DNA barcoding approach to characterize microalgae isolated from freshwater systems in Ecuador

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ABSTRACT
The use of suitable DNA barcodes and the generation of databases with reference sequences have been considered a promissory approach for the identification of Chlorophyta and Cyanophyta microalgae. In this study, we carried out a molecular characterization and identification of strains isolated from freshwater systems in Ecuador using a dual barcode method. The target sequences for Chlorophyta were 18S rDNA and rbcL genes, and 16S rDNA and 16S–23S rDNA intergenic spacer (ITS) for Cyanophyta. We reported these DNA barcodes for 20 different Molecular Operational Taxonomic Units (MOTUs) for Chlorophyta and 10 for Cyanophyta. Our results show that the 18S V4 hypervariable region (300 bp) is sufficient for differentiating between isolates, but rbcL is a determinant for genus identification in Scenedesmaceae and Chlorellaceae strains. In Cyanophyta, both barcodes enabled the genus-level assignment of 9 out of 10 MOTUs. These results highlight the necessity of a second barcode additional to small ribosomal subunit sequences to improve molecular identification. Furthermore, the present study significantly contributes to the body of Ecuadorian barcode sequences of microalgae that are currently documented, making them available for future comparative diversity studies.

Introduction
Chlorophyta microalgae, also known as green microalgae, are eukaryotic microorganisms that comprise a diverse lineage, with approximately 8,000 described species [1]. Cyanophyta microalgae or Cyanobacteria are prokaryotic microorganisms also known as blue-green algae. However, many Cyanobacteria are not blue-green, but purple, brownish, or just green [2,3]. Chlorophytes and Cyanobacteria are important primary producers in marine, freshwater, and terrestrial ecosystems [4]. Both microorganisms have been used as biological indicators for monitoring and protecting aquatic environments [5], and as platforms for the production of a wide variety of bioproducts ranging from biofuels – such as hydrogen, alcohols, and isoprenoids – to high-value bioactive and recombinant proteins [6] and dietary supplementation [7]. They are also regarded as attractive laboratory models for the genetic studies of fundamental processes [8,9]. There are even two genera of Chlorophyta, Chlorella and Prototheca, which are able to cause disease in humans and other mammals through active invasion and spread within host tissues [10].

Traditionally, Chlorophyta microalgae have been classified based on morphological features observed under the microscope, including shape, chloroplast and pyrenoid position, and the presence of flagella [11–13]. Regarding Cyanobacteria, the morphological traits considered for taxonomic classifications are cell size, trichome width, polarity, shape and arrangement, pigmentation, and the presence of characteristics such as gas vacuoles and a sheath [14]. The subjective interpretation of these features and the fact that some strains may lose some important features – for example, gas vesicles or the form of the colony during long-term laboratory cultivation [15,16] – may lead to misidentification. Furthermore, this approach requires high-resolution equipment and specialist taxonomists [17], particularly when dealing with taxa displaying phenotypic plasticity, species where there are few or difficult to observe structural characteristics, and cryptic species [18]. To overcome these difficulties, both the use of DNA barcodes and the generation of databases with reference sequences have been considered a promissory approach for microalgae identification [17,19–22]. The use of DNA barcodes for biological identification, introduced by Hebert et al. [23,24], is based on short, easily amplified regions of DNA that vary greatly among species, but have a low intra-specific variability [25,26]. The most common quantitative approach in DNA taxonomy is based on a predetermined, fixed
genetic divergence threshold of sequences to identify operational taxonomic units as equivalent to species, named Molecular Operational Taxonomic Units (MOTUs) [27]. Currently, there is no consensus to define a universal threshold for microalgae taxa [28,29]. Moreover, the DNA barcoding of microalgae is limited by the lack of universally applied markers among research groups, and it seems more effective to employ multiple genes for barcoding. Many authors have highlighted that a single barcode marker does not meet the needs of the biodiversity community given the complexity and heterogeneity of microalgae [30,31]. Promising targets for potential Chlorophyll DNA barcodes have included chloroplast (rbcL, tufA, and Cp23S), mitochondrial (COI), and nuclear genes (18S rDNA, nuITS1, and nuITS2) [18,31–33].

The availability of sequence databases, for instance the International Nucleotide Sequence Database Collaboration and Barcode of Life Data System (BOLD), is crucial for allowing DNA barcoding to be useful for comparisons of species diversity and identification. A large number of the small subunit rDNA sequences (18S and 16S rDNA) have been deposited in the public DDBJ/EMBL/GenBank databases. Therefore, these regions are good candidates for identifying species and revealing species diversity in ecological systems [34,35,36]. The V4 18S rDNA region is considered suitable for molecular analysis due to its greater size and variability of inversions, insertions, and deletions [36,37]. Another promising candidate is the rbcL marker; despite the lack of a universal primer pair for successful rbcL amplification, it is reportedly sufficiently variable to be able to distinguish among most chlorophyte species [18,38,39]. In fact, there are currently c. 9,000 rbcL sequences from chlorophyte species deposited in the DDBJ/EMBL/GenBank database. To assess the genetic diversity of Cyanobacteria, the 16S rDNA sequence has traditionally been used [40,41]. The 16S-23S rDNA Internal Transcribed Spacer (ITS) is also broadly employed to genetically characterize Cyanobacterial strains [42–46]. Furthermore, the secondary structure of the RNA transcribed from this sequence is taken into account for the elaboration of phylogenetic trees [47].

