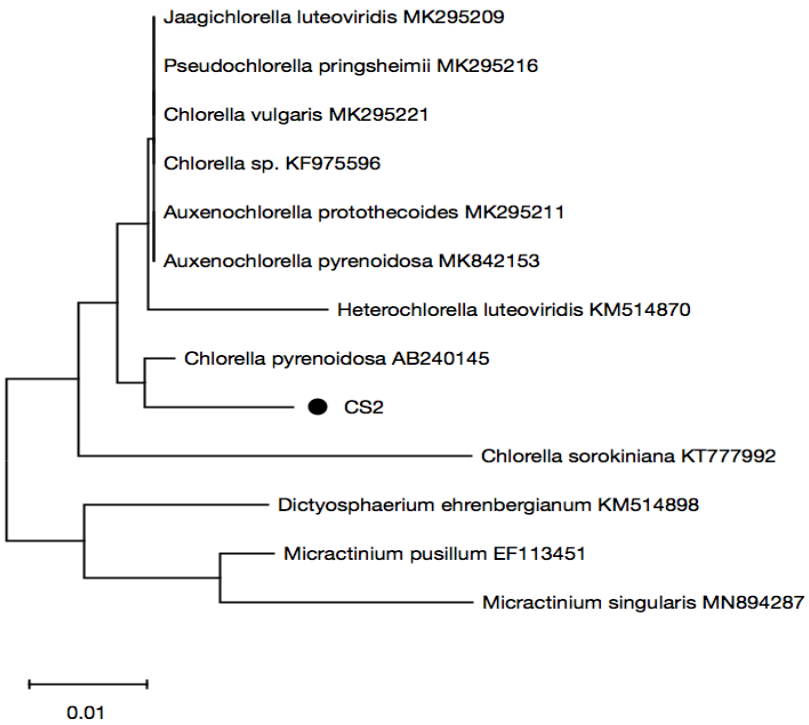

Figure 3: Phylogenetic tree showing the relationship of the CS2 (with black circle) to 12 strains of microalgae based on their rbcL sequences. The DNA sequence accession numbers are shown after the name of the strain. The scale bar corresponds to 10 base substitutions per 100 nucleotide positions.

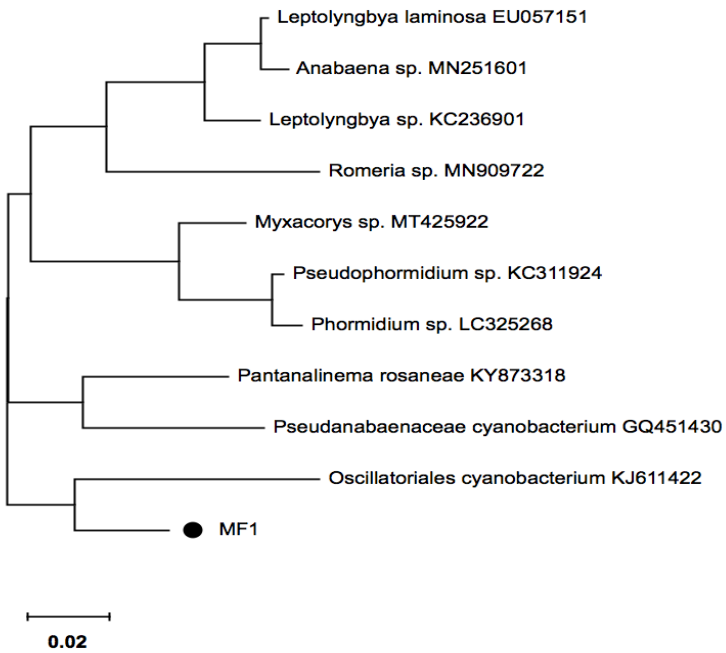

Figure 4: Phylogenetic tree showing the relationship of the MF1 (with black circle) to 10 strains of microalgae based on their 16S rRNA sequences. The DNA sequence accession numbers are shown after the name of the strain. The scale bar corresponds to 20 base substitutions per 100 nucleotide positions.

The rbcL region revealed that the MF1 belonged to the order *Chroococcales*, family *Cyanobacteriaceae*, genus *Oscillatoriales* (Figure 4). The MF1 was closely related to *Oscillatoriales cyanobacterium* (KJ611422). The MF1 and *Oscillatoriales cyanobacterium* were with the same group in a clade with other strains including *Pseudanabaenaceae cyanobacterium* (GQ451430) and *Pantanalinema rosaneae* (KY873318). The other group was in another clade consists of 7 strains including *Leptolyngbya laminosa* (UE057151), *Anabaena* sp. (MN251601), *Leptolyngbya* sp. (KC236901), *Romeria* sp. (MN909722), *Myxacorys* sp. (MT425922), *Pseudophormidium* sp. (KC311924), and *Phormidium* sp. (LC325268).