Ecuadorian continental waters are believed to comprise a biodiversity reservoir of enormous global significance [48]. Nevertheless, studies focused on microalgae from Ecuadorian freshwater systems are scarce and taxonomy studies only take into account its morphological features [11,49].

This study investigated the molecular variability and phylogeny of several Chlorophyta and Cyanophyta microalgae isolated from freshwaters in Ecuador. Dual DNA barcoding was used for molecular identification using 18S rDNA and plastid rbcL markers for chlorophytes, and 16S rDNA and ITS for Cyanobacteria. The present study is the most significant attempt at the DNA barcoding of freshwater microalgae in Ecuador.

**Material and methods**

**Sampling**

Access to freshwater ecosystems was conferred by the Ministerio de Ambiente del Ecuador (MAE) via the permission codes MAE-DNB.CM-2018-0093 and MAE-DNB.CM-2015-0011. Triplicate 1 L water samples were collected from different freshwater systems – including artificial ponds, water storage reservoirs, lagoons, lakes, and rivers – located in the Ecuadorian Andean and Amazon regions (Table S1). The sampling locations span the Andean region from north (Imbabura) to south (Azuay) as well as some water bodies from the Amazon region in order to isolate a good representation of microalgae species present in the country. The samples were kept in coolers on ice and taken to the laboratory for further analysis.

**Enrichment and isolation**

The collected freshwater samples were filtered through glass GF5 grade microfiber filters (pore size 0.7 μm, Chmlab). The harvested biomass underwent an enrichment step through suspension in liquid Bold’s Basal (BB) and Blue-Green 11 Media (BG11) [50]. This was maintained for at least two weeks at an average temperature of 20 ± 2 °C, a relative humidity of 50%, and light intensity of 21.384 ± 1.584 µmol·m⁻²·s⁻¹, with light/dark cycles of 16/8 h inside a germination chamber (Wisd, Korea). Enriched samples were streaked in solidified BB and BG-11 media, with 1% agar, and incubated under the same conditions described for enrichment for at least 4 weeks, until individualized green colonies appeared on agar plates. This process was repeated until unialgal cultures were obtained. The achievement of unialgal cultures was verified by light microscopy using an Olympus BX-40 with 100X phase contrast oil-immersion objective. Microscopic morphologic analysis was performed based on previous studies [11,51,52]. Further identification was accomplished by comparison with the original species descriptions that are available at the AlgaeBase [53].

Unialgal cultures were grown in an appropriate liquid medium for 4 weeks. One milliliter of the culture in a concentration ranging from 2 to 9 × 10⁷ cells/mL was transferred to 2 mL of cryovials and mixed with a DMSO and glycerol solution to a final concentration of 5% (v/v) and 10% (v/v) respectively. After incubation at room temperature for 30 min, cryovials were placed in a freezer at −20 °C for two days and then transferred to a Dewar flask containing liquid nitrogen. Microalgae isolates were kept in a cryopreserved state in an ultralower freeze at −80 °C.
Molecular identification

Isolated microalgae were submitted to DNA extraction with PureLink® Genomic DNA Mini Kit (Invitrogen™), following the instructions provided by the manufacturer with slight modifications in the lysis process. Briefly, microalgae colonies, from unialgal cultures grown for at least 4 weeks, were resuspended in 180 µL of Genomic Digestion Buffer and heated up to 90 °C for 12 to 17 minutes. After cooling, samples were processed as detailed by the manufacturer.

In addition, 16S rDNA and the 16S–23S rDNA intergenic spacer (ITS) were partially amplified for cyanobacterial identification, whereas fragments of 18S rDNA and rbcL were amplified for green algae identification. Primer combinations and their respective annealing temperatures are shown in Table 1. Amplification was performed in reactions of 25 µL containing 2X GoTaq® Green Master Mix (Promega), 0.25 µM of each primer, and 25 to 35 ng of extracted DNA. The reaction consisted of an initial denaturation step at 95 °C for 2 min, followed by 40 cycles as follows: 45 s at 95 °C, 30 s annealing at the proper temperature for each primer pair and 1 min at 72 °C, and a final extension step at 72 °C for 5 min.

Direct sequencing of the PCR amplicon was performed in both directions at the Sequencing service of Universidad de Las Américas (Quito, Ecuador). For ITS amplification with multiple PCR products, the band around 300 base pairs (bp) was extracted from the gel using the Wizard® SV Gel and PCR Clean-Up System (Promega) prior to the sequencing. Sequences were edited with MEGA X software [54] and compared against the GenBank database at the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST). Reference sequences were downloaded from the GenBank database and aligned with barcoding sequences from these work strains using the Muscle Algorithm [55].

Tree topologies and branch lengths were computed separately for the two markers with the maximum-likelihood method (ML). This was performed with 500 bootstrap replicates using the Kimura 2-parameter model with discrete gamma distribution, based on the corrected Akaike Information Criterion (AICc) in the ML model selection feature of MEGAX.

Gloeobacter violaceus (NR074282.1) was used as an outgroup taxon for the Cyanobacteria tree due to its independent taxonomical position at the base of all Cyanobacteria [56].

Results

Genetic diversity analysis among isolates

A total of 82 unialgal strains of Chlorophyta and 10 Cyanobacteria were isolated from freshwater bodies from the Andean and Amazon region of Ecuador (Table S1).

The 18S rDNA V4 hypervariable region was successfully amplified from DNA samples extracted from all the chlorophyte strains isolated using the universal primers described by Zimmerman et al. [37]. This primer set has been extensively used for metabarcoding studies in epilithic diatoms communities [57–59], so our results indicate that it could also be a useful marker for the Chlorophyta microalgae group. The fragments amplified were about 300 base pairs (bp) in length except for the CFU-22-04 strain with 700 bp. The sequences with a similarity of 100% were considered MOTUs. A total of 20 MOTUs were obtained, named from M-01 to M-20. M-01 (17%), M-02 (24%), and M-07 (27%) were the most abundant, followed by M-04 and M-08 (6%–7%). All of them were isolated from different freshwater systems of the Andean region but, in addition, 2 and 4 were also found in the Limoncocha lagoon, located in the Amazon region (Table 2). A second primer set was used to amplify the VI–V3 region of the 18S rDNA gene. All the strains tested were successfully amplified with the expected PCR product size of approximately 600 bp. One representative from each group was amplified with the oligos proposed by Hadi et al. [38] for the rbcL gene. Sixty-two percent of the DNA samples extracted were successfully amplified with primer set Fw_rbcL_357/ Rw_rbcL-1089 and 14% with the Fw_rbcL_192/ Rw_rbcL-657 primer combination. The amplification failed for 5 strains.

Table 1. Primer combinations, the annealing temperature (Ta) and expected amplicon size (bp) for each of set primers used in this study.

| Gene name | Primer ID: sequence (5’-3’) | Ta   | Amplicon size (bp) | Reference |
|-----------|-----------------------------|------|--------------------|-----------|
| 16S rDNA  | CYA106F: CGGACGGTGTAAACCGGTGA CYA781R: GACCTGGGGATATCCTATT | 55°C | 675                | [90]      |
| ITS       | ITSCYA236F: CTGGTCRACGCTCAGGAT ITSCYA225R: TGGACTKTCAGGGTCCTCT | 52°C | Variable           | [78]      |
| 18S rDNA  | DIV4for: GGGGTATCCTCAGCTCAATAG DIVrev3: CTTGCAATAGGATACGATA | 48°C | 329                | [91]      |
| 18S rDNA V4 | A: AACCTGGTTGCTCCTCGGCAG SSU-inR: CACCAAGCTTCGCCCTCA | 55°C | 563                | [63]      |
| rbcL      | rbcL_192: GTTAGCTGGACCAACWGTWWGGAC rbcL_657: GAACCGTCATCICCARCCAT rbcL_375: TTGGTCTCAACGGYTWCGGTG rbcL_1089: ATACACGACRTACGRTCCTTT | 48°-52°C | 465                | [38]      |
Table 2. Molecular Operational Unit Taxonomic (MOTU) identified for Chlorophyta, indicating the number, location of isolates (Table S1), and GenBank accession numbers (AN).

| Strain | No. of isolates | Location | 18S AN | rbcl. AN |
|--------|----------------|----------|--------|----------|
| M-01   | 14             | Colta, Ilincloca, Loretto, Manantial, Mica, Mojanda, Rodeococha lagoon | MT497360 MT550632 |
| M-02   | 20             | Colta, Chinchilla, Ilincloca, Limoncocha, Manantial, Pisayambo lagoon, Yambo lake; Quito pond; Papallacta reservoir | MT497361 |
| M-03   | 1              | Quito pond | MT497362 MT550633 |
| M-04   | 5              | Manantial lagoon; Sumaco Napo Galeras National Park pond; Pita river | MT497363 MT550634 |
| M-05   | 1              | Chinchilla lagoon | MT497364 MT550636 |
| M-06   | 1              | Pita river | MT497365 MT550637 |
| M-07   | 22             | Antequos, Chinchilla, Manantial, Mica, Mojanda, Rodeococha, Toreadora lagoon; Quito pond, Pita river | MT497366 MT550638 |
| M-08   | 6              | Antequos, Colta, Manantia, Mica lagoon; Pita river | MT497367 MT550639 |
| M-09   | 1              | Quito pond | MT497368 MT550640 |
| M-10   | 1              | Rodeococha lagoon | MT497369 MT550641 |
| M-11   | 1              | Yasuni National Park pond | MT497370 MT550642 |
| M-12   | 1              | San Pedro river | MT497371 |
| M-13   | 1              | Manduraciucu Reservoir | MT497372 MT550643 |
| M-14   | 1              | Negra lagoon; San Pablo lake | MT497373 MT550644 |
| M-15   | 1              | Pisayambo lagoon | MT497375 |
| M-16   | 1              | Pintag pond | MT497376 |
| M-17   | 1              | Negra lagoon | MT497378 MT550645 |
| M-18   | 1              | Kuyuk lagoon | MT497377 MT550646 |
| M-19   | 1              | Sumaco Napo Galeras National Park pond | MT497379 MT550647 |
| M-20   | 1              | Sumaco Napo Galeras National Park pond | MT497380 MT550635 |

The 16S rDNA and ITS amplification for Cyanobacteria strains was 100% successful. The sequence analysis of 16S rDNA amplicons revealed 10 molecular variants. Table 3 shows the location and accession number for each Cyanobacteria MOTU. The ITS amplification retrieved fragments with different sizes among the samples, ranging from 100 bp to 300 bp. Three of the samples amplified multiple PCR products. In these cases, the band close to 300 bp was sequenced.