4. Discussion

Microalgae are very common with hundreds of thousand exist and are distributed everywhere: in salt, brackish or freshwater, in tropical to cold regions and sometimes as symbionts with other organisms, [31, 32]. Microalgae are not included in the higher plants because they do not have the characteristics and functions of such plants, they have no roots, stems and leaves [33]. In many countries, the food industries consume a wide range of algae, which are well known to have high contents of fibers, minerals, vitamins and different antioxidants [34] High protein content of various microalgal species is one of the main reasons to consider them as an unconventional source of protein. In addition, the amino acid pattern of almost all algae compares favorably with that of other food proteins. As the cells are capable of synthesizing all amino acids, they can provide the essential ones to humans and animal [35]. With the vast potential, isolation, growth requirements and proper identification of this species must be done. An important consideration for the identification of microalgae was the isolation and separation of the identical cell to obtain monoclonal strain. Isolation is the key activity before the tasks of identification. After isolation, the molecular description of freshwater microalgae for identification using a microscope has been widely used. However, the morphological description alone is insufficient because of the diverse features of microalgae, since microalgae are diverse it was expected that the sample collected in a specific environment consists of different species with various morphological features. Combined with morphological characteristics, DNA barcoding is now being used and found to be effective for fast and accurate species identification [36, 37, 38, 39, 40]. DNA barcoding in microalgae involves ideal markers and several approaches since microalgae are diverse not only in their morphological but also in their genetic make-up. Thus, an appropriate and promising universal marker must be used on specific microalgae to make molecular identification effective. Every universal primer has its discriminatory power and there has been no ideal marker gene that served as an ideal barcode for all life forms either always invariant within species but different among species [15]. Previous undertakings on the isolation and identification of five groups of microalgae in the Philippines is based on morphological information, particularly the abundance of microalgae in Lake Mohicap in San Pablo Laguna, Philippines[41]. The Philippines, being a tropical country, is naturally rich in algal flora. Recently, Tayaban and his colleagues [42] used the 16S rRNA gene to detect and identify microalgae causing algal bloom in Central Luzon, Philippines such as *Oscillatoria agardhii, O. princeps, Microcystis aeruginosa, M. wesenbergii,* and *Anabaena circinalis.* Several corticolous microalgae and cyanobacteria from Mt. Makiling Forest Reserve found in Southern Luzon Philippines have been identified and characterized using morphological characterization [43]. A total of 21 algal taxa were found on submerged parts of water hyacinth in Laguna de Bay and were identified using its morphology mainly using the filaments, the

size, and shape of vegetative cells as well as specialized cells [43]. Baloloy and his colleagues [44] collected and identified a total of 29 species of phytoplankton belonging to five major taxonomic groups: the diatoms, green algae, cyanobacteria, eustigmatophytes and dinoflagellates around Lake Buhi in Camarines Sur Philippines using the classical or morphological approach. In this study, the *C. pyrenoidosa* has been morphologically described based on its microscopic observation and was confirmed to the description provided elsewhere [20], and verified properly using its rbcL gene. The isolate was most similar to the genus *Chlorella* in terms of shape, size, and color. The colonies disintegrated into solitary cells without numerous bristles, making them look similar to *Chlorella* [45]. The *C. pyrenoidosa* is unicellular freshwater green algae that have rich in macronutrients and has many healthful benefits [46]. The *C. pyrenoidosa* are most commonly used in research. It has been found out nutritional supplementation derived from *C. pyrenoidosa* helps relieve the symptoms of fibromyalgia [47], reduces the risk of hypertension [48], and has an immunoenhancing effect [49]. It has been receiving much attention for its increasing scientific and commercial interest because it has a rich oil content. Besides, it proves to be beneficial for the bioremediation of contaminated wastewater. On the other hand, the morphological description of *Oscillatoriales cyanobacterium* was conformed to the description as provided elsewhere [20, 50] and also verified with identity using the 16S rRNA region. The species of *Oscillatoriales* were commonly found in freshwater and some are in the marine environment. It belongs to different species of cyanobacteria and few are associated with planktonic cyanobacteria blooms [51]. On the other hand, it was found out that 58% of marine cyanobacterial sources under the *Oscillatoriales* contain secondary metabolites [52]. Cyanobacteria are among the most widespread and morphologically distinct organism, the phylogenetic relationship belong to this group was also relatively poorly understood [53]. There have been research undertakings that species belonging to this group should be reappraised because of its vague morphological characteristics and no enough evidence on its phylogenetic character. Recently, there are 32 identified strains based on core morphological traits and 16S rRNA from the GenBank database and suggested that the taxa should be replaced with monophyletic assemblages [54]. The *O. cyanobacterium* is a new species that has not been studied and explored for its medicinal, industrial, agricultural, and environmental potential due to the identity biases because of its questionable taxonomic classification. The classification and identify remains problematic and the use of different taxonomic resolution to clarify its status and to resolve its position in the taxonomic key. This study provides the first report on the isolation, description, and identity of the two microalgae collected independently from the two waterfalls located at Paracelis, Mountain Province, Philippines by utilizing two gene markers.

5. Conclusion

Two freshwater microalgae were collected, isolated and molecularly identified from two independent waterfalls in Paracelis, Mountain Province, Philippines. Specifically, one of the isolates was collected from Canabo Falls and was successfully molecularly identified as *C. pyrenoidosa* using rbcL gene marker. On the other hand, isolates from Malinsawang Falls were successfully molecularly identified as *O. cyanobacterium* using the 16s rRNA gene marker.

6. Recommendation

The *C. pyrenoidosa* was a very important microalga because of its potential benefits in terms of health and industrial application. The *O. cyanobacterium* on the other hand is a new species that has not been studied and explored for its medicinal, industrial, agricultural, and environmental potential. So far, this is the first report of microalgae collected from the Mountain Province, Philippines and identified using a molecular approach. Future works on the validation supported with genetic background and studies on the variability and ecology of microalgae in nature must be done.

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