Table 3. MOTU identified for Cyanobacteria, indicating the number, location of isolates (Table S1), and GenBank accession numbers (AN).

| Strain | No. of isolates | Location | 16S AN | ITS AN |
|--------|----------------|----------|--------|--------|
| C-01   | 2              | Loretto, Mojanda lagoon | MT497339 MT497359 |
| C-02   | 1              | Quito pond | MT497341 MT497358 |
| C-03   | 1              | Chimborazo Wildlife Reserve stream | MT497340 MT497350 |
| C-04   | 1              | Pintag pond | MT497343 MT497355 |
| C-05   | 1              | Negra lagoon | MT497344 MT497356 |
| C-06   | 1              | Limoncocha lagoon | MT497345 |
| C-07   | 1              | Chimborazo Wildlife Reserve stream | MT497346 MT497354 |
| C-08   | 1              | Mojanda lagoon | MT497347 MT497353 |
| C-09   | 1              | Mojanda lagoon | MT497348 MT497352 |
| C-10   | 1              | Manduraciucu reservoir | MT497349 MT497351 |

Similarity search based on 18S and rbcl markers for Chlorophyta

In order to perform the molecular identification, the 18S rDNA sequences obtained were submitted to searches against the GenBank database at the NCBI. The closest matches for the 18S rDNA V4 region had a similarity of up to 99–100%, as compared with the sequences available in the Genbank database, excepting M-05 (96%) and M-015 (98%) (Table 4). Most of the MOTUs obtained belong to the Scenedesmaceae and Chlorelaceae families. Six out of the twenty MOTUs matched with only one species, four matched with one genus, and the rest matched with more than one species with 100% similarity belonging to the same family or genus. Sequencing of the V1-V3 region was performed in order to improve molecular identification, and it also retrieved more than one identical sequence from different species for several MOTUs (Table S2). The M-02 MOTU for this region was the only strain that matched 100% with a sequence from an Ecuadorian microaqla (MF677854.1 Coelastrella sp.). The 18S V4 sequence of M-05 was about 400 bp higher than the others; however, the V1-V3 region had the same length as the other strains. The BLAST results showed three sequence entries with the same length as M-005, that is for Scenedesmaceae sp. Tow 9/21 P-14 w (AY197639.1) was the closest match with a 100% query cover and 96% similarity. Nonetheless, it has a 100% identity with the sequence entries of Desmodesmus sp., Acutodesmus deserticola, and Scenedesmus armatus, with a 40% query cover, which is consistent with the results obtained for the 18S rDNA V1-V3 region. The same similarity searches were carried out for the rbcl sequences that were successfully amplified. The similarity with the Genbank sequences was lower than those obtained for the 18S rDNA barcodes. The closest matched identities ranged from 92% to 100% with a smaller number of hits per species (Table 4).

The results presented in Table 4, based on the 18S and rbcl sequences, suggest that species-level identification was achieved for eleven out of the twenty-one MOTUs (M-01, 03, 04, 07, 08, 10, 11, 13, 14, 16, 17, and 19), and four (M-05, 12, and 20) were determined at the genus level. In the case of M-09, the identification was not possible given that both barcodes matched two different species with a 100% identity. However, microscopic visualization showed spherical green cells, which were more similar to Parachlorella kessleri than the ellipsoidal cells of Chlororidium saccharophilum. The same scenario occurred for M-18, and so a change of name was proposed from O. amblystomatis (with the accession number KY091671) to Chlorococcum amblystomatis [60]. These authors recently reported about a novel Chlorococcum strain isolated from a pond in
Table 4. Molecular identification of the Chlorophyta strains used in this study, including the percentage of identity, accession number, and the name of the identified species in the GenBank database, based on the 18S V4 and rbcL marker sequence. * indicates query cover below 100%.

| Strain                  | 18S V4 % id | No. of Hits | Closest match                      | rbcL % id | No. of Hits | Closest match                      |
|-------------------------|-------------|-------------|------------------------------------|-----------|-------------|------------------------------------|
| M-01 Scenedesmus sp.    | 100         | 16          | MN630585.1                         | 99        | 2           | KT777984.1                         |
| Tetradesmus obliquus    | 100         | 15          | KY637056.1                         |           |             |                                    |
| Acutodesmus bajocatalonicus | 100       | 9           | KYS87455.1                         |           |             |                                    |
| Acutodesmus deserticola | 100         | 4           | MG597607.1                         |           |             |                                    |
| Tetradesmus dimorphus   | 100         | 2           | MK764915.1                         |           |             |                                    |
| M-02 Coelastralla sp.   | 100         | 23          | MH716102.1                         |           |             | Not amplified                      |
| Scenedesmus sp.         | 100         | 9           | HE771102.1                         |           |             |                                    |
| Pseudoponiococcus sp.   | 100         | 5           | KU057947.1                         |           |             |                                    |
| M-03 Desmodesmus abundans | 100         | 3           | MH307943.1                         | 98        | 7           | KT777986.1                         |
| Desmodesmus sp.         | 100         | 1           | MH780938.1                         |           |             |                                    |
| M-04 Desmodesmus sp.    | 100         | 1           | M-11                               | 98        | 2           | MK925222                           |
| Scenedesmus sp.         | 100         | 1           | MH879792.1                         | 97        | 1           | EF113451.1                         |
| M-06 Microcystis inermum | 100         | 5           | M-06                               |           |             |                                    |
| Chlorella sorokiniana   | 99          | 30          | M-05                               |           |             |                                    |
| Chlorella vulgaris       | 99          | 12          |                                    |           |             |                                    |
| Chlorella sorokiniana   | 100         | 36          | MN365023.1                         | 99        | 3           | MK842150.1                         |
| Chlorella vulgaris       | 100         | 21          | KU720636.1                         |           |             |                                    |
| Microcystis pusillum     | 100         | 2           | MG597609.1                         |           |             |                                    |
| M-08 Chlorella vulgaris  | 100         | 69          | MT137382.1                         | 100       | 7           | MK948102.1                         |
| M-09 Parachlorella kessleri | 100     | 27          | KX021356.1                         | 100       | 4           | FJ968741.1                         |
| Dicytothecium sp.       | 100         | 10          | G0487245.1                         | 100       | 4           | MK295212.1                         |
| M-10 Gloeotrichis sp.    | 100         | 30          | M-10                               |           |             |                                    |
| Stichococcus cf. deasonii | 100       | 2           | KF142233.1                         | 94        | 1           | EF589147                           |
| M-12 Stigeoclonium helveticum | 100   | 4           | EU123941.1                         |           |             |                                    |
| Stigeoclonium tenuum     | 100         | 2           | MN209224.1                         |           |             |                                    |
| M-13 Chlorolobion braunii | 100        | 4           | MK203111.1                         | 99        | 3           | KT355757.1                         |
| Monoraphidium sp.       | 100         | 2           | KF805717.1                         |           |             |                                    |
| M-14 Caespitella sp.     | 100         | 2           | LN870281.1                         | 92        | 1           | EF113437.1                         |
| M-15 Trebouxia sp.       | 98          | 2           | MG493307.1                         |           |             |                                    |
| Heterochlorella sp.      | 98          | 2           | KM116462.1                         |           |             |                                    |
| M-16 Haematococcus sp.   | 100         | 19          | MF992170.1                         |           |             |                                    |
| M-17 Neochnorococcus sp. | 100         | 3           | KR807495.1                         | 97        | 1           | MK257153.1                         |
| M-18 Chlorococcum sp.   | 100         | 5           | MTO26583.1                         | 97        | 2           | KC810302.1                         |
| Ophiopila sp.            | 100         | 1           | KY091671.1                         | 95        | 1           | KJ63544.1                          |
| M-19 Anakistrodesmus sp. | 99          | 3           | KF733838.1                         | 94        | 1           | EF113406.1                         |
| M-20 Desmodesmus sp.    | 99          | 13          | MH307951.1                         | 97        | 1           | KF975595.1                         |

Portugal that was 100% identical to M-18 and KY091671. The 18S sequence-based identification assigned M-06 to Microcystis inermum, and matched 100% to the M. inermum strain NIES-2171 (AB731604) described by Hoshina & Fujiwara [61]. Under a light microscope, we observed solitary spherical cells without mucilaginous coverings, consistent with the description of M. inermum, but we were not able to distinguish the chloroplast features. Nevertheless, the closest match for the rbcL sequence was Chlorella sp. The nucleotide search for M. inermum rbcL sequences retrieved zero entries. The rbcL amplification for M-02 and M-15 was not successful, and the 18S barcode did not allow for species-level identification. The morphological analysis using light microscopy showed that the cells of M-02 are grouped and broadly ellipsoidal to spherical, the chloroplasts are hollow, spherical and entirely cover the cell sphere, and they contain a pyrenoid similar to the Coelastralla species. No characteristic features were observed in M-15 that permitted differentiation between the genera.

A phylogenetic analysis was carried out to resolve the genus-level identification for the Scenedesmaceae strains. Genetic distances (p-distance) among Scenedesmaceae family sequences for 18S V4 ranged from 0.011 to 0.102. Nevertheless, the variability found among Scenedesmaceae sequences for 18SV1-V3 fragments was lower. For the V1-V3 region, the p-distance between M-01 and M-02 was 0.006, while for the V3 region was 0.018. The phylogenetic tree using reference 18S rDNA sequences from the GenBank database (Figure 1) clearly demonstrates that sequences from these strains group together with their closest matches from the GenBank database (Table 4) in well-supported clades. M-01 and M-02 are clustered with Tetradesmus and Coelastralla sequences, respectively, and separated from the other Scenedesmaceae strains (M-03, M-04, M-05 and M-20) that are grouped in a separated clade. The genetic distances for rbcL sequences ranged from 0.064 to 0.107 among M-01, 03, 04, and 20, with the lowest distance when comparing M-03 and M-04 with M-20. The rbcL region
obtained for M-05 matched only in terms of 63 bp with the sequence amplified from the other strains; therefore, it was not included in the rbcL comparison. Hadi et al. [38] reported that Desmodesmus rbcL interspecific distances ranged from 0.015 to 0.086. Thus, according to the BLAST results and genetic distances, M-04, M-05, and M-20 belong to the Desmodesmus genus and they are probably different species. This idea is supported by the phylogenetic tree constructed using rbcL and 18S joined sequences, which separated M-04 and M-20 into two different clusters (Figure 2). Figure 1 also shows M-18 and Oophila amblystomatis (KY091671) clustered with the Chlorococcum genus in a monophyletic clade, as reported by Correia et al. [60], thus confirming the identification of M-18 as Chlorococcum amblystomatis.

The phylogenetic tree of Trebouxiophyceae class strains using reference 18S rDNA sequences showed two clades corresponding to the Chlorellaceae and Trebouxiaceae families. The genetic distances between Chlorellaceae strains ranged from 0.007 to 0.014 for 18SV4 fragments, and from 0.002 to 0.019 for the 18SV1-V3 region, with M-09 being the most different. Chlorellaceae Ecuadorian sequences were separated into three clusters: M-06 with Micractinium, M-07 and M-08 with Chlorella spp., and M-09 with Parachlorella and Chloroidium spp. (Figure 3). The analysis carried out with both barcodes joined together (Figure 2) also

![Figure 1. Phylogram constructed using 18SV4 rDNA sequences from Ecuadorian strains and sequences in the GenBank database belonging to the Chlorophyceae class. The phylogram was constructed using the maximum-likelihood method with a Kimura 2-parameter model using a discrete Gamma distribution (+G). Numerical values at the nodes of the branches indicate bootstrap values above 50%.](image-url)
indicated that M-08 is closer to M-07 than M-09. The phylogenetic tree did not resolve the genus identification for the M-15 sequence.

**Similarity search based on 16S and ITS markers for Cyanobacteria**

In order to perform the molecular identification, the 16S sequences obtained were submitted to searches against the GenBank database. The closest matches for the 16S rDNA region ranged from 98% to 100% similarity with sequences available in the GenBank database (Table 5).

Two MOTUs (C-02 and 06) matched with only one species, four (C-04, 07, 08, and 09) matched with one genus, and two (C-01 and 10) matched with more than one species with a 100% similarity. The GenBank sequences similar to C-03 and C-05 correspond to undescribed Cyanobacteria species isolated from the Rhine basin (KP143908.1) and Antarctica mats (HQ 827,392.1), respectively. The same similarity searches were carried out for ITS sequences. The similarity with GenBank sequences was lower than those obtained for the 16S barcode. The closest match identities ranged from 92% to 100% with a smaller number of hits per species (Table 5). There were no closest matches corresponding to more than one species. Moreover, the query cover was lower than 100% for C-04 (76%), C-05 (50%), and C-08 (50%).

The species-level identification based on both barcoding sequences (Table 5) was achieved for three MOTUs (C-02, 06, and 10), and six could be determined at the genus level (C-01, 03, 04, 07, 08, and 09). In the case of C-05, the sequence similarity for ITS was too low to even assign the genera of the strain. Light microscopic observation did not allow a species- or genus-level assignment for C-05. However, morphological features observed for C-03 matched those of the *Pseudanabaena* genus.

The phylogenetic analysis for Cyanobacteria strains was accomplished based only on 16S rDNA sequences due to the lack of homogeneity in ITS fragment length. The genetic distances between sequences ranged from 0.03 to 0.25. The most similar sequences were C-07, C-09, and C-03, and the greatest distance was noted between C-05 and C-09. The phylogenetic tree constructed using reference 16S rDNA sequences from the GenBank database (Figure 4) reflects the BLAST results for Cyanobacteria strain identification (Table 4). The unidentified strain C-11 grouped into the *Pseudanabaena* cluster. However, the C-05 strain did not cluster into any of the genera assigned to the other strains or with the closest genera matched with the ITS sequence.

**Discussion**

This study focused on the dual barcoding characterization of microalgae isolated from Ecuadorian freshwater environments. However, with the data presented, we did not intend to perform an ecological biodiversity assessment of Chlorophyta and Cyanobacteria taxa present in Ecuador. The molecular analysis was conducted in order to obtain the DNA barcodes from strains present in the country and compare them to sequences present in the Genbank database. Moreover, it can help to differentiate between isolates that are morphologically indistinguishable. This will
Figure 3. Phylogram constructed using 18SV4 rDNA sequences from Ecuadorian strains and similar sequences in the GenBank database belonging to the Trebouxiophyceae class. The phylogram was constructed using the maximum-likelihood method with the Kimura 2-parameter model using a discrete Gamma distribution (+G). Numerical values at the nodes of the branches indicate bootstrap values above 50%.

Table 5. Molecular identification of the Cyanobacteria strains used in this study, including the percentage of identity, accession number, and the name of the identified species in the GenBank database, based on the 16S and ITS marker sequence. * indicates query cover below 100%.

| MOTU   | 16S     | % Id | No. of Hits | Closest match                | ITS         | % Id | No. of Hits | Closest match              |
|--------|---------|------|-------------|------------------------------|-------------|------|-------------|------------------------------|
| C-01   | Synechocystis sp. | 100  | 35          | AY224195.1 Synechocystis sp.  | 97          | 30   | MH683823.1  |
| C-02   | Aphanocapsa sp.  | 100  | 2           | JQ070058.1                   | 100         | 5    | CP003597.1  |
| C-03   | Chroococcidiopsis thermals | 100  | 11          | NR102464.1 Chroococcidiopsis thermals | 93  | 1    | LT600730.1  |
| C-04   | Unidentified cyanobacterium | 99   | 7           | HQ827392.1 Pseudanabaena sp.  | 92*         | 1    | MK953016.1  |
| C-05   | Nostoc sp.      | 96   | 16          | KP143908.1 Synechococcus sp.  | 95*         | 5    | AJ519834.1  |
| C-06   | Unidentified cyanobacterium | 99   | 99          | EU078548.1 No determined      | 93          | 3    | LC314140.1  |
| C-07   | Cylindrospermopsis raciborskii | 99   | 4           | MN145867.1 Pseudanabaena sp.  | 93          | 3    | LC314140.1  |
| C-08   | Leptolyngbya sp. | 99   | 2           | KX495135 Leptolyngbyaceae    | 97*         | 2    | MK861878    |
| C-09   | Pseudanabaena sp. | 99   | 2           | KR872397.1 Pseudanabaena sp.  | 90*         | 7    | LC314124.1  |
| C-10   | Limnothrix planktonica | 100  | 13          | JQ004021.1 Limnothrix sp.     | 95          | 2    | MN551904.1  |
| Anagnostidinema amphibia | 100  | 2           | MN128963.1 Limnothrix planktonica | 92          | 4    | LT600739.1  |
facilitate the identification and selection of strains for further studies.

Several DNA barcode systems have been proposed as a potential solution for tackling the great diversity of Chlorophyta and Cyanobacteria, yet there is no consensus about which marker should be used \[18,38,62\]. In this work, we followed the nested strategy proposed by the Consortium for the Barcode of Life (CBOL) protist working group using the variable V4 region of 18S rDNA as the universal eukaryotic pre-barcode and second group-specific barcode \[31\]. Our results show that the 18S V4 region is equally effective as the V1 and V3 regions, which are commonly used for identifying Chlorophyta microalgae \[19,63,64\]. In fact, our results show that this marker has a higher nucleotide diversity in a smaller region than the V1-V3 marker. Furthermore, the primer universality achieved for this barcode is higher than the rbcL marker, as described in previous studies \[18,38\]. The V4 region allowed us to differentiate between isolates of

**Figure 4.** Phylogram constructed using 16S rDNA sequences from Ecuadorian cyanobacteria and similar sequences at the GenBank database. The phylogram was constructed using the maximum-likelihood method with Kimura 2 distance. Numerical values at the nodes of the branches indicate bootstrap values above 50%.
Scenedesmaceae and Chloraceae families that were alike under light microscopic visualization. It is noteworthy that the BLAST results, when compared with the GenBank database, allowed for the identification of 38% of 18SV4 sequences from the strains isolated at the species level. This rate increased to 52% when the rbcL BLAST results were taken into account. Several studies highlight the necessity of using several barcodes to properly identify strains at the species level, especially in the Scenedesmaceae [65–67] and Chloroellaceae families [68]. The use of rbcL is not very common in Chlorophyta microalga phylogenetics, but it was useful for characterizing our collection, despite the rate of unsuccessful amplifications reported by others [31,38].

Simple morphology, phenotypic plasticity, and the presence of cryptic species in the Scenedesmaceae and Chloroellaceae families have contributed to taxonomic complications [69] resulting in constant reassignments [70]. Regarding the Scenedesmaceae family, the Desmodesmus and Acutodesmus genera were considered to be Scenedesmus subgenera by Hegewald [71] until they were separated as genera of their own by An et al. [72]. This could explain why strains with identical 18S rDNA gene sequences are assigned with different genera, as was the case for the closest matches M-01 and M-03. On the other hand, the same scenario occurs for the Chloroellaceae family. Most of the Chlorella-like species possess few morphological characteristics useful for species characterization, and many studies often report “little green balls” for Chlorella spp [33]. For instance, the Parachlorella genus was reassigned from Chlorella kesleri (Fott and Nováková) [68] and Chloroidium was reassigned as a new genus from Chlorella saccharophila and C. ellipsoida by Huss et al. [73]. Some authors reported a lack of variability in the 18S rDNA gene when discriminating between the genera of these families [33], thus they suggest the use of a second marker for proper identification, such as rbcL and the ribosomal RNA ITS regions. The plastid-encoded rbcL gene has been widely used for phylogenetic inference in green algae [18,31,38], but it has described as taxon-specific, which is consistent with the lack of amplification in some of our isolates. In spite of this, the rbcL barcode was a determinant in resolving the identification of the M-01, M-03, M-07, M-09, M-013, and M-019 sequences. The rbcL gene is considered to be evolving more rapidly than the 18S rDNA gene [33]; this may be the reason for the lower percentage of identifications with the rbcL sequences deposited in the GenBank database and the strains in this work, as compared to the 18S V4 region. Heterochlorella and Jaagichlorella are very close taxa with a general Chlorella-like morphology, and they belong to the Trebouxiaaceae family. Previous studies reported the necessity of an integrated approach using Small Subunit (SSU) and ITS rDNA sequences and including ecological distribution to resolve the taxonomical status in this group [74]. This could explain the lack of resolution of 16S rDNA barcoding to identify the strain corresponding to MOTU M-15. Darienko et al. [74] proposed the transfer of Heterochlorella luteoviridis to Jaagicheloria. Additionally, it seems that Heterochlorella (Jaagichlorella) luteoviridis and J. africana have different distribution patterns. Strains of J. luteoviridis were commonly isolated from aquatic systems while the others were collected from terrestrial habitats. According to this differentiation, our strains could be named Jaagichlorella luteoviridis; nevertheless, we would have to sequence the ITS region to confirm this assignment. Our results support the necessity of a second marker for DNA barcoding in microalgae, and highlight the lack of reference sequences linked to a precise morphological identification.

The strains we isolated are distributed worldwide. They are all found in freshwater systems from different geographic regions, and their sequences match previously published sequences. Scenedesmaceae and Chlorella strains have been found to be the most common aquatic algae isolated from the aquatic systems sampled in our study, as they are the most commonly reported genera of freshwater cocoid green algae with a ubiquitous distribution [33,39]. Some of these species, like Tetrasdesmus dimorphus, Coelastrella sp., Chlorella vulgaris and C. sorokiniana, have been found throughout the Andean region and present no variability in the barcode sequences. Hadi et al. [38] performed a similar study focussed on the assessment of DNA barcodes for neotropical freshwater Chlorophytes. They analyzed a subset of green microalga strains isolated from Brazilian inland water bodies and most of them also belonged to Scenedesmaceae and Chloraceae species.

It has been demonstrated that the differences in genetic distances for the 16S rDNA barcode are useful in defining taxonomic units in Cyanobacteria [75,76]. Consistent with that, nine of our isolate sequences clustered with Leptolyngbya, Pseudanabaena, Nostoc, Cylindrospermopsis, Synechocystis, Limnothrix, and Chroococcidiopsis genera, based on the 16S rDNA barcode. However, the 16S sequence of two of these strains (C-01 and C-10) matched with more than one species with a 100% identity. This could be due to the lack of genetic interspecific differentiation reported between taxonomic units in prokaryotes [76,77]. Another explanation could be the misidentification of the strains based on morphological characteristics, as it has been reported that many Cyanobacteria strains in culture collections are wrongly classified [78,79]. The C-05 MOTU did not group with any other genera and the sequence only matched (99%) with an unidentified cyanobacterium isolated from
aquatic systems (KP141908.1 in Germany; KJ766163.1 in Taiwan) and humid soil (KF358628.1 in China). The C-03 strain, that grouped with *Pseudanabaena* genera, matched 100% with an unidentified cyanobacterium isolated from mats present in maritime Antarctica. It is worth highlighting that the origin of the C-03 sample is the Chimborazo Volcano (6,300 masl), which has a permanent glacier and is the source of the stream from which the strain was isolated.

Nevertheless, an accurate taxonomy can hardly be achieved using only one barcode, since it is based on nucleotide substitutions of a single gene [76]. Thus, we also sequenced the ITS region of 16S-23S rDNA, as it has been broadly used as a gene marker to examine cyanobacterial community structures [80–82]. Nevertheless, we did not construct a phylogenetic tree based on this barcode because of the heterogeneity of the size of the amplicons, which makes it difficult to align the sequences. Despite this, the ITS barcode allowed us to disambiguate the C-01, C-03, and C-10 strains, and corroborate the 16S matches of C-02 at a species level and C-04, C-07, C-08, and C-09 at a genus level. The ITS region is an effective molecular marker for characterizing intra-species diversity [80,82]. The sequences of our isolates for this marker are more variable than the 16S rDNA BLAST results, and in some cases, the low percentage of query cover hampered the molecular identification of the strain.

Many of the strains isolated have been described to be of biotechnological interest to the food industry, biofuel production, and pharmaceutical and environmental activities. The *Chlorella*, *Chlorococcum*, *Nostoc*, and *Synechococcus* species are of great interest to the first two sectors due to high lipid accumulation and the production of fatty acids and polyunsaturated fatty acids (PUFAs) [60,83,84]. It has also been shown that these species play a role in the biodegradation of pesticide pollutants and metal removal [85]. Cyanobacteria species belonging to the *Nostoc* and *Synechococcus* genera have been used for the development of antiviral, antibacterial, and anti-inflammatory agents, and are of interest to pharmaceutical researchers [83].

The few reports that have focused on the diversity of Chlorophyta and Cyanobacteria in Ecuador were conducted based on morphological identification [11,49]. In spite of the increasing application of molecular phylogeny in the floristic study of microalgae, there are scarcely any barcode sequences available for Ecuadorian strains. Scientists have only reported the ITS2 sequences for *Stichococcus* sp. and *Dipllophaera* sp. strains [86], and 18S rDNA sequences for *Chlorella* sp., *Coelastrella* sp., *Scenedesmus* sp., and diatom strains [87,88]. Furthermore, there are sequences for ribosomal genes (16S and 23S) available in the GenBank database (popset MH090926–32 and MH101455–61) for seven cyanobacteria from
geothermal springs in Ecuador (unpublished) and for the *rbcl* gene of *Nostoc* sp. from the lichen genus *Peltigera* [89]. However, our study considerably increases the molecular characterization of the Chlorophyta and Cyanobacteria strains present in Ecuador, thereby contributing to knowledge regarding the biodiversity of these taxa. All barcode sequences in this work are being reported for the first time for Ecuadorian Chlorophyta and Cyanobacteria strains, except for 18SV1V3 of M-02 MOTU (*Coelastrella* sp.), which were reported previously.

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No potential conflict of interest was reported by the author(s).

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